Food fortification and supplementation

Technological, safety and regulatory aspects

Edited by Peter Berry Ottaway
Food fortification and supplementation
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Food fortification and supplementation
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Edited by
Peter Berry Ottaway
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1 Principles of food fortification and supplementation
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1.1 Introduction

Food fortification and supplementation with nutrients has been carried out for centuries, often before the scientific rationale became available. There are a number of examples of food fortification from different parts of the globe. In Central Europe during the middle ages, mothers were known to push iron nails into apples, leave them there for a while and then feed the apples to listless or ailing daughters. There are reports as early as 1824 of the indigenous Indian population of Columbia, South America, treating goitre with a specific source of salt which was later found to have a high iodine content. In the making of traditional tortillas in Mexico, the corn was first soaked in lime water, and a pinch of ground limestone was added to the tortilla itself; we now know this was to provide an intake of calcium.\(^1\)

One of the first recorded suggestions for the fortification of food was that of Boussingault in 1831, who advocated the iodisation of salt for the reduction of goitre. The first official addition of an iodide to domestic salt started in Switzerland in 1900, and the practice has continued in a number of countries to the present day.

Other foods where micronutrient additions have been officially sanctioned or legally required are margarine and flour. Apparently, only six years after vitamin A (retinol) had been identified, a leading doctor in London wrote to the Chairman of Unilever to the effect that if the company wanted its margarine to resemble butter they would have to add the ‘new-fangled vitamins’. A vitamin A deficiency was reported among children in Denmark in 1917. This was apparently due to Denmark exporting a large proportion of its butter production and replacing it in the home market with margarine. As butter
2 Food fortification and supplementation

was a major source of vitamin A in the Danish diet, the fortification of margarine with fish liver oils was introduced. The legal requirement for the addition of vitamins A and D to margarine continues in many countries.\textsuperscript{2}

During the Second World War, the United Kingdom introduced legislation making it compulsory to add vitamin B\textsubscript{1} (thiamin), niacin and an approved source of iron to all flour used for cooking or baking. Some flours were also required to have added calcium carbonate. These requirements are still in force over 60 years later.

1.2 Definitions

Whilst the terms ‘fortification’ and ‘supplementation’ are widely used, a number of terms are often used indiscriminately. Some agreement on specific definitions was reached within Codex Alimentarius and this was published in 1987 in the ‘General Principles for the Addition of Essential Nutrients to Foods’.\textsuperscript{3} Based on these definitions:

- **Fortification** usually refers to the addition of nutrients to foods from which they were either absent, or present in unimportant amounts. This is carried out because deficiencies or potential deficiencies have been shown in the population. It also includes additions to fulfil the role of another food in the diet. Thus margarine is fortified with vitamin A in many countries to replace that ‘lost’ when margarine is substituted for butter. Vitamin D is added at higher levels than are found in butter as a public health measure, since extra is considered necessary for the population as a whole.

- **Restoration** is self-evident, involving the replacement, in full or in part, of losses incurred in processing (e.g. loss of B vitamins and iron in milling of cereals to low extraction rates; loss of vitamin C in the preparation of instant potato).

  It should be noted that, up to now, restoration has been limited; for example, white bread has thiamin, niacin and iron added back, although other vitamins such as pantothenate, folate, pyridoxine and tocopherol are also partly lost in the milling process. Similarly, fruit juices might have vitamin C added back, but juices also supply folate and thiamin. In some countries, calcium and riboflavin are also added to bread: this is fortification, since wheat contains only small amounts of these two nutrients.

- **Enrichment** is increasing the level of nutrients present to make the food a ‘richer’ source. Enrichment has frequently been interchanged with restoration and fortification as a general term. However, for labelling purposes the use of the term ‘enriched’ is controlled.

- **Standardisation** is sometimes used to mean additions to compensate for natural or seasonal variations in nutrient content.
• **Substitution** is the nutrient addition to a substitute product to the levels found in the food which it is designed to resemble in appearance, texture, flavour and odour and which it is intended to replace partially or completely. An example is the addition of vitamins A and D to fat replacers.

• **Supplementation** is the supply of nutrients (normally micronutrients) singly or in combination in a quantified dose form. Supplements can take a variety of forms such as tablets, capsules, pastilles, measured amounts of liquid or small sachets of powder.

The term ‘nutrification’ has been occasionally used in the past to mean the addition of nutrients to formulated or fabricated foods which are marketed mainly as meal replacements.

### 1.3 Food fortification

Food fortification and restoration has, for more than a century, played an important role in helping to achieve specific health policies and ensuring the nutritional health of populations across the world. In addition to the enrichment of salt with iodine and the fortification of margarine and bread mentioned previously, there have been programmes to combat rickets with vitamin D fortified milk; the addition of thiamin (vitamin B$_1$), niacin and folic acid to cereals to reduce the incidence of beriberi and pellagra; and also the addition of an iron source to cereals to help reduce anaemia.

The fortification of food has the great advantage that it can often be accomplished within the context of an indigenous diet and requires little change to the consumer’s dietary behaviour and food habits. In most cases, effective fortification can be achieved with little or no effect on the organoleptic properties of the food due to the very small amounts of the added micronutrients.

In the developing countries, where there is the greatest need for micronutrient fortification, the vitamins and minerals can be added to the foods or condiments most regularly consumed by a significant proportion of the population at risk. In many cases this is likely to be the staple cereal such as rice, wheat or maize flour, but there have been reports where condiments such as iron-fortified fish sauces and soy sauces have been successfully used in programmes aimed at reducing iron deficiency, mainly in Asia.\(^4\),\(^5\)

Food fortification can have a positive effect on the health of a population at a very low cost. A report published by UNICEF in 2005 based on an evaluation of 80 developing countries gives a vivid picture of the consequences of micronutrient deficiencies in those countries.\(^6\)

• Iodine deficiency is estimated to have lowered the intellectual capacity of almost all of the nations reviewed by as much as 10 to 15 percentage points.

• Iron deficiency in the 6–24 month age group is impairing the development of approximately 40–60% of the developing world’s children.
Food fortification and supplementation

- Severe iron deficiency anaemia is responsible for the deaths in pregnancy and childbirth of more than 60,000 young women a year.
- Vitamin A deficiency is compromising the immune systems of approximately 40% of the developing world’s under-five-year-old children, leading to the death of about one million young children each year.
- Folate deficiency is responsible for an estimated 200,000 severe birth defects each year in the 80 countries assessed.

The above examples are not exhaustive but are indicative of the scale of the problem faced by a large proportion of the world’s population. To put it into perspective, over two billion people, or a third of the global population, are at risk of vitamin A, iron or iodine deficiency.2

The General Assembly of the United Nations in May 2002 agreed that the key micronutrient deficiencies should be one of the global development goals to be achieved in the first decade of the new millennium. Specifically, the UN targets were for the virtual elimination of iodine deficiency by 2005; the elimination of vitamin A deficiency by 2010 and a reduction of at least 30% of iron deficiency anaemia, also by 2010.8

The mandatory fortification programmes needed to achieve these objectives require a political will and commitment at a national level. The scale and severity of micronutrient deficiency is not yet fully appreciated by the politicians, public or press in most nations of the world. The UNICEF report states that ‘the goals will not be achieved, and the impact of vitamin and mineral deficiency will not be substantially reduced without a more ambitious, visionary and systematic commitment to putting known solutions into effect on the same scale as the known problems’. It also points out that progress is only likely to be achieved by dynamic alliances involving governments, the private sector, health and nutrition professionals, academics and researchers, civil society and international agencies.

While some of the more persuasive arguments for food fortification relate to micronutrient deficiencies in developing countries, fortification has a long history of use in the developed countries. Micronutrient addition to breakfast cereals has been a common practice in many countries for decades, as has the addition of vitamin C to fruit juices.

The addition of micronutrients to food can be justified if either a food is generally accepted as being a good source of the nutrient(s) but suffers losses during processing or storage, or if the introduction of a new food concept could be expected to replace an existing food. An example of this was the widespread replacement of full-cream milk as part of a policy to reduce the intake of fat. The removal of the fat also significantly reduced the amounts of the fat soluble vitamins A and D in the milk, thus removing a common source of these vitamins from the diet.
The introduction of meat substitutes in the form of textured vegetable protein (TVP) caused significant debate in the 1960s and 1970s, with differing national requirements for the addition of vitamins, minerals and specific amino acids to replace those found in meat.

The introduction of the fat-replacer Olestra was another good example of the need for fortification. This was due to some of the fat-soluble vitamins (particularly vitamins A, E and the pro-vitamin carotenoids), present in the intestine at the same time as Olestra, being preferentially dissolved in the Olestra and being lost to the body. In 1996 the Food and Drug Administration in the United States of America granted approval for the limited use of Olestra subject to fortification with fat-soluble vitamins. 9

A rapidly increasing area of fortification is that of functional foods, where many contain added substances with a physiological or health effect as well as micronutrients, such as the antioxidant vitamins.

However, when considering fortification of a food it must be borne in mind that the range of foods suited to fortification is considerably limited by a number of factors including technological and organoleptic restrictions, cost and consumer expectations.

Properties inherent in the food such as moisture, pH and oxygen permeability can lead to unacceptable taste, appearance and reduced stability, which can counteract the benefits of fortification. The problems are normally more serious with mineral fortification, where some of the minerals (such as iron and zinc) can adversely affect the vitamin content of the food.

A consumption survey based on the five largest countries of the European Union (France, Germany, Italy, Spain and the United Kingdom) indicated that, where fortification is permitted, such foods are unlikely to comprise more than 3% of the diet on a per capita basis. An exception is the United Kingdom where flour for bread making has mandatory fortification, but the increase is not that great. The study concluded that, in reality, the majority of high-level fortified food consumers are unlikely to obtain more than 4% of their diet from fortified food even though a theoretical calculation could bring the level up to 10%. 10

An area of fortification which has been relatively heavily researched in the developed countries towards the end of the 20th and early 21st centuries is that of folic acid. The addition of folic acid to staple foods was instigated by research on the effects of folic acid on the reduction of the incidence of neural tube syndrome (spina bifida). Since January 1998, the Food and Drug Administration of the United States of America has required folic acid fortification of all enriched cereal grain products. The aim was to increase the intake of folic acid by women of child-bearing age. 11

A study on the effects of this programme by researchers at the University of California Berkeley found that the mandatory folic acid fortification led to significant increases in both serum and erythrocyte concentrations in all sex and age groups. 12 However, less than 10% of women of child-bearing age reached the recommended erythrocyte folate concentration of greater
than 906 nmol/L that has been shown to be associated with a significant reduction in neural tube defect risk. After fortification, the category of ‘bread, rolls and crackers’, became the single largest contributor of total folate to the American diet, contributing 15.6% of total intake.

Another study by the Texas Department of State Health Services considered the epidemiological evidence from a number of states for the period 1995–1996 (pre-fortification) and 1999–2000 (post-fortification). The results indicated the previously reported reductions in the birth prevalence of neural tube defects. In addition, there were modest yet statistically significant decreases in birth prevalence for the transposition of the great arteries, cleft palate, pyloric stenosis, upper limb reduction defects and omphalocele. The researchers concluded that the results suggested some modest benefit from the folic acid fortification on the prevalence of a number of non-NTD birth defects.13

Mandatory folic acid fortification programmes have been started in 38 countries across the world. However, many European countries have deferred the introduction of compulsory fortification, mainly due to concerns about the possible masking of vitamin B₁₂ deficiency.

The Food Standards Agency in the United Kingdom has been considering mandatory fortification of bakery products with folic acid for a number of years. Progress was impeded by the Scientific Advisory Committee on Nutrition (SACN) in 2002, because of its concern that folate consumption in excess of 1000 μg (1 mg) per day could delay the detection of vitamin B₁₂ deficiency, with possible severe neurological consequences in some older people. Although more recent research indicates that vitamin B₁₂ deficiency would be masked only with folate consumption of more than 5000 μg/day, the SACN was still not in a position to give a positive recommendation for folate fortification in mid 2006. On the 17th May 2007, the Food Standards Agency Board unanimously agreed that ‘mandatory fortification’ of either bread or flour with folic acid should be introduced, alongside controls on voluntary fortification and advice on the use of supplements.14 In reaching its decision, the Board considered the risks and benefits to both specific groups of the population as well as to the whole population. Evidence presented to the Board included assessments of the impact that mandatory fortification has had in other countries. Following the Board’s agreement, the Food Standards Agency was mandated to recommend fortification to the UK Health Department and to undertake the legal process required for its introduction.

1.4 Supplementation

Unlike food fortification, where conventional foods or ingredients are used to carry the added nutrients, supplementation relies on specifically designed products to deliver the nutrients in unit dose form. That is, each dose or serving of the supplement is designed to supply a quantified amount of the nutrient.
In contrast to the diet-based approaches, which involve the population in general, supplementation can be used to provide a rapid improvement in the nutritional status of target groups.

In terms of its effect on vulnerable sections of the population, such as children and women of child-bearing age in developing countries, it is accepted that supplementation has a significant role in alleviating deficiencies. However, there are still those who question whether it should play such a role. This is due mainly to the perception that only food-based approaches should be used, as the consumption of supplements does not encourage moves towards good dietary habits.

There are some micronutrients which are best delivered through supplementation. One of these is vitamin A, where there is already evidence that vitamin A supplementation is effective in reducing child mortality at low cost. According to UNICEF, a vitamin A capsule is effective for up to six months and can cost as little as 2 cents (US). A World Development Report by the World Bank in 1994 found that micronutrient programmes in general, and vitamin A supplementation in particular, have been among the most cost effective of all health interventions.15

The use of supplements by consumers in the developed countries to maintain health can be traced back in the USA to the early decades of the 20th century. In 1938, a publication under the auspices of the American Medical Association commented that people were consuming ‘quantities of vitamin pills and capsules to prevent colds, to ward off a long list of dreaded diseases, to give themselves beauty and strength’.16 Surprisingly, this statement could be reiterated unchanged in the first half of the 21st century.

In the intervening years, the consumption of supplements in the USA by the general population has grown enormously. Surveys conducted by the National Health and Nutrition Examination Surveys (NHANES) in the USA show that supplement usage among the older age groups increased from 30% in the 1972–1974 survey to 63% in the 1999–2000 survey. Adult usage (all age groups) in 2000 was 52%. The 2000 survey also shows that although a very wide variety of supplement compositions containing a multiplicity of components were available on the market, by far the most commonly used supplement (35% of adults) was a multivitamin, with or without minerals. The next most popular supplements were those supplying vitamin C, vitamin E and calcium. Each of these were consumed by only 10–13% of adults. It was found that about 17% of supplement users took four or more different supplements a day.17–19

Surveys in other countries across the world show that the USA has the highest use of supplements per head of population of all the developed countries. This could be due to a number of reasons, including the fact that the USA has the most liberal legislative regime and a large degree of market freedom with less control on product composition, distribution and sales outlets. In addition, consumers in the USA appear to be more enthusiastic in their embrace of emerging science, innovation and topical health issues.
By contrast, in many areas of the world supplements are still regulated as quasi-drugs or have strict controls on composition. For example, in the European Union (EU) there are strict controls on the sources of micronutrients and many ingredients, including a number of herbs and other substances which can be added freely to supplements in the USA, are regarded in the EU as medicines.

Supplement usage in developed countries falls into two distinct categories. These can be classified as ‘nutritional insurance’ and ‘therapeutic’. Nutritional insurance is the primary reason for the consumption of the majority of supplements in most countries. They are usually taken on the precept that, for various reasons, the diet is inadequate in that it does not provide the desired quantities of micronutrients or substances with a physiological effect such as the botanical sourced antioxidants. For most of the products falling into this category, the quantities of micronutrients consumed from supplements on a daily basis normally have a relationship to the national recommended daily intakes.

The idea of supplements being used for therapeutic or quasi-therapeutic purposes is one which is difficult for legislators to accept, particularly as many, if not most, regulatory regimes have a prohibition on statements attributing prevention, treatment or cure to food products. This is particularly relevant where supplements are regulated under food law, as in the EU.

Until the early 1980s most supplements contained vitamins and minerals in various combinations. Following the adoption of the Dietary Supplement and Health Education Act (DSHEA) in the USA in 1994, the American market saw an upsurge in products containing herbs. This was due to the inclusion of ‘a herb or other botanical’ in the definition of a dietary supplement ingredient in the Act.

The definition of a supplement and supplement ingredients in DSHEA made no distinction between medicinal and non-medicinal herbs and, as a result, the majority of the herbal supplements on the American market from the late 1990s onwards were regarded as medicinal products in most other countries.

Food supplements have had a chequered history and have been treated with suspicion by a number of national authorities across the world, particularly in relation to actual and perceived safety issues. There has been a major antipathy when considering the use of supplements in nutritional support programmes, even though in some cases the evidence is strongly in favour of micronutrient delivery from supplements.

An area where supplements could possibly have an effective role in developed countries is the nutrition of the elderly. In many countries, demographic changes and an increasing elderly population have already impacted on national health services. The older end of this population sub-group has been shown to be nutritionally vulnerable.20

The low cost of food supplements, the inherent safety of low-dose micronutrient products and the ease of intervention combine to give nutritional
support to the healthcare of the elderly. For example, a calcium and vitamin D supplement taken daily is relatively cheap whereas the costs to the health services of osteoporosis and bone fracture are high.

If existing prejudices can be overcome, supplementation could play a much larger part in healthcare programmes in both the developed and developing countries.

1.5 References

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Part I

Technological aspects
Food fortification and supplementation
2

Forms of food supplements

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2.1 Introduction

Food supplements are normally designed to deliver nutrients and other substances in a measured dose form, with a guaranteed amount of intake per dose. A key element of the definition of a food supplement in a European Union Food Supplements Directive is that the products are ‘… marketed in dose form … designed to be taken in measured unit quantities’ (European Directive 2002/46/EC). This definition also gives examples of forms of supplements such as capsules, pastilles, tablets, pills and other similar forms, sachets of powder, ampoules of liquids, drop dispensing bottles and other similar forms of liquids and powders. This list embraces almost all the examples of supplements in the market. The ways of delivering measured amounts of nutrients and other substances have followed the same development paths as those for oral medicines where there is the essential requirement for the accurate delivery within narrow ranges of medicinal substances to a patient.

Early medicines relied on the use of powders and liquids which were hard for the individual to measure with any accuracy. Liquids usually contained a high proportion of water and were invariably unstable. In the middle of the 19th century the first compressed tablets were developed as a means of delivering medicines in a form in which they could be guaranteed to be correct and in the right proportions. Over the past one and a half centuries there have been major advances in medicine delivery and most have been replicated in food supplements.
2.2 Tablets

Compressed tablets have been on the market for over 150 years. The first tablet compression machines are believed to have developed from experiments made by an English academic to compress graphite for use in lead pencils. The compression of the powder into tablets was seen as an improvement on the methods of pill making where the active ingredients and suitable carriers and binders were formed into a firm paste and cut or rolled into pills of a given weight to ensure the correct delivery to the patient. Most of the pills were hand-made in small quantities.

Developments in tablet compression allowed tablets to be made on a commercial scale with the result that today there are rotary presses containing 75 stations and producing from 114,000 to around 801,000 tablets per hour. The key criteria that a powder needs to fulfil for use in tablet making are:

- It must be free flowing to feed the punches and dies.
- It must have binding properties to retain the compressed form.
- It must not stick to the punches or dies.

As only a few substances possess all three of these qualities, the powders normally need to be processed to make them fit the criteria. There have been two main ways of accomplishing this. The first is to make a compressible granulation out of the mix or the majority of the mix and the second is to use pre-granulated major components that represent the bulk of the tablet.

The granulation of the whole mix normally requires a wet process whereby the powder containing the active ingredients, carriers and excipients is mixed to ensure homogeneity. It is then ‘wetted’ with a solution, which can contain binding substances, and mixed to give moist lumps of agglomerated granules. These lumps are forced through a mesh to give a more consistent size and the resulting granules dried. If the process is applied correctly, the final granules should be easily compressible with each granule containing the required proportions of the ingredients.

A similar process can be applied to the production of compressible carriers or major ingredients such as dicalcium phosphate or ascorbic acid. The pre-granulation of ingredients has a number of advantages as it facilitates direct compression of the mixed powder and eliminates the need for the wet granulation of each batch. Direct compression using pre-granulated ingredients has become the preferred method for the production of food supplement tablets.

2.2.1 Tablet coatings

Tablets need to be coated for a number of reasons, the main ones being:

- Organoleptic, to cover unpleasant taste from some active ingredients.
- To prevent or reduce deterioration of active ingredients such as vitamins and act as a barrier to oxygen and moisture.
- To cover unavoidable mottle effects on a tablet due to different coloured ingredients.
- To colour the tablets to provide differentiation between tablets with different active ingredients but with the same size and weight.

Up until the late 1970s the main coating was of sugar. To achieve the coating, the tablets are tumbled in a coating pan (similar to a concrete mixer) and a sugar syrup applied. The process is continued until a smooth, even coat develops. The tablets can be coloured by adding food colours to the syrup. Once the desired thickness of sugar coating has been achieved the tablets are transferred to a wax-lined polishing pan, normally containing beeswax or beeswax and carnauba wax. The polishing provides both a shiny surface and a final barrier on the tablet. For very hygroscopic tablets, a gelatin sub-coating can be applied to form a barrier between the tablet and the coating (Little and Mitchell, 1968).

Sugar coating predominated until the late 1970s when film coating became commercially available. Film coating is the addition of a thin coat of a film-forming substance to the surface of the tablet. The process is normally carried out in a rotating cylinder with an offset feeder bar containing spray nozzles. The nozzles project a fine spray of the film coating mix onto a bed of tumbling tablets. As with the sugar coating, colours can be added to the film coating mix. The main film forming substances tend to be the cellulose ethers, particularly hydroxypropyl methyl cellulose (HPMC).

### 2.2.2 Types of tablets

Through the second half of the 20th century, tablets evolved from simple sugar-coated swallowable pills into a plethora of forms and functions. Improvements in both compression and ingredient technology allowed the development of chewable, effervescent and laminated tablets, all utilising different techniques. Figure 2.1 gives an indication of the different forms of tablets used for food supplements.

![Forms of tablets](image-url)

**Fig. 2.1** Forms of tablets used for food supplements.
tablets now used for supplements. The bulk of the supplement tablet market is in the form of film-coated, swallowable tablets made using directly compressible major ingredients. The use of sugar-coating has reduced, mainly due to the higher labour costs and processing times when compared to film coating.

**Swallowable tablets**

The main criteria for a swallowable tablet are that the size, shape and weight are consumer friendly. This means that the design of the product must be such that it can be easily swallowed by all intended consumers. Many multivitamin and multimineral tablets have to be swallowed whole due to astringent and metallic tastes contributed by a number of the active components. For some tablets, breaking or crushing before swallowing is not an option which is acceptable to many consumers.

When a tablet exceeds about 1.5 g in weight, the selection of the right shape becomes an important consideration and may mean a move from round to ovoid tablets. Tablet punch design now allows for a variety of shapes to be manufactured at high speed. The maximum practical weight for a swallowable tablet is under 2 g, with the actual weight being governed by the bulk density and compression characteristics of the mixed powder.

When the product specification requires a large tablet due either to the number of active ingredients or the quantity of ingredients, care needs to be taken with the formulation and compromises often have to be made on the original brief. This is particularly the case with the addition of nutrient minerals where the choice of source is often dictated by the weight/volume restrictions of the tablet. For example, the highest proportion of calcium in a source is in calcium carbonate where it constitutes 38–39% of the mass. To meet a label claim of 500 mg calcium per tablet, 1300 mg of the carbonate is required. The organic salts of calcium have a very much lower calcium content, with that of calcium gluconate being only 8% of the mass. A 500 mg claim using the gluconate requires 6250 mg (6.25 g) of the salt. Using a direct compression version of calcium carbonate, a 500 mg calcium tablet is feasible, but five similar size tablets would be required to deliver the same amount from a gluconate.

A tablet formulator requires a good knowledge of the ingredients together with the skill of assessing the appropriate quantities of the non-active components. All tablets require a minimum proportion of compressible material (granulate) together with a number of excipients. The excipients, or additives, are required to ensure that the powder feed to the punches and dies is free-flowing and does not cake or bridge across the punch dies. This is normally achieved by anti-caking agents such as silicon dioxide or one of the silicates. Another additive, normally a stearate, is required to ensure clean ejection of the tablets from the dies and to stop powder sticking to the punches and dies. Although these excipients represent a small proportion of the tablet, they have to be taken into consideration when calculating tablet weights and sizes.
Chewable tablets

Chewable tablets have become popular for the delivery of nutrients required in relatively large quantities (i.e. hundreds of mg) and which have tastes which are benign or pleasant. Examples of such nutrients are calcium or ascorbic acid (vitamin C).

With chewable tablets, the basic principles of formulation apply and the key to a successful product is the correct selection of the non-active carriers and excipients. The aim with a chewable product is to obtain the best organoleptic properties, particularly taste, texture. When chewed by the consumer, it should impart a pleasant taste, with no residual metallic or bitter after-taste. The texture should be smooth and not gritty and the hardness of the tablet has to be carefully judged to ensure the integrity of the tablet during production and transportation but also to make it soft enough to chew without the risk of damaging teeth.

One of the advantages of a chewable tablet is that the product is not so contained by the size and shape limitations that apply to swallowable tablets. This means that it can contain higher quantities of carriers with a sweetening effect, such as dextrose or one of the polyols. Xylitol also has an interesting mouth-cooling effect and, in combination with other bulk sweeteners, can give an unusual and very acceptable taste. It is sometimes necessary to add small quantities of artificial sweeteners. If artificial sweeteners are used, it is essential that those selected and their levels are researched with a representative spectrum of consumers. It is also essential that chewable tablets are subjected to long-term stability trials to ensure that the formulation does not harden during storage (a common problem) and that there are no interactions between the components which can have an adverse effect on the taste.

Effervescent tablets

Effervescent tablets are a very specialised sector of the tablet industry. These are tablets designed to effervesce and dissolve in a liquid, normally water, to give a micronutrient-rich drink with a short-term carbonation effect. The effervescence is produced by the reaction between a bicarbonate and a food acid, such as citric acid, in the presence of water. The necessity for these two chemicals to be present in the tablet in an uncoated form so that they can rapidly react in the water requires very stringent humidity control throughout the operation, from the storage of the raw materials to the packed product. Any ingress of moist air during the processing can have a deleterious effect on the product.

As the tablet is dissolved in water there are a number of challenges for the formulator. The quantity of the acid and base must be sufficient to provide the desired effect and taste in the suggested quantity of water, normally 200 ml or 250 ml. Colour and sweetener levels need to be adjusted to the product as consumed (i.e. drink). Cold-water-soluble forms of the oil-soluble vitamins and carotenoids are required to avoid an oily scum on the surface of the drink.
Sustained release tablets
Developed on the theory that some micronutrients are more effective if delivered and absorbed over a period of time rather than all at once, tablets have been designed to provide a sustained or slow release of the micronutrients. The controlled release has normally been achieved by coating the particles of the nutrient with various thicknesses of a soluble coating. The key to a successful product is the ability to selectively coat the nutrient to a range of coating thicknesses and to blend them to achieve the desired release pattern. The coatings normally consist of mixtures of food grade additives such as gums and waxes and many of these combinations are patented.

Enteric coated tablets
Enteric coatings are used to delay the release of the active ingredient until an advanced stage of the digestive process. They are most commonly used in supplements for active ingredients with a strong flavour or odour, such as garlic. Enteric coatings can be applied only to swallowable tablets and are normally achieved by the application of an undercoat or membrane to the tablet. Whilst a number of polymers have been developed for the enteric coating of tablets delivering drugs, there are very few options for supplements sold under food law, the one normally used being shellac.

Multi-layer tablets
While multi-layer tablet technology has been available in a limited way for many years, a recent mass market exploitation of this technology has been in detergent tablets. In nutritional products, one international company in particular has a range of multi-layer tablets containing vitamins, minerals, pre- and probiotics and phytonutrients.

There are two principal benefits with this dose form; consumer appeal and a technological advantage. For the consumer, the use of distinct layers (particularly if these are of different colours) differentiates the product from others. It also provides strong visual clues that the product is composed of different groups of ingredients (e.g. vitamins and minerals) rather than a ubiquitous multi-vitamin, multi-mineral mixture. Also the use of two, or even three, layers can help impart a visual attraction to what would otherwise be an ordinary tablet. Novel functions can be introduced, aimed at the younger consumer, for example, when an effervescent tablet can be made from two different coloured layers that ‘react’ when the tablet is added to a glass of water, giving a further colour as well as effervescence.

Technologically, multiple layers may be used to keep incompatible ingredients apart until they are digested by the consumer, although there will be some contact at the interface of the layers. Each layer may also be formulated to offer different release characteristics, which would not be possible in a conventional monolayer tablet.

The above benefits do come at a cost. In addition to requiring more sophisticated tablet presses with multiple feeds and potentially slower operating
speeds, different powder/granule mixes have to be prepared for each layer. Care has to be taken with certain ingredients as those in the first tablet layer may undergo compression up to three times during the tabletting phase. The finished tablet may have an increased risk of delaminating and, during the coating operation, discolouration can occur if material is abraded from one layer and deposited on another. If the tablets are to be blister packed, consideration has to be given to the acceptability of different layers being visible to the consumer (Burrows and Sidani, 2007).

2.3 Capsules

Capsules are a convenient method of supplying supplements in a powder, oil, oil-based suspension or paste form. There are two types in common use: hard capsules, which consist of two shells that slot together to form a tight seal and are normally used to hold free-flowing powders or granules and occasionally oil-based suspensions; and soft capsules, hermetically sealed, which tend to be used to hold ingredients in the form of an oil, oily suspension or paste (Council for Responsible Nutrition, 2002).

The first gelatin capsules were produced for medicinal use as far back as the early 1830s (Leino, 2004), and consisted of single-piece capsules that were produced in individual iron moulds, filled by hand and sealed with a drop of gelatin solution. By 1835, gelatin capsules were being produced in many different countries, including France, Germany and the USA. The first two-piece gelatin capsules were patented in the late 1840s (Honkanen, 2004; Stegemann, 2002), the process involving dipping silver-coated metal pins into a gelatin solution and then drying them. One shell would then be filled with a weighed powder dose, via a small funnel, and then the second shell would be slotted on to seal in the medication.

Figure 2.2 displays the forms of capsules which are used in the supplement industry today.

2.3.1 Hard capsules

Hard, two piece capsules are traditionally made from gelatin, but vegetarian forms, generally produced from starch and cellulose mixtures, are now available. The capsules consist of two prefabricated, cylindrical shells, each with one rounded, sealed end and one open end. One of the shells is of a smaller diameter to the other allowing them, once one shell has been filled with the active ingredients, to be slotted together by pressing the open end of the smaller shell (the body) into the open end of the larger shell (the cap) to form a tight fit. Hard capsules can be manufactured with a self-locking, tamper evident closure system that prevents easy opening of the shells; they can be produced in a range of sizes and colours and can be printed on. The hard capsule shell masks the organoleptic properties of the active ingredients,
Fig. 2.2 Forms of capsules used for food supplements.
whilst the capsules themselves are easily swallowable due to their slim shape and, once swallowed, disintegrate within minutes, releasing the active fill.

Due to the technical difficulties in manufacturing separate sections which fitted together, there was little work on the two-piece form of capsule from the time of the first patent in the 19th century until the early part of the 20th century. It was in 1931 that a machine was designed which manufactured both capsule shells at the same time and fitted them together to form a hard gelatin capsule (Stegemann, 2002). Modern production of hard, two piece gelatin capsules is still based on the process first patented in the late 1840s and the machine built in 1931. Highly polished stainless steel mould pins are lowered into the gelatin solution, which is maintained at a constant temperature. The dipping, dwelling and withdrawal of the pins is timed so as to pick up just enough of the gelatin according to the size of the capsule being manufactured. The mould pins are raised and rotated, to promote an even distribution of gelatin, and the capsule halves are dried by having temperature- and humidity-controlled air blown over them. The capsule cap and body sections are removed from the pins, trimmed and then joined together for storage and shipment. Modern capsule machines can produce as many as 81 000 hard gelatin capsules per hour.

Hard capsules are generally filled with powders or granules, though active ingredients in the form of specific oil-based suspensions can also be used. Filling of the capsules is achieved using filling machines ranging in size from small hand operated units to high performance rotary machines with filling speeds in excess of 170 000 capsules per hour. For optimum filling performance, the powder or granule fill needs to be free flowing, so flow aids such as magnesium stearate and silicon dioxide may need to be added. The weight of fill in a capsule is directly related to the bulk density of the powder or granules: the greater the density, the smaller the capsule size required.

In a typical automated filling machine, the empty capsules are loaded into a capsule hopper where they are fed into the magazine. From here they pass into the sorting block where the capsules are correctly oriented with the smaller shell, the body, on the bottom. The capsules are separated by a vacuum and the upper portion is examined to ensure the capsule is separated. If any problems are detected, the faulty capsule is removed from the system. The capsule body is filled with the active ingredients, the two capsule halves are rejoined and the capsule is ejected into a collection container, whilst the filling station is cleaned with an air blast or by suction.

During capsule filling, the temperature and humidity should be maintained in the range 20–25 °C and 45–55%, respectively, in order to keep the moisture content of the shells within the required range of between 13–16%, as the capsules’ moisture content can vary according to the temperature and humidity to which they are exposed. Large variations in temperature or humidity can have an effect on the shape and hardness of the capsule shells. Similarly, the
filled capsules must be properly packaged and stored so that they are protected from large variations in the ambient relative humidity.

### 2.3.2 Soft capsules

Up until the end of the 20th century, soft capsules were known as ‘soft gelatin capsules’ or ‘softgels’, as the outer shell was made using a gelatin base. However, since the early part of the 21st century they have tended to be referred to simply as ‘soft capsules’ as the shell can now be produced from either a gelatin or a vegetarian (e.g. cellulose or carrageenan) base.

Soft capsules can contain active ingredients in oil, oily-suspension or paste form and thus are ideally suited for vitamins and other actives that naturally occur as oils. The capsules can be produced in a range of sizes, in a round, oval or oblong shape, and in various single colours or two-tone combinations; they can be opaque or transparent and can be printed on. As they are hermetically sealed, soft capsules protect ingredients that may be subject to oxidation, whilst protection for photosensitive products can be provided by using an opaque capsule. As with hard capsules, other benefits of the soft capsules include the fact that the capsule shell masks the organoleptic properties of the active ingredients, is easily swallowable and, once swallowed, usually disintegrates within 3 to 5 minutes, thus rapidly releasing the active fill.

The manufacture of soft capsules continued on a small scale from the first patented methods of the 1830s to processes that used sets of plates with pockets to form the capsules. However, in 1933 a rotary die was developed that produced the capsules, filled them by a process of injection moulding and thus enabled the production of uniform capsules with a consistent dosage. Commercial production of soft capsules for supplement use began in the late 1930s for the marketing of cod liver oil and became widely established from around the 1970s.

Modern methods of soft capsule production are based on the original rotary die process. To make the shell mixture, the gelatin or vegetarian base is combined with a plasticiser, or mix of plasticisers, and water, and may have added preservatives, colouring and opacifying agents. This mixture is pumped through two heated tubes into the encapsulating machine, where it is spread onto dual rollers to form two thin films that are then passed between rotating dies containing small pockets in the required shape and size of the capsules (which is dependent upon the quantity of fill required and customer preference). At the same time the fill mixture (containing the active ingredients) is pumped into the die cavity roller and a calibrated amount of fill is injected into the capsule shell as it is formed and sealed. A rotary die machine can produce 10,000 to 100,000 soft capsules per hour, depending on their size. The completed capsules fall onto a conveyor that moves them straight into a tumble dryer, in the case of the gelatin shell, or through a blow drier en route to the tumble dryer in the case of the vegetarian shell. After tumble
drying, the soft capsules are placed on special trays for drying in the drying room, during which time moisture from inside the capsule is drawn to the surface and evaporated. Temperature and humidity must be accurately controlled in order to ensure the appropriate quality of the soft capsules. If the capsule shell is to contain printed text, this can be applied when the shell mixture is spread on the rollers, i.e. prior to the capsule formation, or after manufacture once the capsule has dried (Egberts, 2002; Reich, 2004).

Although soft capsules are ideal for active ingredients that naturally occur as oils, or are oil-soluble, in the 1970s and 1980s the desire grew to use soft capsules to market non-oil soluble vitamin and mineral actives. In this situation a suspending agent such as beeswax or silicon dioxide must be used to create a fill mixture of the correct viscosity and to ensure homogeneity of dosage. The suspending agent that is used will depend upon the specific formulation that is being developed. Potential incompatibilities with the shell mixture have to be considered; for example, gelatin can be cross-linked by certain substances such as aldehydes in herbs, thus increasing the disintegration time of the capsule, whilst too high a moisture content of the fill ingredients can corrode the capsule shell. Consideration must also be given to the ratio for the oil and solids content of the fill, as there is a maximum ratio that must not be exceeded.

2.3.3 Chewable capsules
Chewable capsules are a form of soft capsule, in that the manufacturing process is very similar but the ‘chewability’ is conveyed by the addition of various sugars and other additives to the capsule shell mixture. Because of the commercial implications, the precise combinations of sugars and other additives used to produce the chewable attribute tend to be proprietary information; however, effective additives for producing chewable capsules are hydrogenated oligosaccharides such as maltitol in combination with glycerol, as they improve the taste, chewability and disintegration of the shell upon chewing, hence improving the mouthfeel. Chewable capsules may contain active ingredients in an oil, oily suspension or paste form, whilst some (inexpensive) active ingredients may be included within the shell mixture.

The majority of chewable capsules are currently manufactured using a gelatin base for the shell. However, production using capsules based on vegetarian materials such as hydroxypropyl methyl cellulose (HPMC) is being investigated.

2.4 Liquid supplements
Although liquid supplements have been available for a very long time and are still on the market, particularly as products for young children, there are
a number of formulation problems which can limit the active content of the products. Liquid products can have a syrup or aqueous base, be an emulsion, or be in the form of an oil.

2.4.1 Syrup and other aqueous-based liquid supplements
Syurops have been popular for many decades, mainly as vitamin products for children. Syrups and the other aqueous-based supplements have two major limitations: the possibility of microbiological growth, both during storage and after opening, and the poor stability of some of the vitamins. Preservation of the products can be achieved by reduction of the water activity (a_w) to a level below which most microorganisms cannot grow. It can also be achieved by chemical preservation using permitted preservatives. In many cases, the preservation tends to be a combination of both methods.

In syrups with a high sugar content and an a_w of less than 0.80, it is possible to achieve a product with a low risk of microbiological growth. In aqueous products with an a_w greater than 0.80, chemical preservation is essential, particularly if the product is not expected to be completely consumed within a few days of opening. In most countries there is strict legislation controlling both the substances that can be used as chemical preservatives and their maximum levels in the product.

Vitamin stability during the storage of the product, both before and after opening, has to be given serious consideration. For example, vitamin C is very susceptible to rapid degradation in an aqueous medium, especially if oxygen or certain metallic ions are present. To reduce the risks, the water used in the manufacture of the product may have to be both deaerated and deionised (Institute of Food Science and Technology, 1997). The thiamin (vitamin B_1) molecule is cleaved by sulphite and bisulphite and this reaction can be very rapid, particularly at a high pH. As the sulphites/bisulphites are commonly present in the sugar syrups and fruit juices used in the formulation of liquid supplements, extra care must be taken in the selection of ingredients if the product is required to contain vitamin B_1 (Berry Ottaway, 1993). Similar problems can be found with some of the other vitamins and there is also the risk of known vitamin/vitamin interactions.

It is essential that new formulations for aqueous-based supplements are subjected to rigorous stability trials which assess both the microbiological and chemical stability of the product.

2.4.2 Emulsions
Emulsions, although less common than syrups or oils, are a means of delivering a mixture of oil-soluble and water-soluble ingredients. In addition to the formulation issues discussed for aqueous-based supplements, there is a complex technology associated with the achievement of shelf-stable emulsions.
2.4.3 Oil-based supplements
A well-established oil supplement, particularly in the British market, is cod liver oil. This was a popular supplement for most of the 20th century. With the increase in public awareness of omega-3 fatty acids, blends of cod liver oil and other fish oils have become available. Unlike the encapsulated fish oils, the liquid fish oil supplements cannot contain non-oil-soluble components. However, it has been common practice to restore the levels of vitamins A and D in cod liver oil by the addition of these vitamins after the refining of the oil.

There are a few products on the market based on vegetable oils and, more recently, oils derived from fungal and algal sources. These are normally marketed for their specific fatty acid content.

2.5 Supplements in powder form
Most powdered supplements are nutrient concentrates intended to have water, milk or fruit juice added to make up a drink. Many products include small scoops or measuring devices to achieve an accurate dose.

Although powdered supplements would appear to be the simplest supplement products in technological terms, there are a number of potential problems that need to be addressed. Accurate mixing of the powders to achieve a homogeneous mix is essential as they normally contain very low levels of micronutrients. What is often not appreciated is that powders can de-mix during subsequent handling and packaging after the mixing stage. Unlike tablets, supplement powders have a very large number of interstitial spaces with trapped air. This can lead to stability problems, particularly if the product is not manufactured in a de-humidified atmosphere. Vitamin C is particularly susceptible to moist air due to the oxygen content of the air. Vitamin C degradation is such that 11.2 mg of vitamin C (ascorbic acid) is destroyed by 1.0 mg oxygen. Hence, spaces in the product containers should be minimised to reduce the amount of air. Nitrogen flushing of the containers can remove most of the air and improve stability but is also expensive.

2.6 References
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3

Vitamin and mineral fortification of foods

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3.1 Introduction

Micronutrient deficiencies are common worldwide and many individuals, especially women and children, suffer from the serious and widespread negative health consequences. Decreases in learning capacity and work productivity may severely lower income for the individual, family and country. Over the last years, micronutrient deficiencies have become increasingly recognised as serious public health problems by governments, industry and non-governmental decision makers.

Food fortification is recognised as the most cost-effective long-term strategy for prevention of micronutrient deficiencies, and national programmes have been introduced to fortify widely consumed staple foods such as cereal flour, salt, sugar and soy sauce. Such mass fortification programmes are usually mandated by governments and are most appropriate for developing countries. Although they also exist in some industrialised countries, targeted food fortification is also common. Target products are usually manufactured foods consumed by population groups most at-risk of micronutrient deficiencies. Examples would be infant formulas and complementary foods, but also breakfast cereals and chocolate drinks predominantly consumed by children.

In addition, in the industrialised countries, ‘functional foods’ have been fortified with specific micronutrients to prevent diseases such as osteoporosis, cancer and heart disease. Other fortified products include foods for pregnant and lactating women, and enteral and parenteral feeds for hospital patients.

While fortification of foods is relatively easy for some micronutrients (e.g. iodine), it is not so easy for others (e.g. iron). Certain knowledge is necessary to prevent unacceptable changes in colour, flavour or texture of
the food product caused by some mineral compounds, and to ensure adequate absorption, utilisation and health benefit to the consumer. This chapter reviews the need for mineral and vitamin fortification of foods. Common micronutrient deficiencies, including those of iron, vitamin A, iodine, zinc, selenium, calcium and folate, are summarised, focusing on the prevalence, the risk factors and the negative health consequences of deficiencies.

3.2 The need for fortification

Mineral and vitamin deficiencies are a serious public health problem in many developing countries and often occur in industrialised countries. Common mineral and trace element deficiencies include iron, zinc, selenium, iodine and calcium. The most important vitamin deficiencies today are probably vitamin A, vitamin D and folic acid, although niacin deficiency in maize-eating populations, thiamine deficiency in rice-eating populations, and scurvy caused by lack of fresh fruits and vegetables, have claimed many lives in the past and still appear occasionally in refugee camps. Various strategies have been developed to prevent micronutrient deficiencies. The main approaches are daily or periodic supplementation with pharmacological doses of micronutrients, or fortification of staple foods or condiments. A third approach is dietary diversification, although this is only possible if economy and availability permit. In the future, we can perhaps expect a range of biofortified foods but these will still take many years to develop and be tested for safety, especially if they are genetically engineered.

Food fortification is considered to be the most cost-effective, long-term, population-based strategy to combat micronutrient malnutrition. During the fortification process, micronutrients are added to the food to give concentrations which are higher than those originally present in the native food. Food fortification programmes organised at the national level are most often the mass fortification of staple foods or condiments. Such fortified foods reach all population groups. Alternatively, a targeted fortification programme can be developed. In such a programme, a foodstuff commonly consumed mainly by the population at risk of deficiency is fortified. Mass fortification programmes are usually organised and mandated by governments, whereas the targeted programmes can be mandatory or voluntary and could include market-driven fortification of manufactured foods.1

Around one third of the world’s population suffers from micronutrient malnutrition. Two billion people worldwide are reported to be iron deficient, 1.9 billion are at risk of iodine deficiency and 250 million school-children are at risk of blindness due to vitamin A deficiency (Table 3.1).1

The major risk factors causing micronutrient deficiencies are low dietary intake, impaired absorption or utilisation, and increased requirements during infancy, adolescence and pregnancy. Iron, zinc and vitamin A deficiencies often occur in populations consuming mainly cereal- and legume-based diets,
with low amounts of meat, fruits and vegetables. Such diets are commonly consumed by the lower socio-economic population groups in Africa, South Asia and Latin America. They are high in phytic acid, a potent inhibitor of iron and zinc absorption, and low in preformed retinol and pro-vitamin A carotenoids. Low selenium and iodine intakes are due to consumption of plant and animal foods grown on low selenium and iodine soils, these having lower selenium and iodine concentrations.

Even a theoretically adequate amount of micronutrients in the diet cannot guarantee meeting the requirements. This is because other dietary and physiological factors may negatively affect micronutrients absorption and/or utilisation. Severe illnesses such as protein energy malnutrition, infectious diseases and deficiencies of other micronutrients can affect micronutrient absorption and metabolism. Multiple micronutrient deficiencies occurring in the same individual are now recognised as of particular importance and multi-micronutrient fortification of foods as opposed to single fortification may be necessary for optimising impact.

The health of many individuals, particularly women and children in developing countries, can be improved by food fortification. Industrial food products should be designed to provide a fixed amount of the dietary reference value for the nutrient. Mass fortification programmes, however, should base the fortification level on the nutrient requirements of the at-risk population, the current intake of the nutrient, the amount of micronutrient lacking in the diet and the consumption of the food vehicle. This has been described in detail in the new WHO guidelines.1

### Table 3.1 Global prevalence of anaemia (iron deficiency), and iodine and vitamin A deficiency

<table>
<thead>
<tr>
<th>WHO region</th>
<th>Anaemia (iron deficiency) (Million)</th>
<th>Iodine deficiency (Million)</th>
<th>Vitamin A deficiency (Million)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Africa</td>
<td>244</td>
<td>260</td>
<td>53</td>
</tr>
<tr>
<td>Americas</td>
<td>141</td>
<td>75</td>
<td>16</td>
</tr>
<tr>
<td>E. Mediterranean</td>
<td>184</td>
<td>229</td>
<td>16</td>
</tr>
<tr>
<td>Europe</td>
<td>84</td>
<td>436</td>
<td>57</td>
</tr>
<tr>
<td>South East Asia</td>
<td>779</td>
<td>624</td>
<td>127</td>
</tr>
<tr>
<td>Western Pacific</td>
<td>598</td>
<td>365</td>
<td>42</td>
</tr>
<tr>
<td>Total</td>
<td>2030</td>
<td>1989</td>
<td>254</td>
</tr>
</tbody>
</table>

Note: WHO has no prevalence data for iron deficiency. In developing countries it can be estimated that 50% anaemia is due to iron deficiency. In industrialised countries virtually all the anaemia is due to iron deficiency.

### 3.3 Iron deficiency

Iron deficiency (ID) is the most common and widespread nutritional disorder in the world, and is widespread in both industrialised and developing countries. The most recent WHO report suggests that about 2 billion people in the world are anaemic, of which 1 billion are affected by iron deficiency anaemia.
Food fortification and supplementation

(IDA). A further 1 billion are thought to suffer from ID without anaemia. IDA, the end result of ID, is caused by a long-term negative iron balance, which is usually due to low bioavailability of dietary iron from cereal and legume based diets, little or no meat consumption resulting in low intake of the more highly absorbable haem iron, or increased iron losses due to helminth infections. The increased needs of infants, adolescents and pregnant women, and the high menstrual blood losses of some young women, can also lead to negative iron balance.²

All iron in the human body is linked to proteins. The most abundant iron-containing protein is haemoglobin, which together with myoglobin represents 95% of the functional iron in the body. The major role of both molecules is binding oxygen, respectively either in the blood or in the muscles.³ Other important iron proteins are the haem enzymes, such as cytochromes, and the non-haem enzymes such as NADH hydrogenase, many of which are involved in energy metabolism. Other iron enzymes are involved in the immune defence.⁴ The remainder of body iron is stored as ferritin in the liver or bone marrow.

It should be stressed that anaemia can have causes other than iron deficiency, which is assumed to be responsible for approximately 50% of anaemia worldwide, although this can vary due to age and location.¹ In industrialised countries, anaemia will be predominantly due to ID. Several laboratory methods are used to measure iron status, including serum ferritin (SF), serum transferrin receptor (sTfR) and zinc portoporphyrin (ZnPP).¹

The consequences of ID are dependent on the severity of the condition. The spectrum includes fatigue, weakness, increased susceptibility to infections, diminished work capacity, increased maternal and perinatal mortality, increased prevalence of preterm and low-birth weight infants, and reduced cognitive development, as well as reduced learning ability of children.⁵–⁸

Good sources of dietary iron showing a high bioavailability, such as meat and meat products, are not available in many countries and particularly not in developing countries. There are two types of food iron: non-haem iron from plant sources and haem iron coming from the haemoglobin and myoglobin in animal products. In industrialised countries, haem iron can contribute 30–40% and more of the absorbed iron, although it represents only 10–15% of the dietary iron. While haem iron absorption is not significantly influenced by the composition of the meal or iron status, non-haem iron absorption is strongly affected by both iron status and dietary factors inhibiting or enhancing iron uptake. In particular, phytic acid from cereals and legumes and polyphenols from beverages such as tea and coffee can significantly inhibit non-haem iron absorption.⁹ When fortifying these foods, or when fortifying foods that are consumed with these foods or beverages, it may be necessary to add enhancers of iron absorption such as ascorbic acid and/or ethylenediaminetetraacetic acid (EDTA) to ensure adequate iron absorption.¹⁰¹¹

Combating iron deficiency includes being aware of potential risks and possible adverse effects of excess iron in relation to haemochromatosis,
Vitamin and mineral fortification of foods

thalassaemias, prooxidant activity and increased risk of incidence and severity of infections in areas of endemic infections.

Although iron is the most difficult nutrient to add to foods in an efficacious form without causing sensory changes, much progress has been made in recent years in fortification technology and improved iron status has been demonstrated in women or children fed iron-fortified wheat flour, rice, salt, fish sauce, soy sauce, maize, milk and complementary foods.

3.4 Iodine deficiency

As a leading cause of intellectual impairment, iodine deficiency can be a serious public health problem in vulnerable population groups such as pregnant women and young children throughout the world. The WHO estimates that 36.5% of school-age children have an insufficient iodine intake and are at risk of iodine deficiency. The highest prevalence of insufficient iodine intake was found in Europe (59.9%), the lowest in the Americas (10.1%). Surprisingly, only a few countries were completely iodine sufficient before 1990 but after the extensive introduction of iodised salt, the prevalence of iodine deficiency was greatly decreased.

Iodine is an essential component of thyroid hormones, which are needed for pre- and postnatal brain development during gestation and early infancy. Inadequate intake of iodine leads to impaired thyroid hormone synthesis, hypothyroidism and goitre. This results in a series of functional and developmental abnormalities which have been called the iodine deficiency disorders (IDD). The most serious disorder is cretinism, which is due to severe iodine deficiency during pregnancy. It causes severe neurological damage in the foetus, and may include dwarfism, being mute, deaf and/or spastic. Mild to moderate deficiency can also cause mental impairment, poor school performance, reduced intellectual ability and impaired work capacity in children and adults. Major risk factors for iodine deficiencies are low iodine in soil and water, particularly in high altitude regions and river plains where iodine has been leached from the soil. Low iodine utilisation, and thus iodine deficiency, can be aggravated by lack of iron and selenium, which are both required for efficient production of thyroid hormones, and by the consumption of non-detoxified cassava and other goitrogenic substances. Urinary iodine excretion is the main indicator for assessing iodine deficiency. Other indicators include measuring thyroid size, thyroid hormone concentrations and thyroid-stimulating hormone levels.

Iodised salt is one of the best examples of successful food fortification. Iodisation of salt began in 1922 in Switzerland and today the WHO recommends universal salt iodisation, including all salt for human and livestock consumption, to control iodine deficiency disorders. Recently published data show that substantial progress has been made to eliminate IDD worldwide.
Food fortification and supplementation

study, iodine supplementation was reported to improve cognition in school-aged children, and earlier human studies describe the improved motor and cognitive performance of children born to iodine-deficient mothers after iodine administration. Other foods, such as wheat flour, sauces or water, may be alternatives to salt iodisation.

3.5 Vitamin A deficiency

Together with iron and iodine deficiency, vitamin A deficiency (VAD) represents one of the most common micronutrient deficiencies. VAD is a major public health problem in many developing countries, particularly those in Africa and South-East Asia. VAD is the leading cause of preventable blindness in children. VAD also significantly increases the risk of severe illness and death from childhood infections such as diarrhoea and measles. Each year, up to 500,000 children become blind and 50% of these children die within 12 months.

In almost every cell in the human body, there are nuclear receptors for retinoids, demonstrating the essential nature of vitamin A for many important metabolic processes. In addition to the specific need for vitamin A in rhodopsin in the visual system, retinoic acid is essential for growth and development, maintenance of epithelial cellular integrity, immune function and reproduction. Because of its importance in vision, vitamin A deficiency can be easily assessed by indicators related to changes in the eye. However, changes in vision are signs of severe vitamin A deficiency and milder forms of vitamin A deficiency are best identified by monitoring plasma or serum retinol levels.

Excess vitamin A is stored in the liver, so unlike many minerals and water-soluble vitamins, infrequent high doses can be stored for future use. Good sources of preformed retinol are milk products; however, meat itself is low in retinol content. Because of its storage function, liver is the best source of vitamin A. Some carotenoids occurring in plant tissues can be converted to retinol. This conversion depends on the form of the carotenoid. β-Carotene is suggested to have a conversion factor of 12:1, and other carotenoids 24:1, however the exact conversion depends on other various factors, including release from plant cells during digestion. Cereals and legumes are low in vitamin A and therefore vitamin A deficiency is common in populations living on a cereal- and legume-based diet with insufficient carotenoid intake from fruits and vegetables and little access to preformed retinol in meat products.

Although vitamin A supplementation during national immunisation days is the strategy recommended by WHO, for many years vitamin A has been added to margarine and the fortification of sugar and cooking oil are alternative approaches for combating vitamin A deficiency. Several countries in Africa have recently begun national programmes to fortify cooking oil with vitamin A.
3.6 Zinc deficiency

Due to the lack of reliable status indicators, zinc deficiency is difficult to identify and quantify. Plasma zinc concentration, and perhaps other methods such as hair zinc concentration, can detect severe but not moderate zinc deficiency.46–48 There is therefore no global prevalence data for zinc deficiency; however, because of low dietary zinc intake and the high prevalence of growth stunting, zinc deficiency is expected to be high in Southeast Asia, Africa and the Western Pacific, with perhaps mild to moderate deficiency in some European populations. The International Zinc Nutrition Consultative Group (IZiNCG) has estimated the risk of zinc deficiency based on the calculated amount of absorbable zinc (based on food balance sheets) and the prevalence of growth stunting (WHO database 1997).48

Zinc is necessary for the activity of over 100 specific enzymes which are involved in major metabolic pathways, including physical growth, immune competence, reproductive function and neurobehavioural development.49,50 Several factors can contribute to zinc deficiency, although low intake and low bioavailability from high phytate diets are the major reason. Increased requirements for growth and pregnancy, malabsorption, increased losses and impaired utilisation can also contribute. Zinc deficiency, like ID, is expected to be common in lower socio-economic populations consuming cereal- and legume-based diets with little meat and milk products.51–54

The negative health effects of zinc deficiency depend on age and include low weight gain, diarrhoea, anorexia and neurological disturbances in infancy. Skin changes or impairment of linear growth are more common in school-age children.55,56 The influence of zinc deficiency in pregnancy is not clear and there are contradictory findings. Recent supplementation trials have shown a reduced prenatal mortality rate57 but have shown no effect on size of infants at birth or pregnancy duration.58,59 In a recent meta-analysis,60 zinc supplementation was shown to have a beneficial effect on linear growth and weight gain in children, especially in those who were stunted or underweight at baseline. Zinc supplementation has been reported to reduce the incidence of diarrhoea infections and acute lower respiratory infections.61–64 Although zinc supplementation has also been recommended for the treatment of diarrhoea since 2004,65 no therapeutic effect has been shown for other infections such as measles56 or malaria therapy.57 A small number of studies have reported that zinc supplementation reduces mortality.57,68,69

With the exception of commercial infant foods, zinc fortification of foods is not widely practiced. There is a need to better evaluate the possible sensory problems in the food vehicle, the bioavailability of different zinc compounds in human subjects and to demonstrate the efficacy of zinc-fortified foods in improving zinc status. Recent studies with zinc-fortified foods have failed to demonstrate efficacy.70
3.7 Selenium deficiency

The global prevalence of selenium deficiency has not been mapped. Nevertheless, moderate to severe selenium deficiency has been reported in regions of China, Siberia, New Zealand, Korea, Scandinavia and Japan. The occurrence of selenium deficiencies, with serious consequences for human health, has been linked to areas where the soil selenium content is low and, as a consequence, the diet has low selenium content.

Selenium is an essential component of several major metabolic pathways, including antioxidant defence systems, thyroid hormone metabolism (selenium-containing deiodinase and selenoperoxidases) and immune function. The existing laboratory methods to evaluate selenium status include measuring serum concentrations in blood, hair and nails or measuring the glutathione peroxide activity in the red blood cells. These methods are technically difficult and not suitable for national surveys. They have un-defined cut-off levels and thus little accuracy to detect moderate to mild selenium deficiency.

Keshan disease and Kaschin–Beck disease are well known forms of selenium deficiency. Keshan disease is a juvenile cardiomyopathy or alternatively a multifocal myocarditis. It occurs primarily in children and less in women of child-bearing age. It is manifested as an acute or chronic insufficiency of cardiac function, cardiac enlargement, arrhythmias, and electrocardiographic and radiographic abnormalities. Recent findings suggest that not only selenium deficiency but also an infection with the Coxacki virus is a necessary factor for the pathogenesis. Kashin–Beck disease is an osteoarthropathy which is manifested as enlarged joints (especially the fingers, toes and knees), shortened fingers, toes and extremities and, in severe cases, dwarfism. It would seem unlikely that selenium deficiency is the main cause of Kashin–Beck disease and it is assumed that selenium deficiency is a predisposing factor for the pathogenic effect of some other agent(s).

As selenium is a component of glutathione peroxidase, an important enzyme of the antioxidant defence system, selenium deficiency, and especially low selenium intake, has been linked to increased cancer risk. Dietetic products offer good possibilities for selenium fortification with a view to improving immune defence and perhaps preventing cancer. Selenium is added to several food vehicles worldwide, including infant formula and milk products. Several studies have reported a positive impact of selenium fortification in infant foods on selenium status and fortifying salt with selenite in China led to a significant reduction of Keshan disease.

3.8 Calcium deficiency

Low intakes of calcium are common in non-milk consuming countries worldwide. However, reliable and cost-effective indicators of calcium status do not exist, so it has not been possible to determine calcium status of
populations. The only way to estimate calcium status is by comparing current
dietary calcium intakes with those that are recommended. There are, however,
different opinions on calcium requirements and the dietary reference values
for calcium recommended by different national nutrition boards vary widely.

Over 99% of the body calcium pool (approx. 1000 g) exists as hydroxyapatite
in the skeleton. It is especially important to maintain adequate calcium intake
in life periods of rapid skeletal growth and in women after menopause, to
help compensate increased bone losses. Low calcium intake does not affect
the metabolic function of calcium at the cellular level because serum calcium
levels are tightly controlled by using calcium from the bone pool. Bone
mineral density in healthy subjects increases until the age of about 20, there
is consolidation until maybe 30 and then a steady calcium loss in both men
and women through the rest of their lives. After the menopause, women have
a greatly increased bone loss and are at increased risk of osteoporosis, leading
to bone fractures. In growing children, low calcium and vitamin D intakes
can lead to a decreased bone mineralisation. If calcium is deficient
during the period of bone formation, the genetically predetermined peak
bone mass will not be reached, thus increasing the risk of osteoporosis in
later life.

Dairy products are the major food sources of calcium in industrialised
countries, where they usually account for 60–70% of the calcium intake.
Such products are not available in many developing countries. In such countries,
calcium absorption may also be decreased due to high phytate intake from
cereals or high oxalate intake from green leafy vegetables. Calcium absorption
is also strongly dependent on adequate vitamin D, and poor bone health can
be due to vitamin D deficiency, especially in Northern climates where vitamin
D production from the sun is low. There is therefore great potential for
calcium-fortified foods to increase calcium intake in Asia and Africa, and
even in industrialised countries. Dietary intake of calcium in adolescent girls
and women is often low if milk products are not consumed regularly. Calcium
and vitamin D fortified foods help young people reach their genetically
predetermined peak bone mass and could help adult and elderly people in
reducing the rate of physiological bone loss.

### 3.9 Folate deficiency

Global prevalence of folate deficiency is not available yet but it is assumed
that folate deficiency could be prevalent in countries with high intakes of
refined cereals and low intake of leafy green vegetables and fruits. Estimates
of folate intake are also imprecise since methodological problems still exist
in relation to the measurements of food folate concentrations. Good indicators
of folate status are serum folate and erythrocyte folate measurements. Serum
folate is a good indicator of recent dietary intake and erythrocyte folate is a
better measurement of long-term folate status. Although other B-vitamin
deficiencies can contribute to elevated homocysteine levels, the measurement of homocysteine is a good predictor of inadequate folate status.\textsuperscript{1}

The term ‘folate’ is used for compounds showing similar structure and function to folic acid. Folate is a B-complex vitamin playing a key role as coenzyme in one-carbon-transfer reactions facilitating the transfer of carbon-units. Cell multiplication and tissue growth depend on adequate supply of these transfer reactions. Inadequate folate, or a dysfunction in folate metabolism, has been linked to neural tube defects (NTD), megaloblastic anaemia, neurological degeneration, cancer and cardiovascular disease.\textsuperscript{88–91} Folate deficiency (and other B-vitamin deficiencies) leads to increased homocysteine in the blood. Homocysteine has been reported to be a risk factor for coronary heart diseases (CHD).\textsuperscript{92}

Folate supplementation has been shown to decrease NTD in new born babies.\textsuperscript{93,94} Folate fortification of cereals is one of the most promising approaches to combat NTD and has become mandatory in many countries including Canada and the US.\textsuperscript{95} NTDs have greatly decreased in the US and Canada since cereal fortification.\textsuperscript{96} The potential risk of masking vitamin B\textsubscript{12} deficiency and cognitive problems in the elderly by adding folate to foods has been reported. Currently, this is being closely monitored but is not considered as a major concern.

3.10 References

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4

Fortification with substances other than vitamins and minerals (polyphenols, carotenoids, fatty acids and phytosterols)

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4.1 Introduction

As the pace of life gets ever faster, health and convenience are among the key trends driving product innovation in the food and beverage sectors. With consumers continually seeking new ways to help them lead healthy, active lives, functional food ingredients offer new opportunities to capitalise on these important market trends. Consumers are increasingly well-informed and adventurous about healthy ingredients and are moving from being ‘nutritionally curious’ to ‘nutritionally active’.

There is particular interest in fortified/functional foods with innovative new ingredients – not just the ‘established’ vitamins and minerals, but secondary plant products, oils and/or amino acids which provide health benefits. The number of functional food consumers in major European markets is growing each year by around 6–7%.

Healthy ingredients are being added to foods such as biscuits and sweets, as well as ‘healthy image’ products such as dairy foods and fruit juices.

The challenge, from a technological point of view, is to develop formulations of food matrices with innovative functional food ingredients which both maintain colour, odour and taste that are acceptable to the consumer, as well as sufficient product stability. To develop successful functional food products, two different technological criteria have to be fulfilled. First, suitable active ingredients must be developed which guarantee both efficacy in support of a health claim and also the necessary properties for application in different food matrices (e.g. water-soluble functional food ingredients are preferable for the beverage industry). Second, incorporating these active ingredients into the food matrix requires knowledge and experience. The development of special technologies such as encapsulation is also required.
4.2 Polyphenols

4.2.1 Health benefits
Polyphenols, also known as flavonoids, form a huge group of 6000+ secondary plant products. Their polyphenolic nature, i.e. their hydroxyl groups, makes them very efficient antioxidants, and this is believed to be the basis for their health benefits. Flavonoids are grouped into six categories: flavonols, flavones, catechins (or flavan-3-ols), flavanones, anthocyanidins, and isoflavones (see Fig. 4.1 and Table 4.1).

There is extensive literature on the health benefits of flavonoids and the mechanisms via which these effects may be exerted. In addition to their antioxidant properties, flavonoids can modulate the activity of various key enzymes (cyclooxygenase, phospholipase A2, glutathione reductase and many more). Both these properties – antioxidant and non-antioxidant – may contribute to the vasodilatory, anticarcinogenic, anti-inflammatory and immune-modulating effects of flavonoids. The main food sources of flavonoids are fruit juices, tea, coffee, red wine, onions, apples and berries. The principal dietary flavonoids, therefore, include catechin and catechin gallates, and quercetin, kaempferol and their glycosides.

Research in nutrition and medicine has hitherto concentrated on the ‘disease-prevention’ angle (cardiovascular disease, cancer), recently, the focus has shifted to ‘wellness’ and ‘balance’-related claims for these flavonoids and extracts, which originate partly from the traditional use of plants for general well-being as well as for medicinal purposes. These additional properties of flavonoids and flavonoid-rich extracts are attracting increasing interest in the food fortification industry. Extracts typically used in foods include proanthocyanidin-rich extracts such as grape seed or pine bark, lemon balm, green tea, olive and rooibos.

Fig. 4.1 Flavonoids: General structures of the six main flavonoid categories.
Fortification with substances other than vitamins and minerals

Table 4.1 Polyphenols: Food sources of the main dietary polyphenols

<table>
<thead>
<tr>
<th>Class</th>
<th>Main flavonoid</th>
<th>Important food sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavonols</td>
<td>Quercetin</td>
<td>Apples</td>
</tr>
<tr>
<td></td>
<td>Kaempferol</td>
<td>Onions</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Red wine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tea (black)</td>
</tr>
<tr>
<td>Flavan-3-ols</td>
<td>Catechin</td>
<td>Red wine</td>
</tr>
<tr>
<td></td>
<td>Epicatechin</td>
<td>Chocolate (dark)</td>
</tr>
<tr>
<td></td>
<td>Epigallocatechin gallate</td>
<td>Tea (black)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tea (green)</td>
</tr>
<tr>
<td>Flavones</td>
<td>Apigenin</td>
<td>Celery</td>
</tr>
<tr>
<td></td>
<td>Luteolin</td>
<td>Oregano</td>
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<td></td>
<td></td>
<td>Parsley</td>
</tr>
<tr>
<td>Flavanones</td>
<td>Narigenin</td>
<td>Grapefruit juice</td>
</tr>
<tr>
<td></td>
<td>Hesperetin</td>
<td>Orange juice</td>
</tr>
<tr>
<td>Anthocyanidins</td>
<td>Malvidin</td>
<td>Red wine</td>
</tr>
<tr>
<td></td>
<td>Cyanidin</td>
<td>Blueberries</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Raspberries</td>
</tr>
<tr>
<td>Isoflavones</td>
<td>Daidzein</td>
<td>Peas</td>
</tr>
<tr>
<td></td>
<td>Genistein</td>
<td>Soy beans</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Soy milk</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tofu</td>
</tr>
</tbody>
</table>


- **Grape seed extract**, *Vitis vinifera L.* seed extract, has a high content of polyphenols, including up to 25% oligomeric proanthocyanidins (OPCs). OPCs in grape seed occur as dimers, trimers and so on, up to polymers, and this oligomerisation is unique among all polyphenols.\(^7\) Grape seed OPCs are potent antioxidants, and further lines of evidence support their vascular effects – the cardiovascular benefits of red wine have been attributed to OPCs (‘French paradox’). They are also thought to help improve and preserve the skin’s elasticity by stabilising collagen and elastin.\(^8\) Recently, grape seed extract has been associated with effects on body composition because it may limit dietary fat absorption and accumulation of fat in adipose tissue.

- **Maritime pine bark extract**, *Pinus maritima L.* bark extract, has many times been compared to grape seed extract. Both extracts contain procyanidins and therefore the same effects such as antioxidant efficacy and lipid peroxidation inhibition are being found and described.\(^9\)\(^–\)\(^12\)

- **Lemon balm extract**, *Melissa officinalis L.*, is rich in antioxidant polyphenol compounds, including phenolic acids, such as rosmarinic acid. Lemon balm has sedative effects, with possible indications including nervous disorders, stress and sleep disturbance. Studies suggest that low doses may have a beneficial effect on mood and higher doses may act as...
a mild sedative. Research results show that lemon balm may improve mood, resulting in significantly increased ‘calmness’. When cognitive performance was evaluated, positive effects on attention were noted, a benefit which seems to be the most popular reason for its use in innovative functional food products. However, attention must be paid to the regulatory perception of claims in this area in individual countries, since such claims can either be perceived to relate to drugs or to foods. Using a recent example from Germany, a product with claims relating to ‘improving cognitive performance’ is considered a food, as opposed to a product with claims such as ‘eliminating clinical symptoms’. Examples of claims, if substantiated, include: ‘improves cognitive performance’, ‘increases concentration’, ‘improves mental power’.

- **Green tea extract**, *Camellia sinensis* L. leaf extract, is rich in catechins, particularly epigallocatechin gallate (EGCG). Preliminary research suggests it may have a beneficial role in body composition by speeding up fat oxidation. It also has antioxidant and anti-inflammatory properties. All these effects are related to its catechin content. Green tea also contains significant levels of alkaloids (caffeine being the most abundant), which may be responsible for its potential stimulant effects such as increased alertness and concentration. Daily consumption of tea containing catechins may be useful in weight management as well as in the prevention and improvement of lifestyle-related diseases. Green tea and epigallocatechin gallate have been reported in several studies to reduce body weight and adipose tissue. This may be related to suppression both of lipid metabolism and of fat accumulation and body weight increase.

- **Rooibos extract**, *Aspalathus linearis* L. leaf extract, is rich in antioxidant polyphenol compounds, including flavonoids and phenolic acids, which are potent free radical scavengers and inhibitors of oxidative stress. Research *in vitro* and in animals suggests that the high level of antioxidants present in rooibos may help in promoting health, delaying ageing and combating disease.

### 4.2.2 Technological aspects

There is increasing demand in functional foods for natural active ingredients such as plant extracts, including green tea, rooibos tea, lemon balm and Aloe Vera, as well as many more. Purified EGCG is now also available. However, not every plant extract complies with the regulatory and application requirements governing their use in foods. Extracts must have food status, which differs even between individual European countries. Food status depends on the traditional food use of the plant, the presentation of the consumer product, and its physiological or pharmacological function, as well as on the manufacturing process of the plant extract.

Plant extracts are concentrated preparations, usually obtained from dried vegetable plant materials. Extraction has to be performed with a food grade...
Fortification with substances other than vitamins and minerals

solvent that is allowed for use in the respective category of foods. If the traditional food use of the chosen plant is an infusion, an aqueous extract should be selected in order to guarantee that the final composition of the extract is similar to the traditionally-consumed infusion. Solvents other than water may change the composition of an extract to an extent that the resulting extract will be considered a Novel Food according to EU Regulation (EC) No. 258/97. Plants and plant extracts contain hundreds of different compounds which work synergistically as a whole. Sometimes one or more active ingredient or marker substance is described, but the complete composition is never fully known.

A number of contaminants are regulated in foods, such as heavy metals, mycotoxins, polycyclic aromatic hydrocarbons (PAH), dioxins and polychlorinated biphenyls (PCB), but also secondary plant constituents such as cumarine, aloin or pulegone. Depending on the type of food, these contaminants must be carefully monitored. The target is to define a quality for a plant extract and guarantee reproducibility of that quality from batch to batch. To ensure consistent high quality, it is necessary to have particular guidelines in place for the selection of the raw material, a validated production process to guarantee the right content of the active component, and appropriate analytical control parameters. High performance liquid chromatography (HPLC) fingerprints should be available both for the extract, to allow batch-to-batch quality comparison, and also for the raw material, to demonstrate that the natural composition of the plant was not modified by the extraction process. In addition, properties such as pH-stability and solubility are important in selecting the right plant extracts. The extracts should be easy to formulate in the desired food matrix in order to avoid colour shifts or precipitations which may decrease consumer acceptance and thus the success of the final market product.

Depending on the type of extract, its colour and taste may have an effect on the fortified food – it may introduce a degree of brown colouring or a bitter and astringent flavour. Plant extracts for use in fortified food should therefore be checked for their sensorial impact. An example is green tea extract which has a strong bitter taste. As illustrated in Fig. 4.2, 600 ppm of a green tea extract (≥120 ppm EGCG) was the upper limit for addition to various beverages. However, the addition of sugar or flavours, as well as the process used (homogenisation, pressure) may mask some of the bitter and astringent taste of the extract, enabling higher amounts to be added. Furthermore, the colour of the fortified food may also change as a result of adding plant extracts. For example, grape seed extracts are difficult to incorporate into functional foods due to their dark brown-red colour and low water-solubility. Using green tea extract with ≥20% EGCG added to water for example, the colour remained a very pale amber up to 0.05% of extract added, but became darker with higher concentrations. Addition of 0.1–0.2% resulted in whisky-like colour, and further increases to 1.0% resulted in an unattractive brown solution. Further, the colour of food products containing
Food fortification and supplementation

Grape seed extract may darken during storage due to oxidation. Development of encapsulation technologies is required.

High concentrations of green tea extract (≥ 0.5%, ≥ 20% EGCG, i.e. 1000 ppm) may result in sedimentation of the extract in water. The addition of some hydrocolloids, such as xanthan or pectin (depending on pH of the solution) may result in a better distribution of the extract in the fortified food.

### 4.2.3 Examples of foods fortified with polyphenols

Plant extracts containing polyphenols are used in beverages including water-type or tea-based drinks, as well as in dairy products and innovative formulations such as ‘smoothies’, which could be defined both as beverages and dairy products. The most popular plant extract used in this type of beverage is green tea extract followed by rooibos tea. Aloe Vera is also quite common in dairy products.

### 4.3 Carotenoids

#### 4.3.1 Health benefits

Carotenoids are a large class of natural plant pigments which are responsible for much of the colour in our lives. More than 600 of these yellow to red lipophilic pigments have been identified in nature. As carotenoids are synthesised only by plants, algae and bacteria, animals and humans have to obtain them from the diet. The human diet contains about 50–60 different carotenoids, of which about 30 have been identified in human blood and
tissues. The most obvious example of a carotenoid-rich food in the human diet is the carrot, which derives its colour from the best known carotenoid, \( \beta \)-carotene (also the ‘classic’ provitamin A-carotenoid). As such, \( \beta \)-carotene has a long history of use in food fortification, for example, in multivitamin juices.

The colour of carotenoids is the basis for their best known use in foods: as food colourants. \( \beta \)-Carotene, lutein, lycopene and some others are approved food colourants in Europe, listed as E 160a–f, E 161b and E 161g.

Mounting evidence of the benefits of carotenoids to human health is generating increasing interest in their use in food fortification: diets high in fruit and vegetables, and thus carotenoids, have been associated with a reduced risk of various diseases, most of them in some way related to oxidative stress.\(^{25}\) It has been suggested that carotenoids supplied by fortified foods and/or food supplements can help ‘bridge the gap’ between the desirable dietary intake of ‘five a day’ servings of fruit and vegetables, and the actual, much lower, fruit and vegetable intake typical in most western countries.

Of the many carotenoids present in the human diet, six are the most significant: these are the provitamin A carotenoids \( \beta \)-carotene, \( \alpha \)-carotene and \( \beta \)-cryptoxanthin, and the non-provitamin A carotenoids lutein, zeaxanthin and lycopene (see Fig. 4.3).

Benefits attributed to carotenoids derive from their antioxidant properties, which are generic for all carotenoids. They also have more specific roles – as precursors for vitamin A, for example. In this case, \( \beta \)-carotene is certainly the most prominent, but other carotenoids also display provitamin A activity. Lutein, zeaxanthin and lycopene are associated with specific benefits too: lutein and zeaxanthin in eye health\(^{26}\) and lycopene in relation to prostate

![Fig. 4.3 Structural formulae of the main dietary carotenoids.](image-url)
cancer.\textsuperscript{27} The latter is based on epidemiological studies which show that high intakes of lycopene-rich foods – tomatoes and especially tomato-products – are associated with a lower risk of prostate cancer. These results are supported by data from in vitro and animal studies, strengthening the biological plausibility. Results of some smaller human trials are also available, and further supportive data is expected from ongoing research. Lutein and zeaxanthin are highly and selectively accumulated in the macula lutea section of the retina of the eye, where they may act as antioxidants and blue light filters, protecting the retina at its site of highest visual acuity, from life-long, light-induced damage and thus from eye diseases such as age-related macular degeneration (AMD). Data from animal and smaller human trials support such a view. Currently, lutein and zeaxanthin are being tested in AREDS II, a large human intervention trial conducted by the US National Eye Institute (NEI).\textsuperscript{28}

Astaxanthin, a pink carotenoid without provitamin A activity, present in animal foods such as shrimp and lobsters, has recently been receiving attention, and various benefits to human health have been claimed. However, data from human trials supporting such effects is still scarce.\textsuperscript{29} Further, often only astaxanthin was tested, but none of the other major dietary carotenoids, so that it is usually not clear whether the effects observed in in vitro, animal or human studies are specific to astaxanthin or generic to carotenoids. Due to the limited dietary sources of astaxanthin, and thus low dietary intake, it is usually not detectable in human blood.

4.3.2 Technological aspects
Carotenoids are derived from plant sources or from algal cultures. The carotenoids used most commonly for food applications are in oil or powder form, depending on the food matrix. The oil form is applied to fat-containing food, such as spreads, milk, yoghurt and cheese. The powder form is applied mainly to beverages and food with little or no fat. The mode of application varies according to the food to be fortified:

- To spreads consisting mainly of fat and water, the carotenoids are added to the oil phase together with emulsifiers, flavours and antioxidants. Water is then added and the emulsion is formed.
- To cereal products, baked goods and nutrition bars, an oil preparation can be added during the dough preparation step, but it is more convenient to add a powder form directly to the dry blend before dough preparation.
- For beverages, dairy drinks and fruit preparations, the powder form is the most effective way to apply the carotenoids to these non- or low-fat type products. Efficient dispersion and stabilisation in liquid foods is required to prevent separation during storage. This can be done by either high-speed mixing or additional high-pressure homogenisation to maintain physical stability.
The dosage of carotenoids depends mainly on the desired colour and health claim for the fortified food. Further, the natural colour of a beverage affects the amount of a carotenoid which can be added: the colour of a rich orange multivitamin juice will not be affected by the addition of 40 to 60 ppm (4–6 mg/100 ml) lutein esters, while the colour of orange juice, typically lighter and more yellowish than the colour of a multivitamin juice, will become noticeably darker, therefore limiting the use of lutein esters in this application.

4.3.3 Examples for foods fortified with carotenoids

β-Carotene and lycopene are widely used as colourants in the food industry. β-Carotene is mainly used in spreads, cheese, yoghurts and ice cream, but also in beverages, bakery products, soups, sauces, salad dressings and sweets. In terms of food fortification, the most common products are multivitamin and ACE drinks and juices.

Lycopene is used mainly as a colourant in soups and sauces, and less commonly in bakery products and sweets. The use of lycopene in fortified food is not yet widespread, but is reported in instant chocolate drinks (along with other carotenoids) and in vegetable juice blends.

The use of lutein for food fortification is seen in vegetable (carrot) juice and in instant chocolate drinks (together with other carotenoids).

4.4 Oils

4.4.1 Health benefits

Today, lipids have lost their overall ‘bad’ image as ‘fats promoting weight gain and heart disease’, and it is accepted that the picture is more complex. Several lipids are recognised as having beneficial effects to human health, the best known probably being plant sterols for their cholesterol-lowering effects, and fatty acids with anti-inflammatory properties. The latter are often summarised as ‘PUFAs’ (polyunsaturated fatty acids) or ‘Omega-3s’ – thus overlooking the complexity of the substances and their efficacy, as explained further below. More recently, conjugated linoleic acid (CLA) has attracted interest for its effects on weight management and immune modulation.

Plant sterols (esters)

Many names exist for the plant equivalents to cholesterol: plant sterols, phytosterols, plant stanols or phytostanols, all also being available in the form of their fatty acid esters. They comprise a class of natural compounds with chemical structures very similar to cholesterol. The most common plant sterols are sitosterol, campesterol and stigmasterol. Plant stanols can be regarded as saturated plant sterols, since they have no double bonds in the sterol ring structure. Plant sterols are cell wall components and thus occur
naturally in plant foods such as vegetables, fruit, legumes, nuts, grains and cooking oils (including corn, soy and olive oils). Dietary intakes are usually between 100–400 mg/day, while clinical trials have consistently demonstrated that a daily consumption of 1–3 g of plant sterols is needed to lower LDL-cholesterol by 10–15%. Generally, plant sterols do not affect triglyceride and HDL levels. Thus, with unchanged HDL and reduced LDL-cholesterol, plant sterols improve the ratio between LDL and HDL in a less atherogenic direction.30

As a result of the consistency of the evidence and the importance of cholesterol-lowering for reducing the risk of heart disease, the US Food and Drug Administration (US FDA) approved the following health claim in 2000: ‘Foods containing at least 0.65 g per serving of vegetable oil sterol esters, eaten twice a day with meals for a daily intake of at least 1.3 g, as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease.’31 Recently, this claim was extended to include plant stanols and their esters. The claim can be used on the packaging of spreads, dressings, snack bars and dietary supplements. In the USA, plant sterols and stanols are mainly used in orange juice, yoghurt, spreads, nutrition bars and dietary supplements.

In Europe, the first approval under the Novel Foods Regulation for the use of plant sterols in yellow spreads was granted in 2000 in conjunction with a mandatory label claim that the products ‘are exclusively intended for people who want to lower their blood cholesterol’,32 and has subsequently been extended to milk-type products, fermented milk-type products, yoghurt type products, soy based beverages, cheese type products, dressings, mayonnaise, spicy sauces and rye bread.

Polyunsaturated fatty acids (PUFAs)

The term ‘polyunsaturated fatty acid (PUFA)’ refers to all fatty acids (FA) with at least two double bonds. Relevant to human nutrition are long-chain PUFAs (LC-PUFAs), i.e. C18 and longer. Linoleic acid (C18:2 n-6) and linolenic acid (18:3 n-3) are defined as essential FAs, i.e. they cannot be made by the human body itself but must be obtained from the diet.

Depending on the position of the first double bond from the methyl end of the molecule, PUFAs are categorised into omega-3 (or ω-3, or n-3) and omega-6 (or ω-6, or n-6) FAs. Both are used by the human body as structural components and for the synthesis of the so-called eicosanoids – hormone-like substances which modulate cardiovascular, pulmonary, immune and reproductive functions. Eicosanoids play a key role in inflammation and thus in conditions with an inflammatory component, such as cardiovascular diseases, chronic inflammatory diseases such as rheumatoid arthritis, and many more. The main classes of eicosanoids are leukotrienes, prostaglandins, prostacyclins and thromboxanes. Eicosanoids made from ω-3 FA have weaker and sometimes opposite effects than their counterpart made from an ω-6 FA. Therefore, the ratio between the two is most important. The ratio of eicosanoids
made from ω-6 vs. ω-3 is largely determined by the availability of the respective FAs for eicosanoid biosynthesis, and thus by dietary intake of these FAs. Ratios of ω-6:ω-3 of about 4:1 are found in countries such as Japan and are believed to be desirable, compared to ratios of 7:1 and 17:1 as reported for the UK and the USA, respectively.33 Sources of PUFAs for food fortification may include specific vegetable oils, such as evening primrose oil (EPO) or flaxseed oil, each characterised with a typical ratio of ω-6:ω-3 FAs, and especially fish oil high, or enriched in, eicosapentanoic acid (EPA, C20:5 n-3) and/or docosahexanoic acid (DHA, C22:6 n-3).

Data on health benefits come from animal studies as well as from epidemiological and intervention trials. Epidemiological data may appear inconsistent, but this may be due to difficulties in assessing intakes in a sufficiently reliable manner and in a way to enable proper comparison between results of different studies, especially when it comes to intakes of specific fatty acids. Intervention trials such as the GISSI-trial have confirmed the benefits of PUFAs in cardiovascular disease.34 However, intervention trials with PUFAs may be hampered by the general issues currently being discussed within the scientific community, such as correct substance and dosage, choice of placebo, assessment of compliance and confounding factors, choice of study population in terms of disease stage, duration of intervention, and so on. Nevertheless, the US FDA considered the evidence sufficient to issue a Qualified Health Claim for food supplements in 2000 and for conventional foods in 2004.35 In the UK, the Joint Health Claims Initiative (JHCI) issued a claim in 2004.36

Evidence for benefits of ω-3 FAs in other conditions, such as asthma, neurodermatitis and eye diseases such as AMD, is emerging and is an area of active research.

**Conjugated linoleic acid (CLA)**

Conjugated linoleic acid (CLA) refers to a class of linoleic acid isomers found in meat and dairy foods derived from ruminant animals – cattle, sheep and goats. In the early 1980s, researchers at the University of Wisconsin-Madison almost accidentally discovered the effects of CLA on body composition while investigating its anti-carcinogenic effects: they found that CLA-fed animals had significantly less body fat than controls. Animal and mechanistic studies conducted since then have found that CLA acts by decreasing the amount of fat stored after eating, decreasing the total number of fat cells, and by increasing the rate at which fat is used for energy. The effects on body composition appear to have been confirmed in humans. However, CLA is not a weight loss agent. Rather, it improves body composition by decreasing body fat mass while maintaining lean body mass, thereby supporting body weight management and helping to prevent the so-called ‘yo-yo’ dieting effect of losing and regaining. Recently, the immune-modulating effects of CLA have been attracting interest. Data from both animals and
humans appear to confirm effects on cell-mediated immunity such as responses to vaccination. Also, modulation of cytokine levels has been observed, which may play a role in inflammatory responses.\textsuperscript{37}

The two bio-active isomers of CLA are the cis-9, trans-11 and trans-10, cis-12 isomers. Most commercially available CLA preparations are derived from safflower oil and contain a 50:50 mixture of the two. CLA is the ‘newest’ and most innovative of the ingredients discussed in this section, with many more potential applications and high interest within the Functional Foods and Nutraceuticals industry.

### 4.4.2 Technological aspects

#### Sterols: Solid fat content

Plant sterols are isolated from vegetable deodoriser distillates (VODs) of soft seed oils such as soybean, sunflower or rapeseed oil, or from tall oil pitch which is obtained during the manufacture of paper from wood, particularly pine wood. Sterol esters are produced via esterification with fatty acids or transesterification with fatty acid methyl esters based on sunflower or rapeseed/canola oil. Sterol esters are paste-like products with a melting range of 38–47 °C. The solid fat content of the products depends on the fatty acid part of the sterol ester. This is shown in Table 4.2.

#### CLA: Isomeric purity

The technological challenge in the manufacture of CLA is to obtain a product with high isomeric purity. Isomerisation of linoleic acid under alkaline heat treatment will lead to a technical grade CLA, which contains several different isomers, as shown in Fig. 4.4. Therefore, mild reaction conditions have to be chosen followed by an optimised work-up, in order to get the desired 50:50 mixture of cis-9, trans-11 and trans-10, cis-12 isomers.

#### Antioxidants

Unsaturated products are sensitive to oxidation, and very often the secondary oxidative products bring an unacceptable flavour to the final product. Therefore, antioxidant systems have to be used to overcome these problems. Very often,

<table>
<thead>
<tr>
<th>Table 4.2 Solid fat content of sterol esters</th>
</tr>
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<tbody>
<tr>
<td>Sunflower-based sterol ester</td>
</tr>
<tr>
<td>SFC 10°C</td>
</tr>
<tr>
<td>SFC 20°C</td>
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<tr>
<td>SFC 25°C</td>
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<tr>
<td>SFC 30°C</td>
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<tr>
<td>SFC 35°C</td>
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<tr>
<td>SFC 40°C</td>
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mixed tocopherols, ascorbyl palmitate and, in some cases, rosemary extracts and salts of citric acids are used as antioxidants. As an example, the development of hexanal and heptanal in Tonalin CLA, stabilised with mixed tocopherols, is shown during storage at 25°C in Fig. 4.5.

In addition to the protection against oxidation, the kind of packaging used, as well as storage and delivery conditions, may influence the final product quality.

Food fortification

The challenge in fortification is to achieve a final product with the desired sensory properties. Very often, aqueous systems are used, such as dairy products or milk-based fruit drinks. In these cases, lipophilic functional
ingredients such as sterols, CLA and PUFAs are difficult to incorporate due to their physical properties. In aqueous systems, it is therefore preferable to add the product in an emulsion form. From a marketing standpoint, water-clear beverages are ideal, but due to the physical properties of the product, they are not always achievable.

Oils, such as LC-PUFAs or CLA, are added to the final product as triacylglycerols. In every case, deodorised products have to be used to ensure good sensory properties of the final product. The oils can be added with the aid of an emulsifier to improve the dispersibility of the oil, but this may not always be desirable, as the emulsifier will bring additional flavour components to the product. It is necessary to homogenise the product to achieve homogeneous distribution and stabilisation of the oil. Sterol esters will be added in the same way; however, use of an emulsifier is usually not necessary. Usual processing conditions, such as pasteurisation, UHT treatment, etc. will not affect the properties of the functional ingredient.

To facilitate the addition of functional lipid components, a variety of powder products have been developed. Very often, spray-dried powders, which are easily dispersible in the aqueous system, are used. The main challenge is to achieve high concentrations of the active ingredient while maintaining a free flowing powder. Matrix encapsulation protects the product against oxidation, but the product has to be stored under the right conditions.

Ground sterols are difficult to add to aqueous systems as they are very hydrophobic. This means that the sterols are not moistened after being added to water and immediately separate. For this reason, high amounts of emulsifiers or high shear forces are needed when adding ground sterols to aqueous products. In addition to their hydrophobic properties, ground sterols belong to a high dust explosion class, necessitating additional safety measures when handling the powder. Furthermore, ground sterols lead to a chalky taste and stick to the teeth. These problems have to be overcome; otherwise the products will not be acceptable to the consumer.

### 4.4.3 Example(s) of foods fortified with conjugated lenoleic acid (CLA), polyunsaturated fatty acids (PUFAs), sterols (esters)

Table spreads were the first products to be fortified with plant sterols. In 2004, approvals for dairy products, soy drinks and dressings opened up the market for further applications in the EU. In the US, beverages and cereals are the most popular products for functional food applications.

One-shot dairy drinks, which deliver the daily requirement of the active ingredient in a single shot, are common in Europe, but do not have a parallel in the US. Daily dose drinks are established in the minds of European consumers as an optimum product form. For example, sterol-containing one-shot drinks are retailed in packs of four 100 g bottles, each bottle containing 2.7 g of plant sterol esters. As well as the one-shot drinks, spoonable yoghurt, milk, milk powder and cream cheese are also fortified with sterols.
In the US, an orange juice with ground sterols (containing 0.4 g plant sterols per serving) is now on the market, as well as chocolate bars (containing 1.1 g sterol ester per serving) and fruit flavoured yoghurts.

Cholesterol-lowering food products in Japan include cooking oils, spreads and mayonnaise. Cooking oils currently account for the majority of sterol-based products in Japan.

CLA is used as an active ingredient in food supplements in the US and Europe. Further, it is already being used in foods and beverages in the dairy market. In Spain, products such as skimmed milk (0.75% CLA), and yoghurt and yoghurt drinks (1.87% CLA) have been launched. Further applications include pineapple and mango fruit juice and fresh cheese fortified with CLA.

As a result of widespread scientific support and almost unanimous recommendation by nutritionists, LC-PUFAs such as DHA and EPA are found in products for children, adults and elderly people. DHA-fortified milk powders are used for brain development in babies, toddlers and young infants. Milk powders, yoghurt or yoghurt drinks are good delivery systems. The content in these dairy products varies between about 80 mg/100 g and 100–200 mg/100 g. The main challenge is to overcome the fishy taste of the products – an issue which limits the concentration in the final product.

Apart from dairy products, a number of omega-breads are found in the market, with typical concentrations of between 30 and 60 mg per serving size. Beverages are also used as a delivery system and contain values of about 80 mg/100 g.

4.5 Future trends

One of the main issues for the future will be to increase the bioavailability of functional ingredients. Poor bioavailability is the result of:

- poor dissolution or low aqueous solubility
- degradation (poor chemical or enzymatic stability) in gastric or intestinal fluids
- poor intestinal absorption
- presystemic metabolism

Therefore, improved solubilisation, enhanced absorption, adequate stability and controlled release of the functional ingredients are required. Nanostructured products or special delivery systems such as molecular encapsulation will be the products of the future. In addition to the actual functional ingredients, it is probable that different products will be fortified in the future, such as functional confectionery – candies, lollipops or chewing gums – or fast dissolving strips for products where smaller amounts are required, for example vitamins, etc.

With regard to plant extracts, there is already a trend towards high quality, standardised ingredients. Plant extracts are required to be as purified as
possible to maintain the food grade status necessary for real functional food concepts supporting efficacy and health claims. There may also be a trend away from products that are functional in name only. The so-called ‘wellness’ products which include an extract simply for marketing purposes and for positioning the product within the ‘well-being’ area will be gradually replaced by genuinely functional food products.

Currently, products usually contain just one functional ingredient. In the near future, there are likely to be products incorporating different active ingredients with an additive or even synergistic effect. An example is green tea and CLA – both showing positive effects in terms of weight management. But these synergistic effects have to be proven by clinical trials in order to convince consumers and authorities that these products are genuinely good for health. ‘Beauty foods’ is an example of a growth area for combinational concepts. The main beauty food claims are anti-ageing, sun protection, moisturising (dry skin), skin tanning and improved digestion which helps to improve impure skin and body composition. The claims are very similar to those made for traditional cosmetic products. Beauty food products can be positioned as ‘daily-use’ or ‘spa-at-home’ products with a recommended period of use. ‘On-the-go’ or ‘one-dose’ products simplify their use and increase consumer compliance. Table 4.3 gives an overview of possible functional ingredients that support these main beauty food claims.

The 21st century will see further advances in nutrition science as a result of the characterisation of the human genome and improved understanding of the potential of nutrients to maintain or improve health. Much scientific work is currently conducted in the ‘omics’ area (proteomics, lipidomics, etc.). Nutrients can participate in the regulation of metabolic pathways. It has been established that these products are able to modify the expression of genes and thus protein levels. In the future, individuals may be able to identify possible predispositions to diet-related diseases. They may be able to map their individual health, based on information about their individual

<table>
<thead>
<tr>
<th>Beauty food claims</th>
<th>Natural functional ingredients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-ageing</td>
<td>Vitamin E, Carotenoids, Ginseng, Green tea, Lemon balm, Rooibos, Alpha lipoic acid, Biotin, Coenzyme Q10, Folic acid, Red clover, Grape seed OPCs</td>
</tr>
<tr>
<td>Digestion and skin</td>
<td>Probiotics, Fibre, Aloe Vera, Honeybush, Lemon verbena</td>
</tr>
<tr>
<td>Sun protection/skin tanning</td>
<td>Vitamin E, Carotenoids, Green tea, Rooibos, Selenium, Bilberry</td>
</tr>
<tr>
<td>Body composition</td>
<td>Conjugated linoleic acid, Phytosterols, Green tea, Grape seed OPCs, DHA, EPA</td>
</tr>
<tr>
<td>Stress reduction and skin</td>
<td>Vitamin E, Carotenoids, Lemon balm, Ginseng, Green tea, Passion flower, Rooibos</td>
</tr>
</tbody>
</table>
Fortification with substances other than vitamins and minerals

genetic code, and use nutrition as a key factor to maintain or improve their health.

4.6 Sources of further information and advice

European Botanical Forum, 50 Rue de l’Association, 1000 Brussels, Belgium
International Life Sciences Institute: ILSI Europe (http://europe.ilsi.org/)
ILSI PASSCLAIM (http://europe.ilsi.org/activities/ecprojects/PASSCLAIM/)
Canadian Library Association: CLA - interest group (http://www.cla.ca/about/intgroup.htm) European Nutrigenomics Organization - NuGO - (www.nugo.org)

4.7 Dedication

This chapter is dedicated to Dr. Gerhard Schwarz, our co-author and valued colleague, who died suddenly and much too early.

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Food fortification and supplementation


15 OVG North-Rhine/Westphalia, judgements of 17.03.2006: Demarcation medicament/food (13 A 197702)


31 US FDA: 21 CFR § 101.83 http://ecfr.gpoaccess.gov/cgi/t/text/text-idx?c=ecfr&sid=77ed7da9463357d9a09892213e5c74db&rgn=div8&view=text&node=21:2.0.1.1.2.5.1.14&idno=21


35 US FDA: http://www.cfsan.fda.gov/~dms/qhc-sum.html#omega3


5

Healthy polyunsaturated fatty acids (PUFAs) for food enrichment

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5.1 Introduction

During the past thirty years there has been an increasing interest in polyunsaturated fatty acids (PUFAs) for food, nutritional and pharmaceutical applications. This is due to the increasing evidence that PUFAs have a wide range of nutritional benefits in the human body. There are two distinct families of PUFA, namely the n-3 and the n-6 families, and these families cannot be interconverted. The terms ‘n-3’ and ‘n-6’ refer to the position of the first double bond in the carbon chain as counted from the methyl terminus.

The health benefits of n-3 long chain PUFAs have received particular attention during the last decade, and from a nutritional point of view the three most important n-3 PUFAs are α-linolenic acid (LNA, C18:3 n-3), eicosapentaenoic acid (EPA, C20:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3). The molecular structures of EPA and DHA are shown in Fig. 5.1. The potential health effects of EPA and DHA include reduction of cardiovascular disease risk,1–3 anti-inflammatory effects including reduction of symptoms of rheumatoid arthritis4,5 and Crohn’s disease,6 and reduction of the risk of certain cancer forms. DHA is particularly important in the development of brain and nervous tissue in the infant.7

![Molecular structure of EPA and DHA n-3 fatty acids.](image)

Fig. 5.1 Molecular structure of EPA and DHA n-3 fatty acids.
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Evidence for the preventive effect of EPA and DHA on cardiovascular disease is strong. This is also demonstrated by the fact that the US Food and Drug Administration (FDA) in September 2004 announced the availability of a qualified health claim for reduced risk of coronary heart disease (CHD) on conventional foods that contain EPA and DHA n-3 fatty acids. This means that the following claim can be used on food products containing EPA and DHA in the US: ‘Supportive but not conclusive research shows that consumption of EPA and DHA omega-3 fatty acids may reduce the risk of coronary heart disease. One serving of [name of food] provides [x] grams of EPA and DHA omega-3 fatty acids’. The Joint Health Claims Initiative (JHCI) in the UK has also approved the following claim: ‘Eating 3 g weekly (or 0.45 g daily) of long chain omega-3 polyunsaturated fatty acids as part of a healthy life style helps maintain heart health’.

5.1.1 Sources for n-3 PUFA from plants and fish

Plant materials such as flaxseed, canola (rape) and soybean oil contain relatively high levels of n-3 PUFA in the form of LNA. However, n-3 and n-6 PUFA with 18 carbon atoms (LNA and linoleic acid) are competing for the same enzyme systems for conversion of the C18 fatty acids into PUFA with longer chain length (EPA from LNA and C20:4 n-6 from linoleic acid). Therefore, only a minor part of LNA is converted to EPA and DHA. This is particularly a problem if the intake ratio between n-3/n-6 PUFAs is low. This chapter will therefore mainly focus on EPA and DHA and, in the remaining part of this chapter, the term n-3 PUFA refers to EPA plus DHA and not LNA. The main source of EPA and DHA are seafood products, especially fatty fish. The n-3 PUFAs are extracted from fish in connection with the production of fish meal.

The fish that are processed to produce crude fish oil (and fish meal) can usually be categorised as follows:

(i) offal and waste from the edible fisheries, e.g. cuttings from filleting industry;
(ii) fish of a quality that is not high enough to make the fish suitable for human consumption; and
(iii) fish types that are not considered acceptable or aesthetically pleasing for human consumption.

The last are caught especially for reduction to fish meal and fish oil. The most important fish species that are caught commercially and processed into fish oil are shown in Table 5.1. The fatty acid composition of the fish oil depends on the fatty acid composition of the feed and therefore substantial variation is observed within each species. Approximate data for the most important fatty acids are also shown in Table 5.1.

The total annual world production of fish oil during the last 10 years has been approximately 1.25 million tonnes. The main producers are Japan,
Table 5.1  Sources of fish oil and their fatty acid compositions (from Allen\textsuperscript{10})

<table>
<thead>
<tr>
<th>Main sources</th>
<th>Fish species</th>
<th>Capelin</th>
<th>Herring</th>
<th>Norway pout</th>
<th>Mackerel</th>
<th>Sand eel</th>
<th>Menhaden</th>
<th>Sardine/pilchard</th>
<th>Horse mackerel</th>
<th>Anchovy</th>
<th>Sprat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barents Sea</td>
<td>N. Atlantic</td>
<td>7</td>
<td>7</td>
<td>6</td>
<td>8</td>
<td>7</td>
<td>9</td>
<td>8</td>
<td>8</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>N. Atlantic</td>
<td>N. Sea, Norwegian Sea, Pacific Ocean</td>
<td>10</td>
<td>16</td>
<td>13</td>
<td>14</td>
<td>15</td>
<td>20</td>
<td>18</td>
<td>18</td>
<td>19</td>
<td>16</td>
</tr>
<tr>
<td>N. Sea</td>
<td>N. Atlantic, Barents Sea</td>
<td>10</td>
<td>6</td>
<td>5</td>
<td>7</td>
<td>8</td>
<td>12</td>
<td>10</td>
<td>8</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>N. Atlantic</td>
<td>N. Sea, N. Atlantic, Pacific Ocean, N. Sea</td>
<td>14</td>
<td>13</td>
<td>14</td>
<td>9</td>
<td>11</td>
<td>13</td>
<td>11</td>
<td>13</td>
<td>13</td>
<td>16</td>
</tr>
<tr>
<td>N. Sea</td>
<td>USA East Coast, Gulf of Mexico</td>
<td>17</td>
<td>13</td>
<td>11</td>
<td>12</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>8</td>
<td>2</td>
<td>14</td>
</tr>
<tr>
<td>USA, Atlantic, Pacific Coast of Canada &amp; USA</td>
<td>14</td>
<td>20</td>
<td>12</td>
<td>15</td>
<td>16</td>
<td>0.2</td>
<td>3</td>
<td>8</td>
<td>2</td>
<td>14</td>
<td>10</td>
</tr>
<tr>
<td>S. Africa, Pacific Coast of South America</td>
<td>20:5</td>
<td>8</td>
<td>5</td>
<td>8</td>
<td>7</td>
<td>9</td>
<td>14</td>
<td>18</td>
<td>13</td>
<td>17</td>
<td>6</td>
</tr>
<tr>
<td>Off S. &amp; W. Africa, Chile, Peru, and Mexico (Pacific Coast)</td>
<td>22:6</td>
<td>6</td>
<td>6</td>
<td>13</td>
<td>8</td>
<td>9</td>
<td>8</td>
<td>9</td>
<td>10</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Total (Principals)</td>
<td>86</td>
<td>86</td>
<td>82</td>
<td>84</td>
<td>88</td>
<td>75</td>
<td>83</td>
<td>81</td>
<td>83</td>
<td>78</td>
<td></td>
</tr>
</tbody>
</table>

Fatty acids (wt% of total FAs)

| 14:0 | 7 | 10 | 10 | 14 | 17 | 14 | 8 |
| 16:0 | 7 | 16 | 6  | 14 | 13 | 12 | 9 |
| 16:1 | 5 | 13 | 11 | 14 | 13 | 15 | 10|
| 18:1 | 13| 11 | 12 | 15 | 16 | 0.2| 4|
| 20:1 | 12| 15 | 16 | 9  | 5  | 8  | 3 |
| 22:1 | 11| 12 | 14 | 9  | 14 | 18 | 9 |
| 20:5 | 5 | 5  | 8  | 8  | 9  | 3  | 3 |
| 22:6 | 6 | 6  | 8  | 9  | 8  | 8  | 9 |
| Total | 86 | 86 | 82 | 84 | 88 | 75 | 83 | 81 | 83 | 78 |
Scandinavia, Chile, Peru, USA and Russia. Most of the fish oil (56%) is going into the production of farmed salmon in Norway, Chile, Canada and in various European countries. With the current growth in aquaculture, this figure may increase to 80% or more before 2010.11 There may even be a risk that the demand for fish oil for use in aquaculture could exceed the production. However, approximately 25–30 million tonnes of fish are discarded annually. Efforts are being made to increase fish oil production by decreasing the amount of waste and increasing the amount of recycling of the fish waste to fish meal and fish oil production. In addition, efforts are being made to reduce the amount of fish oil used per kg farmed fish produced, e.g. by substituting part of the fish oil with rapeseed oil. However, to obtain a satisfactory n-3 PUFA level in farmed fish at the time of slaughtering it may be possible to substitute fish oil with rapeseed (canola) oil only at the beginning of the feeding period. Due to these efforts, it is expected that fish oil will still be available for human consumption in the years to come, despite the expected growth in aquaculture.

Certain fishing areas are heavily polluted with compounds such as polychlorinated biphenyls (PCBs), dioxins, lead and arsenic. PCBs and dioxin are lipid soluble and therefore they will be extracted together with the fish oil during the fish oil manufacturing process. In July 2002, a regulation was imposed in the EU whereby the limit for dioxin in fish oil was set at 2 pg WHO-PCDD/F-TEQ/g. The legislation was amended in 2006 to include limits for dioxin-like PCBs. Due to the strict rules, new technologies have been developed to remove dioxin from fish oil. The most common method is to remove the dioxin by activated carbon, but new deodorisation techniques, including thin film deodorisation and molecular distillation that efficiently remove these compounds, have also been developed. Such new technologies are required to remove PCBs, as they cannot be removed effectively by activated carbon.

5.1.2 Microbial sources of n-3 PUFAs
Micro-organisms capable of producing n-3 PUFAs with a chain length above C20 include lower fungi, bacteria and marine micro-algae.12–16 The most promising micro-organisms for the production of n-3 PUFA seem to be the marine micro-algae, as they are able to accumulate high amounts of n-3 PUFA. The advantage of algae oil compared with fish oil is thus that the oil contains higher levels of, in particular, DHA than fish oil, e.g. up to 52%.17 Micro-algae are cultivated either in photo-autotrophic or in heterotrophic production systems. The disadvantage of the former is that they require the presence of light, which means that the production is dependent on the weather if carried out in open ponds. If the production is carried out in closed photo-bioreactors, the scale-up of the production is limited by the ability to effectively introduce light.18 The production of EPA by photo-autotrophic growth has been intensively studied.17 The yield of EPA by this production
method is low, and therefore the production is not commercially feasible. EPA content and productivity rates of some of the most promising microalgae are summarised in Medina et al. In recent years, production of DHA by heterotrophic marine micro-organisms has received increased commercial attention and today DHA produced this way is used in several infant formula products. Currently, Martek Biosciences uses Schizochytrium sp. for the production of DHA, which has been used to produce DHA-enriched eggs and as feed for aquaculture. The European Commission has approved the use of DHA-rich oil from Schizochytrium sp. produced by Martek Biosciences in items such as dairy products, spreads, dressings, breakfast cereals and food supplements. Martek Biosciences has also patented a process for the production of DHA-rich oil (25–60%) using Cryptothecodinium cohnii, and this DHA-oil is currently used in several infant formula products.

5.2 Current problems in producing n-3 PUFA and using fish oils in food products

The main problem in relation to the use of n-3 PUFAs in both pharmaceutical and food applications is their susceptibility to lipid oxidation. The chemistry behind lipid oxidation is therefore briefly summarised.

5.2.1 Lipid oxidation and antioxidation chemistry

The basic substrates for lipid oxidation reactions are unsaturated fatty acids with one or more double bond. The susceptibility to lipid oxidation increases with the number of double bonds in the fatty acid. For example, the oxidisability of DHA is five times greater than that of linoleic acid. There are three different types of oxidation: autoxidation, photo-oxidation and enzymatic oxidation. Autoxidation is a spontaneous free radical reaction with oxygen and consists of three main stages: initiation, propagation and termination. Photo-oxidation happens only in the presence of light and when the food system contains photosensitisers. Enzymatic oxidation is due to the presence of certain enzymes such as lipoxygenase in plant and animal systems.

The autoxidation reaction is initiated by initiators (e.g., metal ions, heat, protein radicals), which cause unsaturated fatty acids (LHs) to form carbon-centred alkyl radicals (L*) (Fig. 5.2). In the presence of oxygen, these radicals propagate by a free radical chain mechanism to form peroxyl radicals (LOO*) and, later, hydroperoxides (LOOH). The hydroperoxides are the primary oxidation products of autoxidation. The free radical chain reaction propagates until two free radicals combine and form a non-radical product to terminate the chain. The hydroperoxides can be decomposed by heat or in the presence of traces of transition metals and thereby alkoxy and peroxy radical intermediates (LO* and LOO*) are formed. These radicals propagate the
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Free radical chain reaction. Moreover, these radicals may be further decomposed to form a variety of non-volatile and volatile secondary oxidation products (in Fig. 5.2 aldehydes are given as an example of volatile oxidation compounds). The latter are termed ‘volatiles’ and include a wide range of carbonyl compounds (aldehydes, ketones and alcohols), hydrocarbons and furans that are responsible for flavour deterioration. In contrast to the volatiles, hydroperoxides are essentially tasteless and odourless.

Photo-oxidation leads to oxidation of unsaturated fatty acids due to exposure to light in the presence of photosensitisers. The latter will be activated by absorbing visible or near-UV light. Type I sensitisers then react with the substrate, generating substrate radicals which can react with oxygen. Type II sensitisers react directly with triplet oxygen, transforming it into the short-lived, but highly reactive, high-energy form of singlet oxygen $^{1} \text{O}_2$, which reacts directly with the double bond of unsaturated fatty acids to form hydroperoxides (LOOH). This is not a free-radical process and will lead to the formation of other lipid hydroperoxides and, in turn, also to other volatiles than those formed from free radical oxidation. In food systems, chlorophyll, riboflavin or heme proteins, serve as photosensitisers. The hydroperoxides are decomposed by the same reactions as described under autoxidation.

Lipid oxidation may, to a certain extent, be prevented by the addition of antioxidants, which are usually classified as either primary or secondary antioxidants. Primary antioxidants (AH) are also referred to as free radical scavengers because they act as chain-breaking antioxidants by donating electrons/hydrogen to free radicals such as the lipid, peroxyl or the alkoxyl radical (Fig. 5.2), thereby terminating the free radical chain reaction. Primary antioxidants include hindered phenols, such as the synthetic antioxidants.

Fig. 5.2 Initiation and propagation of lipid oxidation and prevention of oxidation by free radical chain-breaking antioxidants.
BHA (butylhydroxyanisole), BHT (butylhydroxytoluene), propyl gallate, naturally occurring compounds such as tocopherol, and plant polyphenols such as carnosic acid. The secondary antioxidants act by a number of different mechanisms such as metal chelation, oxygen scavenging and replenishing hydrogen to primary antioxidants. Therefore, the secondary antioxidants often exert synergistic effects when used with primary antioxidants.

5.2.2 Lipid oxidation during processing of fish and micro-algae into n-3 PUFA oils

Generally, fish are processed to fish oil by the so-called wet reduction method. The principal operations are cooking, pressing, separation of oil and water by centrifugation to recover the oil, and drying of the residual protein material. The purpose of the cooking step is to coagulate the proteins, which enables mechanical separation of the liquids and solids in the pressing step. Moreover, fat cells are ruptured during the cooking step, whereby the oil is released into the liquid phase. During the pressing operation, two intermediate products are produced, namely the press cake and the press water. The press cake is dried to produce fish meal. The press water passes a screen to remove coarse particles followed by removal of fine particles in a decanter. Subsequently, the oil is removed from the press water in a separator. Impurities are removed from the resulting oil in a polisher. The protein and lipid fractions may also be separated in the step after the heating step by using a three-phase decanter centrifuge. As mentioned in Section 5.2.1, high temperatures, light, metal ions and heme proteins will catalyse lipid oxidation. Thus, the traditional oil extraction method will unavoidably lead to some oxidation of the fish oil. Lipid oxidation will be less severe if fresh raw materials of good quality are used. Thus, efforts should be made on board the fishing vessel to reduce transportation time and temperature, avoid exposure to light and reduce the compression of the fish and thereby decrease the risk of bleeding, which will otherwise expose the lipids to heme proteins.

It is possible to reduce the fish processing temperature by extracting the lipids by an enzymatic hydrolysis process. In this process, proteins are hydrolysed by enzymes, whereby lipids can be released into the liquid phase at a much lower temperature (e.g. 60 °C) with a satisfying yield (Jacobsen et al., unpublished findings). It may therefore be possible to produce fish oil of a better quality by an enzymatic extraction method.

Recently, several studies on the production of fish oil from by-products (including the oxidative stability of these oils) have been reported in the literature. The effect of the processing conditions on the oxidative stability of herring oil when using a three-phase decanter to extract the oil from fresh unsalted herring by-products was reported by Aidos et al. Surprisingly, it was observed that the decanter temperature did not influence the oxidative stability of the fish oil. In contrast, the oxidative stability was influenced by an interaction effect of the speed of the mono-pump and the speed of the
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decanter. The best oil stability was obtained when the oils were processed with the highest mono-pump speed. Aidos et al. compared the stability of herring oil produced from three different herring by-products: only heads, mixed, and headless by-products. Oils from the heads had the highest oxidation level, despite the fact that they contained less PUFA than the other two by-products. It was suggested that a lower \( \alpha \)-tocopherol content in the oils from the heads compared with the other oils and liberation of endogenous enzymes from the skin were responsible for the increased oxidation in the heads. In another study, the oxidative stability and flavour deterioration of herring oil produced from freshly produced or frozen unsalted herring by-products or salted maatjes by-products was compared. As expected, oil produced from fresh unsalted by-product had a higher stability and a better sensory quality than oils produced from the other by-products. This supports the finding that the quality of the fish is of great importance to the quality of the resulting oil. Moreover, the increased oxidation in the oil produced from salted maatjes, which had a higher content of iron than the other by-products, indicates that both the presence of transition metals in the fish and the presence of salt will promote oxidation in the resulting oil.

The extraction of n-3 PUFA from micro-algae is a complicated process that involves the use of organic solvents. To the authors’ knowledge, data on the effect of the processing conditions on the oxidative stability of the oils have not been published.

5.2.3 Lipid oxidation and refining of fish oil

The general objective of processing crude fish oil is to remove impurities that cause the original product to have an unattractive colour or taste, or that cause harmful metabolic effects. At the same time, the processing should retain desirable nutritional components such as the n-3 PUFA and antioxidants such as tocopherol. Before refining, the crude oil is often stored in large bulk storage tanks. Insoluble impurities are precipitated during storage and can be drained off, together with moisture, and thereby reduce the increase in free fatty acids, which may otherwise promote oxidation. To further minimise oxidation during storage, Young recommended that intake pipelines should be extended to the bottom of the tank and that contact with iron, copper and copper alloys should be eliminated. The procedure for refining unhydrogenated and unfractionated fish oil often involves the following steps (the reader may refer to Bimbo for a more thorough review of the refining process):

- Degumming by treatment with phosphoric acid or other acids to remove phospholipids, proteinaceous compounds, trace metals and other contaminants. A high content of phospholipids will lead to emulsion formation in the subsequent refining steps and therefore make separation of oil and water difficult. Fish oils are low in phospholipids and degumming for that reason is not necessary. However, the oil quality (i.e. oxidative
stability) is often improved by the degumming step due to the removal of trace metals.\textsuperscript{39}

- Neutralisation by addition of an alkali solution such as caustic soda to remove free fatty acids, pigments, phospholipids, oil insolubles, water solubles and trace metals. The neutralisation process involves heating and is followed by one or more washing steps with water. Reduction in the content of free fatty acids improves the sensory properties and oxidative stability of the oil. The free fatty acid content of refined fish oils should be as low as possible, preferably not higher than 0.2%.

- Bleaching is performed to improve the colour, flavour and oxidative stability of the oil, and to remove impurities. Activated clay (bleaching earth) is used for the process. Bleaching involves the adsorption of coloured compounds, peroxides and some volatile oxidation compounds as well as other impurities to the bleaching clay. Bleaching can be carried out either at atmospheric pressure or under vacuum. The latter may be performed in a batch or continuous process. The best oxidative stability is obtained by the use of continuous vacuum bleaching.\textsuperscript{39}

- The final step in the refining process is deodorisation, which removes undesirable ingredients from the oil and compounds formed during the preceding steps. The deodorisation process is basically steam distillation, which removes compounds that are more volatile than the triglycerides. Deodorisation of fish oil is often carried out at a temperature around 190 °C. Due to the high temperature, peroxides are decomposed into secondary volatile oxidation products, which are then distilled off. Deodorisation may be carried out in either a batch, semi-continuous, continuous or thin-film deodoriser. The difference between the first three processes and the thin film deodoriser is that the last employs a thin-film concept to strip volatiles from the oil at high transfer rates, whereas deodorisation in the first three processes takes place in one or more consecutive vessels/tanks. The deodorisation time and temperature in the thin film process are lower than in the other processes, and therefore the thin-film deodorisation is more gentle. This leads to a lower loss of tocopherol and a lower formation of undesirable components such as trans fatty acids and polyaromatic hydrocarbons.

\section*{5.3 Improving the sensory quality and shelf-life of n-3 PUFA enriched foods}

The very high susceptibility of n-3 PUFA oils towards oxidative deterioration invariably means that special precautions have to be taken in order to achieve stable and sensory acceptable PUFA-enriched products. When n-3 PUFAs are added as an ingredient in a food product, the product is usually processed further in order to achieve the desired physical stability, functional and sensory properties. Such processing will imply further oxidative stress on the n-3
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PUFA oils. Choice of processing conditions, packaging material and storage conditions are important extrinsic factors, which need to be addressed. Secondly, the intrinsic or physico-chemical properties of the individual food product can affect oxidative stability in both antioxidative and pro-oxidative directions. In the following section, various actions and approaches to achieve and maintain good quality and oxidative stability of n-3 PUFA enriched foods will be discussed.

5.3.1 Quality of the n-3 PUFA oil

The quality, i.e. oxidative status, of the n-3 PUFA oil has a significant influence on the oxidative stability of foods enriched with this oil. The oxidative status of oils has traditionally been measured by the peroxide value (PV) and the anisidine value (AV). PV is a measure of the level of the primary oxidation products (lipid hydroperoxides) in the product, while the anisidine value is an unspecific measure of saturated and unsaturated carbonyl compounds. Several fish oil-producing companies guarantee that their fish oils have a PV lower than 1.0 meq/kg and an AV lower than 5. Recent studies performed with fish oil-enriched milk have corroborated the importance of using a fish oil of high quality for incorporation into foods.40,41 Thus, it was reported that milk emulsions based on cod liver oil with a slightly elevated PV of 1.5 meq/kg oxidised significantly faster than ones with a tuna oil with a low PV of 0.1 meq/kg, despite the fact that the tuna oil was more unsaturated than the cod liver oil.40 It was hypothesised that trace metals present in the milk, in combination with the slightly elevated level of lipid hydroperoxides, were responsible for the rapid oxidative flavour deterioration of the milk based on cod liver oil, due to the ability of trace metals to decompose lipid hydroperoxides. A subsequent study supported these findings and also showed that a sensory panel was able to distinguish milk emulsions produced with fish oil with a PV of 0.1 meq/kg as being less fishy and rancid than those containing a fish oil with a PV of 0.5 meq/kg.41

5.3.2 Emulsion formulation

Emulsifiers

Many n-3 PUFA enriched foods exist in the form of some kind of emulsion (e.g. salad dressing, spreads, milk, ice cream). These food systems require the addition of an emulsifier. Primarily, emulsifiers provide physical stability to the emulsions. However, emulsifiers are able to interact with other components/ingredients of the food product, and the choice of emulsifier can therefore be of significant importance for both physical and oxidative stability of the product. Basically, emulsifiers are surface active molecules with amphiphilic properties, which can interact with the oil–water interface and reduce surface tension. Emulsifiers for food use are thus either macromolecules, such as proteins unfolding at the interface, or smaller surfactant molecules,
such as phospholipids, free fatty acids, monoacylglycerols and synthetic surfactants.

Emulsifiers are able to influence lipid oxidation in various ways. In emulsions stabilised by proteins, pH will generally be either below or above the isoelectric point (pI) of the protein in order to avoid coalescence of droplets. This results in either a positive or negative surface charge of these droplets. Similarly, the use of some surfactants such as charged phospholipids may lead to a charged oil droplet. The surface charge of emulsion droplets is important for lipid oxidation catalysed by the presence of trace metal ions, such as Fe^{2+}. With a negative surface charge, emulsion droplets will attract the potentially highly pro-oxidative trace metals, and bring them into closer proximity to the n-3 PUFA oil, thereby enhancing lipid oxidation. If instead, an emulsifier which creates a positive charge on the droplets is chosen, trace metals are repelled and oxidation is likely to be reduced.42,43 Another aspect is the fact that the solubility of trace metals generally increases at decreasing pH,27 which potentially can promote oxidation. As practically all food products contain some amounts of trace metals, the choice of an appropriate emulsifier for PUFA-enriched foods should consider the pH of the given food.

An example of the effect of pH on oxidation was the finding that in fish oil-enriched mayonnaise, lipid oxidation increased as pH decreased from 6.0 to 3.8.44 The following hypothesis was suggested to explain this phenomenon: the egg yolk used as an emulsifier in mayonnaise contains large amounts of iron, which is bound to the protein phosvitin. At the natural pH of egg yolk (pH 6.0), the iron forms cation bridges between phosvitin and other components in egg yolk, namely low density lipoproteins (LDL) and lipovitellin. These components are located at the oil–water interface in mayonnaise. When the pH is decreased to 4.0, which is the pH in mayonnaise, the cation bridges between the before-mentioned egg yolk components are broken and iron becomes dissociated from LDL and lipovitellin. Thus, iron becomes more active as a catalyst of oxidation.44,45 In contrast, lipid oxidation in salmon-oil-in-water model emulsions (5% oil) was greater and more rapid at pH 7.0 than at pH 3.0.46 These contradicting results demonstrate that in complex multiphase systems, pH may affect lipid oxidation differently through various mechanisms, and it is often necessary to neutralise trace metals by adding metal chelating compounds.

Surfactants can also influence the location of the metal ions and lipid hydroperoxides by forming micelles. This is because under normal conditions surfactants are present in excess in emulsions, and surfactants not associated with the emulsion droplets may form micelles in the continuous phase. Lipid hydroperoxides and/or metal ions could become associated with or solubilised in the micelles. When present in the micelles, these components cannot react with lipid components in the oil phase and this may, in turn, reduce lipid oxidation.47,48

Apart from influencing droplet surface charge, the emulsifier may otherwise affect the oxidative stability of the emulsions.49-51 Protein emulsifiers may
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Affect oxidative stability through the amino acid composition, as some amino acids possess antioxidative properties. For example, the sulphydryl group of cysteine has been reported to have antioxidant activity due to its ability to scavenge free radicals. Other amino acids, such as tyrosine, phenylalanine, tryptophan, proline, methionine, lysine, and histidine, have also been reported to have antioxidative effects. In model emulsions, it has also been suggested that the actual thickness of the interface layer of the droplets is important. A thicker or more dense interface could provide enhanced protection of the emulsified oil by decreasing accessibility of water-soluble pro-oxidants. Finally, the food matrix components may also influence the release of secondary volatile oxidation products, thereby affecting the release of fishy or rancid off-flavour developed during oxidation. Thus, it may be possible to ‘mask’ the rancidity by choosing the right emulsifier.

Antioxidants and metal chelators
The most thoroughly investigated area regarding oxidative stabilisation of lipid systems concerns the addition of antioxidants and antioxidant systems (natural as well as synthetic). However, compared with the number of studies performed in oil-in-water model emulsion, relatively few studies of the antioxidant mechanism in real food emulsions have been reported. In complex food systems, several factors influence the efficacy of the different types of antioxidants, and it is clearly an important issue to address during the manufacture of stable n-3 PUFA-enriched foods. The use of antioxidants in microencapsulated n-3 PUFA oil is dealt with as a special case in Section 5.3.3 concerning microencapsulation of n-3 PUFA oils.

The localisation or partitioning of antioxidants into the different phases of a food system seems to be of major importance. This is probably because the antioxidants need to be located close to where oxidation occurs. Therefore, when choosing an antioxidant for a particular food system, both the mode of action (chain breaking, O₂ scavenging or metal chelating) and the solubility/partitioning properties of the antioxidant should be considered. Several studies have shown that in model oil-in-water emulsions, non-polar antioxidants were more efficient than polar antioxidants. It has been suggested that the non-polar antioxidants were located in the oil droplets, where oxidation would propagate, whereas the polar antioxidants were solubilised in the water phase, far from where the initiation and propagation of lipid oxidation take place. Furthermore, in fish oil-enriched mayonnaise, antioxidants such as Trolox, tocopherol, propyl gallate, gallic acid, ferulic acid, caffeic acid, and catechin have been shown to interact with the interfacial layer of the emulsion. As several authors have proposed that oxidation in emulsions is initiated at the interfacial layer, such interactions with antioxidants could also affect the activity and efficiency of the antioxidants. The antioxidative effect of propyl gallate, gallic acid, tocopherol, ascorbic acid or a mixture of ascorbic acid (8.6% w/w), lecithin (86.2% w/w) and tocopherol (5.2% w/w) (the so-called A/L/T system) in fish oil-enriched mayonnaise has been
determined by sensory profiling, measurements of lipid hydroperoxides and volatiles, and in some cases also by measurements of free radical formation.\textsuperscript{44,45,59–62} Weak pro-oxidative effects of propyl gallate and gallic acid were observed.\textsuperscript{59,62} Tocopherol was inactive as an antioxidant and it even seemed to have pro-oxidative effects at higher concentrations (>140 mg/kg).\textsuperscript{60,61} Ascorbic acid (40–800 mg/kg) and the A/L/T system (200 mg/kg total concentration) were strong pro-oxidants (Table 5.2).\textsuperscript{44,45,60} The pro-oxidative effect of these antioxidant systems was suggested to be due to the ability of ascorbic acid to promote the release of iron from the egg yolk located at the oil–water interface. The released iron would then be able to decompose pre-existing lipid hydroperoxides located near the oil–water interface or in the aqueous phase. The findings that tocopherol, gallic acid and propyl gallate were ineffective as antioxidants could be due either to their interaction with the emulsifier, or due to the fact that these antioxidants are free radical scavengers that cannot prevent metal-catalysed oxidation happening at the oil–water interface.\textsuperscript{59,61,62}

In contrast to these results, it was reported that γ-tocopherol (330 mg/kg), but not α-tocopherol, was able to reduce lipid oxidation in fish oil-enriched milk.\textsuperscript{63} When both α- and γ-tocopherol were present, a slight pro-oxidative effect on oxidation was observed (Fig. 5.3). Likewise, EDTA at a concentration of 5 mg/kg did not have any effect. However, ascorbyl palmitate (300 mg/kg) was able to inhibit lipid oxidation in this food system (Fig. 5.3). It was suggested that ascorbyl palmitate exerted its antioxidative effect either via its ability to regenerate tocopherol, via its ability to act as a free radical scavenger, or from its metal-chelating properties. Ascorbyl palmitate is an amphiphilic molecule and can therefore be expected to be located at the oil–water interface where oxidation takes place. This location may have a positive influence on the antioxidative effect of ascorbyl palmitate.

**Table 5.2** Sensory scores in freshly produced mayonnaise with different addition levels of ascorbic acid illustrating the pro-oxidative effect of ascorbic acid (from Jacobsen et al.\textsuperscript{44})

<table>
<thead>
<tr>
<th>Amount of ascorbic acid added [ppm (mM)]</th>
<th>Fishy/train oil aroma</th>
<th>Rancid aroma</th>
<th>Fishy/train oil flavour</th>
<th>Rancid flavour</th>
<th>Metallic flavour</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (0)\textsuperscript{1}</td>
<td>0.4 ± 0.8\textsuperscript{a,2}</td>
<td>0.5 ± 1.2\textsuperscript{a}</td>
<td>0.2 ± 0.6\textsuperscript{a}</td>
<td>0.3 ± 0.6\textsuperscript{a}</td>
<td>0.3 ± 0.8\textsuperscript{a}</td>
</tr>
<tr>
<td>40 (0.23)</td>
<td>1.1 ± 1.2\textsuperscript{ab}</td>
<td>1.5 ± 1.8\textsuperscript{a}</td>
<td>2.7 ± 2.2\textsuperscript{b}</td>
<td>1.7 ± 2.0\textsuperscript{abcd}</td>
<td>0.4 ± 0.7\textsuperscript{a}</td>
</tr>
<tr>
<td>80 (0.45)</td>
<td>1.9 ± 1.6\textsuperscript{ab}</td>
<td>1.4 ± 1.9\textsuperscript{a}</td>
<td>2.6 ± 1.4\textsuperscript{b}</td>
<td>1.2 ± 1.9\textsuperscript{abc}</td>
<td>0.8 ± 1.3\textsuperscript{a}</td>
</tr>
<tr>
<td>200 (1.14)</td>
<td>1.8 ± 1.4\textsuperscript{ab}</td>
<td>1.4 ± 2.2\textsuperscript{a}</td>
<td>3.2 ± 1.7\textsuperscript{b}</td>
<td>2.5 ± 2.2\textsuperscript{abcd}</td>
<td>0.8 ± 1.3\textsuperscript{a}</td>
</tr>
<tr>
<td>400 (2.27)</td>
<td>2.5 ± 2.2\textsuperscript{b}</td>
<td>1.7 ± 1.7\textsuperscript{a}</td>
<td>4.2 ± 2.4\textsuperscript{b}</td>
<td>3.0 ± 2.1\textsuperscript{d}</td>
<td>1.0 ± 1.5\textsuperscript{a}</td>
</tr>
<tr>
<td>800 (4.45)</td>
<td>1.6 ± 2.2\textsuperscript{ab}</td>
<td>2.0 ± 1.9\textsuperscript{a}</td>
<td>3.5 ± 2.4\textsuperscript{b}</td>
<td>2.3 ± 1.7\textsuperscript{bcd}</td>
<td>1.2 ± 1.5\textsuperscript{a}</td>
</tr>
</tbody>
</table>

\textsuperscript{1}Values in parenthesis show the concentration of ascorbic acid in mM.

\textsuperscript{2}Values in the same column followed by the same letter are not significantly different (p < 0.05).
Healthy polyunsaturated fatty acids (PUFAs) for food enrichment

In contrast to the poor effect of free radical scavengers in fish oil-enriched mayonnaise, the metal chelator EDTA efficiently inhibited lipid oxidation in mayonnaise enriched with fish oil (Table 5.3) or with structured lipid based on sunflower oil. Likewise, EDTA efficiently inhibited lipid oxidation in fish oil-enriched salad dressing. Ascorbyl palmitate was as efficient in reducing lipid oxidation as the addition of rapeseed oil. Addition of tocopherol or EDTA to the milk with ascorbyl palmitate did not reduce oxidation further (from Let et al.63).

Table 5.3 Sensory scores during storage at 20 °C for fishy off-flavour in fish oil-enriched mayonnaise with and without 75 ppm EDTA. Sensory scale from 0 to 9 (from Jacobsen et al.61)

<table>
<thead>
<tr>
<th></th>
<th>0 weeks</th>
<th>1 week</th>
<th>2 weeks</th>
<th>3 weeks</th>
<th>4 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mayonnaise without antioxidant</td>
<td>0.2 ± 0.4</td>
<td>2.1 ± 1.5</td>
<td>2.4 ± 1.6</td>
<td>2.8 ± 1.3</td>
<td>3.4 ± 1.8</td>
</tr>
<tr>
<td>Mayonnaise with 75 ppm EDTA</td>
<td>0.2 ± 0.3</td>
<td>0.2 ± 0.4</td>
<td>0.1 ± 0.3</td>
<td>0.3 ± 0.5</td>
<td>0.2 ± 0.3</td>
</tr>
</tbody>
</table>

In contrast to the poor effect of free radical scavengers in fish oil-enriched mayonnaise, the metal chelator EDTA efficiently inhibited lipid oxidation in mayonnaise enriched with fish oil (Table 5.3) or with structured lipid based on sunflower oil. Likewise, EDTA efficiently inhibited lipid oxidation in fish oil-enriched salad dressing. In fish oil-enriched milk, low levels of EDTA (5 mg/kg) were also able to reduce lipid oxidation significantly, although not completely, when fish oil with a peroxide value (PV) of 1.5 meq/kg was used. However, when fish oil with a PV of 0.1 meq/kg was used, the emulsions were oxidatively stable and no effect of EDTA was observed. These data indicated that trace metal-catalysed lipid oxidation is very important in many food emulsions enriched with n-3 PUFA. Therefore, addition of metal chelating compounds to such foods may be an efficient way of preventing oxidation.

In model emulsions of fish or algae oil in water, it has been shown that EDTA was a pro-oxidant in molar ratios of EDTA to iron of 1:1 or lower, but
otherwise effectively inhibited oxidation at molar ratios of 2:1 and 4:1. In contrast, in fish oil-enriched mayonnaise a significant antioxidant effect of EDTA was found at an EDTA:iron ratio of 1:2. It therefore seems that the ratio between the actual concentration of trace metals and the metal-chelating compound also is of importance for inhibition of lipid oxidation, but that this ratio is additionally influenced by the particular composition of the food system.

Apart from addition of natural and synthetic purified antioxidants, another approach to obtain stable products enriched with n-3 PUFA is to mix these sensitive n-3 PUFA oils with more stable fats and oils. Claims have been made that vegetable oils, such as rapeseed (canola) oil, corn oil, sunflower oil and soybean oil, as well as animal fat, are able to stabilise fish oil against oxidation. Subsequent studies have shown that products, such as milk (Table 5.4) and spreads, containing these stabilised oils were significantly more resistant against oxidation during storage than products containing only fish oil. It was suggested that vegetable oil and fish oil should be co-refined in order to obtain optimum stability, and that the protective effect of the vegetable oils was based mainly on the natural content of antioxidants present in these oils. However, it was also claimed that the protection of unsaturated oils was based on a dilution of the unsaturated fatty acids with saturated fatty acids. Dilution of vegetable oils containing natural antioxidants with animal fats, such as beef tallow, containing no or relatively low amounts of natural antioxidants was claimed to enhance the oxidative stability. A recent study did not, however, find any significant effect on peroxide values or formation of volatiles in drinking yoghurt by using a mixture of rapeseed oil and fish oil as compared to addition of fish oil alone. This is most likely due to the fact that the oxidative stability of fish oil-enriched yoghurt products is very high and much higher than that of fish oil-enriched milk. With this high stability of fish oil-enriched yoghurt drink, it is difficult to detect an antioxidative effect by adding rapeseed oil to the fish oil.

Finally, some carbohydrates have shown antioxidative activity in high concentrations due to their ability to scavenge free radicals. Furthermore, the effect of sucrose is to increase the viscosity of the emulsion and this may decrease the diffusion coefficient of oxygen, metals, and other reaction products and reactants, which may in turn slow down oxidation rates. Fructose has been suggested as an efficient antioxidant in various meat formulations and in emulsions such as salad dressings, both enriched with fish oil.

5.3.3 Process means for optimising quality and stability of n-3 PUFA enriched food

Process and storage conditions

Production of PUFA-enriched foods includes basic operations such as homogenisation and mixing with other ingredients. Generally, the most important issues to address during production and storage of n-3 PUFA-enriched
Table 5.4 Sensory scores during storage at 2 °C in milk drink enriched with fish oil (0.5%) or a mixture of fish oil and rapeseed oil (0.25% of each), illustrating the protective effect of rapeseed oil (from Let et al.69)

<table>
<thead>
<tr>
<th></th>
<th>Fish odour</th>
<th></th>
<th>Fish taste</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 11</td>
<td>Day 4</td>
<td>Day 8</td>
<td>Day 1</td>
</tr>
<tr>
<td>Rapeseed</td>
<td>0.4a ± 0.7</td>
<td>0.0a ± 0.1</td>
<td>0.2a ± 0.4</td>
<td>0.2a ± 0.5</td>
</tr>
<tr>
<td>FR1+A</td>
<td>0.3a ± 0.5</td>
<td>0.1a ± 0.3</td>
<td>0.3a ± 0.5</td>
<td>0.6ab ± 0.8</td>
</tr>
<tr>
<td>FR2+A</td>
<td>0.1a ± 0.3</td>
<td>0.3a ± 0.5</td>
<td>0.2a ± 0.3</td>
<td>0.2a ± 0.2</td>
</tr>
<tr>
<td>FR3</td>
<td>0.7a ± 0.9</td>
<td>0.2a ± 0.4</td>
<td>0.3a ± 0.5</td>
<td>1.0bc ± 0.7</td>
</tr>
<tr>
<td>F1</td>
<td>0.4a ± 0.4</td>
<td>1.3b ± 1.1</td>
<td>1.9b ± 1.7</td>
<td>1.0bc ± 0.8</td>
</tr>
<tr>
<td>F2</td>
<td>0.4a ± 0.4</td>
<td>1.5b ± 1.4</td>
<td>1.4b ± 1.1</td>
<td>1.3c ± 1.0</td>
</tr>
</tbody>
</table>

1 Average of all 12 assessors’ determinations.
The six emulsions were compared at each day (columnwise) in Tukey’s test using 0.05-level of significance, and emulsions followed by same letter are not significantly different.
FR 0.25% fish oil and 0.25% rapeseed oil.
F 0.5% fish oil.
1 and 2 refer to different deodorisation procedures of the same cod liver oil.
3 refers to tuna oil.
A refers to antioxidants added to the oil.
enriched foods are control of oxygen access, control of temperature and reduction of light.

Oxygen is necessary for the propagation of lipid oxidation. It is therefore important to avoid contact between the n-3 PUFA oils and headspace oxygen, dissolved oxygen and trapped air bubbles during both processing and storage. Several studies have shown that a reduction in the access of oxygen retards lipid oxidation. Reduction of dissolved or trapped oxygen can be achieved by processing under vacuum or in a nitrogen atmosphere. In the final product, exclusion of headspace oxygen can be achieved by packaging in an air-tight container impermeable to oxygen, and preferably under modified atmosphere.

The mechanisms of lipid oxidation change with temperature, especially above 60°C. Additionally, lipid hydroperoxides from different fatty acids decompose into secondary volatile oxidation products at different temperatures. Therefore, it is difficult to predict the effect of temperature on lipid oxidation during processing and storage of complex food systems such as n-3 PUFA-enriched foods. However, as temperature affects oxidation rates in an exponential manner, limited temperature increases and otherwise strict control of temperature are required to achieve stability in such foods.

Apart from lipid autoxidation, n-3 PUFA-enriched foods may undergo photo-oxidation. Photo-oxidation requires light, oxygen and the presence of a photosensitising compound in the food, as previously described. Therefore, the access of light to n-3 PUFA-enriched foods should be restricted in order to enhance storage stability.

Finally, the physical structure of the oil-in-water emulsions obtained during processing of the n-3 PUFA-enriched foods may be of importance to lipid oxidation. To obtain a physically stable emulsion, the oil droplet size is reduced during emulsification, which results in the formation of a large interfacial area, increasing the contact between the oil and the water phases. Initiation of lipid oxidation is suggested to occur at the interface, as the oil droplets become exposed to the water-soluble pro-oxidants and dissolved oxygen, via diffusion through the interfacial membrane. However, the potential presence of antioxidants, unsaturated phospholipids and other amphiphilic compounds at the interface, as well as the physical packaging of the interfacial membrane, are also able to affect oxidation and thus the impact of droplet size on oxidation is complex and depends on the composition of the particular food product. In fish oil-enriched mayonnaises with small droplet sizes, lipid oxidation was faster in the initial part of the storage period than in mayonnaise with larger droplets, whereas no effect of droplet size on oxidative flavour deterioration was observed in the later part of the storage period. The following mechanism to explain these findings was suggested: in the initial oxidation phase, a small droplet size, i.e. a large interfacial area, would increase the contact area between iron located in the aqueous phase and lipid hydroperoxides located at the interface, and this would increase oxidation. In the later stage, oxidation proceeds inside the oil droplet and therefore the droplet size is less important. In fish oil-enriched milk, almost the reverse
relationship between droplet size and oxidative stability was observed.\textsuperscript{81,82} In this product, the smallest droplet size and highest oxidative stability was obtained by homogenization at high temperature (72°C) and pressure (225 Bar), whereas homogenization at lower temperature and pressure resulted in lower oxidative stability and bigger droplets. SDS-PAGE and confocal laser scanning microscopy (CLSM) indicated that the conditions of homogenization also affected the protein composition of the oil–water interface in the milk emulsions. High temperature resulted in an increase in β-lactoglobulin adsorbed at the oil–water interface, and this was even more pronounced with higher pressure. In contrast, less casein seemed to be present at the oil–water interface with increasing pressure. It was hypothesized that a combination of more β-lactoglobulin and less casein at the oil–water interface was responsible for the increased oxidative stability. Hence, these results demonstrated that the composition of the interface, including unfolding of proteins at the interface, is very important and that droplet size may have an impact on lipid oxidation only under certain circumstances.\textsuperscript{82}

\textit{Pre-emulsification}

One strategy to produce n-3 PUFA-enriched foods is to prepare a pre-emulsion of the n-3 PUFA oil, which is then to be added to the finished or semi-finished food product. This approach has long been known, for example, regarding fortification with fat-soluble vitamins and fish oil,\textsuperscript{83} and has been attempted in products such as various milk drinks and tofu.\textsuperscript{84,85} A recent study by Park \textit{et al.} has reported a procedure for the production of n-3 PUFA-enriched surimi, using an algal oil stabilised by tocopherols, ascorbyl palmitate and rosemary extract, which was emulsified in water by whey protein isolate (WPI).\textsuperscript{86} This emulsion was subsequently mixed with the semi-finished fish product and mixed into the final surimi product. Djordjevic \textit{et al.} determined the optimum conditions for producing WPI-stabilised oil-in-water emulsions, with a high content of n-3 PUFA and a low viscosity, that could be used for incorporation of n-3 PUFA in foods.\textsuperscript{87} Subsequently, they evaluated the oxidative stability of oil-in-water emulsions (25% oil) stabilised either by casein or WPI.\textsuperscript{88} They found that PV was significantly higher in the WPI-stabilised emulsions compared with the casein-stabilised emulsions, but that there was no significant difference in the formation of headspace propanal. Moreover, they observed that it was difficult to dissolve casein at low pH, which makes it impractical to use this protein from a technological standpoint.\textsuperscript{87} Another problem when using casein was that the viscosity increased steeply at high oil concentrations. Because of these findings, they suggested that WPI-stabilised oil-in-water emulsions (pH 3) could be used to produce oxidatively and physically stable n-3 PUFA delivery systems.

The idea behind the pre-emulsification strategy is, first of all, to reduce the extent of processing of the oil, e.g. to reduce the amount of stresses such as heat, oxygen and access of light, which are otherwise necessary for the production of the final product.
production of the particular food product. Additionally, the contact between the n-3 PUFA oil and the potential pro-oxidant compounds of the food product during processing is reduced by adding the oil in an already stabilised pre-emulsion as the final step of processing. Finally, by using pre-emulsification it is possible to design a stable emulsion by choosing an optimum combination of emulsifier(s), antioxidants and, for example, stabilisers. However, when designing such pre-emulsions, it seems necessary to take into account the composition and physical properties of the final product to which the pre-emulsion is to be added. Complete avoidance of exposure of the n-3 PUFA oil and thus contact with remaining product ingredients in the final product is dependent on the physical stability of the pre-emulsion over time. If the pre-emulsion interacts with other product components, or if diffusion occurs across the emulsion droplet interface, the n-3 PUFA oil might, in time, get into contact with the remaining ingredients of the product. Therefore, it is likely that the pre-emulsification strategy may be more suitable for some products than for others, as also observed by Let et al. 66 They found that yoghurt and salad dressing enriched with neat fish oil were more oxidatively stable than those enriched with a WPI stabilised fish-oil-in-water emulsion, whereas milk enriched with neat fish oil was less stable than milk enriched with the WPI stabilised fish-oil-in-water emulsion. It was suggested that the improved oxidative stability observed in milk enriched with the WPI stabilised fish oil emulsion was most likely due to the higher concentration of whey protein in the fish-oil-in-water emulsion (1.5 wt%) compared to pasteurized milk (around 0.35 wt%), which could lead to an improved surface coverage, previously shown to improve the oxidative stability. Secondly, denaturation of the whey protein exposes the hydrophobic regions, which makes it adsorb better onto the oil–water interface.

**Microencapsulation**

Another approach to reduce contact between the oxidatively susceptible n-3 PUFA oils and atmospheric oxygen, as well as the other ingredients of the food product, is to use microencapsulated oils. This microencapsulation approach is used in a large variety of products, mainly in dry formulations and products such as milk and infant formula powders. Microencapsulation of fats and oils basically consists of an emulsion stabilised by modified starch or hydrocolloids and/or proteins, which is either spray or freeze dried to produce a powder. A non-emulsifying water-soluble material such as sugar or hydrolysed starch is used as filler. 89 Similar to fluid emulsions, the oxidative stability of microencapsulated PUFA oils depends on processing conditions and the choice of emulsifier and antioxidant addition. 89,90 The individual processing steps have been shown to stress the oil, resulting in increased PV. 91,92 Additionally, the oxidative stability of microencapsulated n-3 PUFA oil depends on molecular diffusion through the protective wall matrix and maintenance of the structural integrity that keeps emulsified lipids within each powder particle.
Kagami et al.\textsuperscript{90} investigated the effect of various emulsifiers and fillers and found that encapsulates stabilised by sodium caseinate in combination with highly branched cyclic dextrin produced from waxy corn starch were more stable than encapsulates made with sodium caseinate and maltodextrin, or combinations of whey protein and highly branched cyclic dextrin.

Another study by Keogh et al.\textsuperscript{89} regarding emulsifiers showed that a low level of off-flavour and a shelf-life of 31 weeks at 4 °C can be obtained using only dairy ingredients as encapsulate material of a fish oil powder. The results also showed that the shelf-life increased when the free non-encapsulated fat and vacuole volume of the powder decreased. They did not find any effect of the surface fat. A study by Velasco et al.\textsuperscript{93} on the oxidative stability of fish oil powder stabilised by ascorbic acid, lecithin and tocopherol stored in open Petri dishes found that oxidation was slower in the free oil fraction compared with the encapsulated fraction.

Several studies have investigated the effects of different antioxidants in encapsulates. Hogan et al. investigated the antioxidative effects of tocopherol and its hydrophilic analogue Trolox C in fish oil encapsulates prepared from herring oil, emulsified and stabilised by sodium caseinate and maltodextrin, respectively.\textsuperscript{94} They observed that all antioxidants had reduced oxidation in the powders after 14 days of storage at 4 °C. Similarly, Baik et al.\textsuperscript{92} showed that α-tocopherol inhibited oxidation significantly in microencapsulated menhaden oil, while ascorbyl palmitate was much less efficient. However, it should be noticed that PV was high in both studies, ranging from 10 meq/kg in the freshly produced powders to 60 meq/kg after 1 to 4 weeks of storage. It is possible that the effects of the antioxidants would be less pronounced in powders with lower initial PV.

Heinzelmann et al.\textsuperscript{91} showed that optimum shelf-life of an encapsulated fish oil was achieved by a combination of ascorbic acid, lecithin and tocopherol (A/L/T-system). In the study by Velasco, oxidation of a fish oil powder was slightly delayed by the A/L/T system compared to a non-stabilised powder. The oxidative stability seemed more dependent on the storage conditions, which was either light or dark, with or without air.\textsuperscript{93} Oxidation was stopped in the microencapsulated fish oil stabilised by ascorbic acid, lecithin and tocopherol which was stored under vacuum.

Finally, other storage conditions, such as relative humidity, have been shown to influence oxidation of microencapsulated fat and surface fat differently during storage.\textsuperscript{95} However, this study was performed on encapsulated milk fat. Oxidation of encapsulated fat was maximum at a water activity ($a_w$) of 0.52, and decreased with decreasing $a_w$, minimum oxidation of surface fat being observed at an $a_w$ of 0.52. In the study by Baik et al., the relative humidity had only very slight effect on the oxidative stability of fish oil encapsulate effectively stabilised by α-tocopherol, as determined by thiobarbituric acid reactive species (TBARS).\textsuperscript{92}
5.3.4 Recommendations

On the basis of the above summary of how lipid oxidation can be reduced during production of fish oil and in products enriched with n-3 PUFA the following strategies to avoid lipid oxidation are suggested:

- Reduce transportation time, exposure to heat and light, and minimise bleeding of fish to be used for fish oil production.
- Do not use too high a temperature during refining and deodorisation of the fish oil and reduce exposure to light and oxygen to a minimum.
- Exclude oxygen from the food system, for example by packaging under vacuum.
- Store the enriched products at chilled temperatures.
- Ensure that ingredients have a low content of hydroperoxides, transition metals and other pro-oxidants. It seems to be especially important that n-3 PUFA oils have a low PV. Therefore, these oils should be stored at low temperatures (< 0°C), in the dark, with reduced oxygen, and the fish oils should be used as fast as possible after deodorisation as hydroperoxides will form even at temperatures below 0°C.
- Beware that the choice of emulsifier may significantly affect lipid oxidation rates. Therefore, when applying n-3 oils in a new food product it may be necessary to reformulate the conventional recipe to include other emulsifier types.
- Use metal chelators such as EDTA, citric acid, proteins, polysaccharides and metallic chelating plant polyphenols to prevent lipid hydroperoxide decomposition.
- Addition of free radical chain breaking antioxidants may further reduce lipid oxidation. Select antioxidants that will be located where they are required, i.e. normally near the oil–water interface, where the decomposition of lipid hydroperoxides takes place.
- Optimise the processing conditions. In some food systems the particle/droplet size will affect the oxidation rates, in other foods they may not. Therefore, this issue should be taken into consideration. In addition, the emulsification process may disrupt natural membranes that may protect the fish oil from protein-bound metals. Emulsification processes should be optimised to minimise lipid oxidation.

5.4 Future trends

As mentioned in the introduction to this chapter, the US Food and Drug Administration allowed a qualified health claim on n-3 PUFA-enriched food products in September 2004, and later a similar health claim was also allowed in the UK. It may be expected that this will lead to a growing industrial interest in exploiting the health effects of these oils in the US and in the UK. A new regulation on health claims was adopted in the EU in December 2006.
Whether similar health claims on n-3 PUFA will also be allowed in the rest of the EU is not known at this time. Nevertheless, due to increased public awareness about the beneficial effects of n-3 PUFAs, it is expected that more new food products enriched with these materials will enter the market place in the coming years.

The infant’s requirements for DHA have received substantial attention recently. Therefore, a promising new area is food for the young. For example, several infant formulae enriched with DHA are now on the market. It is likely that efforts will also be made to develop other types of baby foods enriched with n-3 PUFA in the years to come.

Ice cream producers are now also targeting health conscious consumers. Recently, a number of new fat-reduced ice cream formulations, as well as calcium-enriched ice creams, have entered the market. Efforts are currently being made to develop ice cream enriched with n-3 LC PUFA.

Dairy products are the fastest growing product within the functional food area. So far, functional dairy products have mostly been ‘functional’ due to the addition of probiotic bacteria and consumers already perceive some dairy products as being healthy. Therefore, milk drinks and yoghurts may be a good vehicle for n-3 PUFA enrichment and we may see a number of new products in this category in the future.

This chapter has dealt mainly with EPA and DHA from marine sources. However, with the increased focus on the beneficial effects of n-3 PUFA in general, products enriched with LNA will most likely also receive more attention from the industry. Efforts are also being made to develop plants with a high level of EPA and DHA by genetic engineering.

Traditionally, the industry has mainly used free radical chain-breaking synthetic antioxidants for the prevention of oxidation in foods. However, this strategy seems to be less efficient in preventing lipid oxidation in emulsified food systems enriched with n-3 PUFA. With our increased understanding of the important role of trace metals, emulsifiers and processing conditions in the lipid oxidation processes, more efforts will be dedicated to use this knowledge to develop alternative strategies to retard lipid oxidation in real foods with n-3 PUFA oils. One such strategy may be an increased use of both synthetic and natural metal chelators. Another strategy may be to design oxidatively stable oil-in-water emulsion delivery systems for each particular food system.

5.5 Sources of further information and advice

The Omega-3 Information Network at:
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Healthy polyunsaturated fatty acids (PUFAs) for food enrichment

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The stability of vitamins in fortified foods and supplements

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6.1 Introduction

Vitamins, by their definition, are essential to health and have to be obtained from the diet on a regular basis as, with the exception of vitamin D, they cannot be produced by the body. In terms of medicine and nutrition, our knowledge of vitamins is relatively recent. Although Lind discovered an association between lime juice and scurvy in 1753, it was over 170 years later that vitamin C was eventually isolated. The understanding of vitamin B\textsubscript{12} only goes back to the 1950s and new roles for folates were still being discovered in the late 1990s.

Man’s supply of vitamins is obtained from a varied diet of vegetables, cereals, fruits and meats and the quantities of vitamins that are present in the dietary sources can be affected significantly by the processing and storage of the food.

6.2 The vitamins

The vitamins are a heterogeneous group of substances that fall into the classification of being vital nutrients that have to be obtained from the diet. Although a number of substances were termed vitamins between the 1930s and 1950s, nutritional science now recognises only 13 substances, or groups of substances, as being true vitamins. The 13 substances are divided into two categories, the fat-soluble vitamins and the water-soluble vitamins. These are listed in Table 6.1. Even within the two sub-categories, the vitamins have almost no common attributes in terms of chemistry, function or daily...
requirements. In terms of requirements some, such as vitamins C, E and niacin, are needed in tens of milligrams a day whilst others, such as vitamins D and B_{12}, are required only in single microgram amounts. It can be seen from these examples that there is no relationship between the form of delivery (i.e. fat or water soluble) and the daily requirements. The heterogeneity also applies to the chemical structure and the functions of the vitamins. Chemically, there are no similarities between the substances. Some are single substances, such as biotin, whilst others, such as vitamin E, are groups of compounds all exhibiting vitamin activity.

### 6.3 Factors affecting vitamin stability

One of the very few attributes that the vitamins have in common is that none are completely stable in foods. The stability of the individual vitamins varies from the relatively stable, such as in the case of niacin, to the relatively unstable, such as vitamin B_{12}. The factors that affect the stability vary from vitamin to vitamin and the principal ones are summarised in Table 6.2. The most important of these factors are heat, moisture, oxygen, pH and light.

The deterioration of vitamins can take place naturally during the storage of vegetables and fruits, and losses can occur during the processing and preparation of foods and their ingredients, particularly those subjected to heat treatment. The factors that affect the degradation of vitamins are the
same whether the vitamins are naturally occurring in the food or are added to the food from synthetic sources. However, the form in which a synthetic source is used (e.g. a salt or ester) may enhance its stability. For example, the vitamin E (tocopherol) esters are more stable than the alcohol form.

With the increased use of nutritional labelling of food products, vitamin levels in foods have become the subject of label claims that can be easily checked by the enforcement authorities. This poses a number of problems for the food technologist. When more than one vitamin is the subject of a quantitative label claim for a fortified food or supplement, it is very unlikely that the vitamins will deteriorate at the same rate. If the amounts of these vitamins are included in nutritional labelling, the shelf life of the food is determined by the life of the most unstable component. In order to comply with the legal requirements of maintaining the label claim throughout the declared life of a food product, the technologist needs to obtain a reasonably accurate estimation of the stability of each of the vitamins in the product. This has to be evaluated in the context of the food system (solid, liquid, etc.), the packaging, and probable storage conditions, and is achieved by conducting well-designed stability tests.

It is interesting to note that most of the scientific literature on the stability of vitamins in foods and supplements relates to work carried out in the 1970s and 1980s. Whilst ‘in house’ stability studies have been carried out by many larger companies on their specific products, much of this data remains confidential to the companies. There are still a number of unanswered questions on vitamin stability, particularly with regard to the stability in different processed food matrices.

### 6.4 Fat-soluble vitamins

#### 6.4.1 Vitamin A

Nutritionally, the human body can obtain its vitamin A requirements from two sources: from animal sources as forms of retinol, and from plant sources from beta-carotene and related carotenoids. Both sources provide a supply of

<table>
<thead>
<tr>
<th>Table 6.2 Factors affecting the stability of vitamins</th>
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<tr>
<td>Temperature</td>
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<td>Moisture</td>
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<td>pH</td>
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<tr>
<td>Oxidising and reducing agents</td>
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<td>Presence of metallic ions (e.g. copper, iron)</td>
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<tr>
<td>Other components of food such as sulphur dioxide</td>
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<tr>
<td>Presence of other vitamins</td>
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<td>Combinations of the above</td>
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vitamin A, but by different metabolic pathways. In terms of stability the two sources are different from each other.

**Retinol**

Vitamin A is one of the more labile vitamins, with retinol being less stable than the retinyl esters. The presence of double bonds in its structure makes it subject to isomerisation, particularly in an aqueous medium at acid pH. The isomer with the highest biological activity is the all-trans vitamin A. The predominant cis isomer is 13-cis or neovitamin A, which has a biological activity of only 75% of the all-trans isomer; and 6-cis and 2, 6-di-cis isomers, which may also form during isomerisation, have less than 25% of the biological activity of the all-trans form of vitamin A. Natural vitamin A sources usually contain about one third neovitamin A while most synthetic sources generally contain considerably less. For aqueous products where isomerisation is known to occur, mixtures of vitamin A palmitate isomers at the equilibrium ratio have been produced commercially. Vitamin A is relatively stable in alkaline solutions.

Vitamin A is sensitive to atmospheric oxygen, with the alcohol form being less stable than the esters. Decomposition is catalysed by the presence of trace minerals. As a consequence of its sensitivity to oxygen, vitamin A is normally available commercially as a preparation that includes an antioxidant and often a protective coating. While butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are permitted in a number of countries for use as antioxidants in vitamin A preparations, the recent trend has been towards the use of tocopherols (vitamin E).

Both retinol and its esters are inactivated by the ultraviolet component of light.

In general, vitamin A is relatively stable during food processes involving heating, with the palmitate ester more stable to heat than retinol. It is normally regarded as stable during milk processing, and food composition tables give only small differences between the retinol contents of fresh whole milk, sterilised and ultra high temperature (UHT) treated milk. However, prolonged holding of milk at high temperatures in the presence of air can be shown to result in a significant decrease in the vitamin A activity.

**β-Carotene as provitamin A**

A provitamin is a compound that can be converted in the body to a vitamin. There are a small number of carotenoids with provitamin A activity. These compounds are generally found as naturally occurring plant pigments that give the characteristic yellow, orange and red colours to a wide range of fruits and vegetables. Some can also be found in the liver, kidney, spleen and milk. The provitamin A with the greatest nutritional and commercial importance is β-carotene.

The stability of the carotenoids is similar to vitamin A in that they are sensitive to oxygen, light and acid media.
It has been reported that treatment with sulphur dioxide reduces carotenoid destruction in vegetables during dehydration and storage. A study with model systems showed that the stability of β-carotene was greatly enhanced by sulphur dioxide added either as a sulphite solution to cellulose powder prior to β-carotene absorption or as a headspace gas in containers of β-carotene. While it was found that the β-carotene stability was improved by increasing the nitrogen levels in the containers, the stability was even greater when the nitrogen was replaced by sulphur dioxide. Comparative values for the induction period were 19 hours for β-carotene samples stored in oxygen only, 120 hours in nitrogen, and 252 hours in sulphur dioxide.²

There is some evidence of a protective effect from ascorbic acid on β-carotene and other provitamin A carotenoids both in liquid and powder form³,⁴. It would appear that, in these circumstances, the ascorbic acid is acting as an antioxidant protecting the carotenoids from rapid oxidation.

Products containing β-carotene should be protected from light and headspace air should be kept to the minimum.

6.4.2 Vitamin E
A number of naturally occurring substances exhibit vitamin E activity, including the alpha, beta, gamma and delta tocopherols and alpha tocotrienols. Dietary sources of vitamin E are found in a number of vegetables and cereals, with some vegetable oils such as wheatgerm, sunflower seed, safflower seed and maize oils being particularly good sources. Both synthetic and naturally sourced forms of vitamin E are available commercially. Whilst the natural sources of the tocopherols, which also have the highest biological activity, are in the ‘d’ form, the synthetic versions can only be produced in the ‘dl’ form. Both the ‘d’ and ‘dl’ forms are also commercially available as esters.

There is a considerable difference in the stability of the tocopherol forms of vitamin E and the tocopherol esters. While vitamin E is regarded as being one of the more stable vitamins, the unesterified tocopherol is less stable due to the free phenolic hydroxyl group.

Vitamin E is unusual in that it exhibits reduced stability at temperatures below freezing. The explanation given for this is that the peroxides formed during fat oxidation are degraded at higher temperatures but are stable at temperatures below 0°C and, as a consequence, can react with the vitamin E.⁵ It has also been shown that α-tocopherol may function as a pro-oxidant in the presence of metal ions such as iron.

α-tocopherol is readily oxidised by air. It is stable to heat in the absence of air but is degraded if heated in the presence of air. α-tocopherol is readily oxidised during the processing and storage of foods. One of the most important naturally occurring sources of tocopherols are the vegetable oils, particularly wheat germ and cottonseed oils. While deep-frying of the oils may result in a loss of vitamin E of around 10%, it has been found that the storage of fried
foods, even at temperatures as low as –12°C, can result in very significant losses.

dl-α-Tocopheryl acetate is relatively stable in air but is hydrolysed by moisture in the presence of alkalis or strong acids to free tocopherols.

6.4.3 Vitamin D

Present in nature in several forms, dietary vitamin D occurs predominantly in animal products, with very little being obtained from plant sources. Vitamin D₃ or cholecalciferol is derived in animals, including man, from ultra-violet irradiation of 7-dehydrocholesterol found in the skin. Human requirements are obtained both from the endogenous production in the skin and from dietary sources. Vitamin D₂ (ergocalciferol) is produced by ultra-violet irradiation of ergosterol, which is widely distributed in plants and fungi. Both vitamins D₂ and D₃ are manufactured for commercial use. Both vitamins D₂ and D₃ are sensitive to light and can be destroyed relatively rapidly if exposed to light. They are also adversely affected by acids.

Preparations of vitamin D in edible oils are more stable than the crystalline forms, and the vitamin is normally provided for commercial usage as an oil preparation or stabilised powder containing an antioxidant (usually tocopherol). The preparations are usually provided in lightproof containers with inert gas flushing.

The presence of double bonds in the structure of both forms of vitamin D can make them susceptible to isomerisation under certain conditions. Studies have shown that the isomerisation rates of ergocalciferol and cholecalciferol are almost equal. Isomerisation in solutions of cholecalciferol resulted in an equilibrium being formed between ergocalciferol and precalciferol with the ratios of the isomers being temperature dependent. The isomerisation of ergocalciferol has been studied in powders prepared with calcium sulphate, calcium phosphate, talc and magnesium trisilicate. It was found that the isomerisation was catalysed by the surface acid of these additives.⁶

Crystalline vitamin D₂ is sensitive to atmospheric oxygen and will show signs of decomposition after a few days storage in the presence of air at ambient temperatures. Crystalline cholecalciferol (D₃) is also destroyed by atmospheric oxygen but is relatively more stable than D₂, possibly due to the fact that it has one less double bond.

The vitamin D₃ naturally occurring in milk appears to be relatively stable to heat processing.

6.4.4 Vitamin K

Vitamin K occurs in a number of forms. Vitamin K₁ (phytomenadione or phylloquinone) is found in green plants and vegetables, potatoes and fruits, while vitamin K₂ (menaquinone) can be found in animal and microbial materials.
The presence of double bonds in both vitamins $K_1$ and $K_2$ makes them liable to isomerisation. Vitamin $K_1$ has only one double bond in the side chain, in the 3-position, whereas in $K_2$ double bonds recur regularly in the side chain. Vitamin $K_1$ exists in the form of both trans and cis isomers. The trans isomer is the naturally occurring form and is the one that is biologically active. The cis form has no significant biological activity.

The various forms of vitamin $K$ are relatively stable to heat and are retained after most heating processes. The vitamin is destroyed by sunlight and is decomposed by alkalis. Vitamin $K_1$ is only slowly decomposed by atmospheric oxygen.

Vitamin $K$ is rarely added to food products but is found in supplements and the most common commercially available form is $K_1$ (phytomenadione), which is insoluble in water. A water-soluble $K_3$ is available as menadione sodium bisulphite.

### 6.5 Water-soluble vitamins

The water-soluble vitamin group contains eight vitamins collectively known as the B-Complex vitamins plus vitamin C (ascorbic acid).

#### 6.5.1 Thiamin (vitamin $B_1$)

Thiamin is widely distributed in living tissues. In most animal products it occurs in a phosphorylated form, and in plant products it is predominantly in the non-phosphorylated form. Commercially, it is available as either thiamin hydrochloride or thiamin mononitrate. Both these salts have specific areas of application and their use depends on the product matrix to which they are added.

A considerable amount of research has been carried out on the heat stability of thiamin and its salts, particularly in the context of cooking losses. Early work on thiamin losses during bread making showed an initial cleavage of the thiamin to pyrimidine and thiazole. The destruction of thiamin by heat is more rapid in alkaline media. Vitamin $B_1$ losses in milk, which has an average fresh content of 0.04 mg thiamin per 100 g, are normally less than 10% for pasteurised milk, between 5 and 15% for UHT milk and between 30 and 40% for sterilised milk. Between 30 and 50% of the vitamin $B_1$ activity can be lost during the production of evaporated milk. Losses of thiamin during the commercial baking of white bread are between 15 and 20%. Part of this loss is due to the yeast fermentation, which can convert thiamin to cocarboxylase, which is less stable than thiamin.

Thiamin is very sensitive to sulphites and bisulphites, as it is cleaved by sulphite. This reaction is rapid at high pH and is the cause of large losses of the vitamin in fortified juices, drinks and liquid supplements where sulphites
and bisulphites are used as preservatives. Where the pH is low, such as in citrus fruit juices, the bisulphite occurs mainly as the unionised acid, and thiamin losses in such systems are not significantly different from those in products not containing bisulphite. It has also been reported that thiamin is cleaved by aromatic aldehydes.

Thiamin is decomposed by both oxidising and reducing agents. If it is allowed to stand in alkaline solution in air, it is oxidised to the disulphide and small amounts of thiothiazolone. Thiamin becomes increasingly unstable in alkaline solutions as the pH increases. The stability of the vitamin in low pH solutions such as fortified fruit drinks is very good.

A range of food ingredients has been shown to have an effect on the stability of thiamin. In general, proteins are protective of the vitamin, particularly food proteins such as egg albumin and casein. When heated with glucose, either as a dry mixture or in solution, a browning analogous to a Maillard reaction can occur. This reaction is similar to the reaction between sugars and amino acids and may be important in the loss of thiamin during heat processing. Work has shown that fructose, inverts, mannitol and inositol can actually retard the rate of destruction of thiamin.

In common with some other vitamins, the stability of thiamin is adversely affected by the presence of copper ions. This effect can be reduced by the addition of metal-chelating compounds such as calcium disodium ethylenediamine tetra-acetate (EDTA). The heavy metals appear to influence thiamin stability only when they are capable of forming complex anions with constituents of the medium.

The enzymes, thiaminases, which are present in small concentrations in a number of animal and vegetable food sources, can degrade thiamin. These enzymes are most commonly found in a range of seafoods such as shrimps, clams and raw fish, but are also found in some varieties of beans, mustard seed and rice polishings. Two types of thiaminases are known and these are designated thiaminase I and thiaminase II. The former catalyses the decomposition of the thiamine by a base-exchange reaction, involving a nucleophilic displacement of the methylene group of the pyrimidine moiety. Thiaminase II catalyses a simple hydrolysis of thiamin.

A problem associated with the addition of vitamin B1 to fortified food products is the unpleasant flavour and odour of the thiamin salts. The breakdown of thiamin, particularly during heating, may give rise to off-flavours, and the compounds derived from the degradation of the vitamins are believed to contribute to the ‘cooked’ flavours in a number of foods. However, both thiamin hydrochloride and mononitrate are relatively stable to atmospheric oxygen in the absence of light and moisture, and both are normally considered to be very stable when used in dry products with light and moisture-proof packaging.
6.5.2 Riboflavin (vitamin B<sub>2</sub>)
Riboflavin is the most widely distributed of all the vitamins and is found in all plant and animal cells, although there are relatively few rich food sources. It is present naturally in foods in two bound forms, riboflavin mononucleotide and flavin adenine dinucleotide. Plants and many bacteria can synthesise riboflavin and it is also found in dietary amounts in dairy products. Riboflavin is available commercially as a crystalline powder that is only sparingly soluble in water. As a consequence, the sodium salt of riboflavin-5′-phosphate, which is more soluble in water, is used for liquid preparations.

The most important factor influencing the stability of this vitamin is light, with the greatest effect being caused by light in the 420 to 560 µm range. Fluorescent light is less harmful than direct sunlight, but products in transparent packaging can be affected by strip lighting in retail outlets.

Riboflavin and riboflavin phosphate are both stable to heat and atmospheric oxygen, particularly in an acid medium. In this respect, riboflavin is regarded as being one of the more stable vitamins. It is degraded by reducing agents and becomes increasingly unstable with increasing pH. While riboflavin is stable to the heat processing of milk, one of the main causes of loss in milk and milk products is from exposure to light. Liquid milk exposed to light can lose between 20 and 80% of its riboflavin content in two hours, with the rate and extent of loss being dependent upon the light intensity, the temperature and the surface area of the container exposed.

6.5.3 Niacin
The term ‘niacin’ is generic for both nicotinic acid and nicotinamide (niacinamide) in foods. Both forms have equal vitamin activity, both are present in a variety of foods, and both are available as commercial isolates.

Niacin occurs naturally in the meat and liver of hoofed animals, and also in some plants. In maize and some other cereals it is found in the form of niacytin, which is bound to polysaccharides and peptides in the outer layers of the cereal grains and is unavailable to man unless treated with a mild alkali.

Both forms of niacin are normally very stable in foods as they are stable to atmospheric oxygen, heat and light in both aqueous and solid systems.

6.5.4 Pantothenic acid
In nature, pantothenic acid is widely distributed in plants and animals, but is rarely found in the free state as it forms part of the coenzyme A molecule. It is found in yeast and egg yolk and in muscle tissue, liver, kidney and heart of animals. It is also found in a number of vegetables, cereals and nuts.

Pantothenic acid is optically active and only its dextro-rotary forms have vitamin activity. Losses of pantothenic acid during the preparation and cooking of foods are normally not very large. Milk generally loses less than 10% during processing.
Free pantothenic acid is an unstable and very hygroscopic oil. Commercial preparations are normally provided as calcium or sodium salts. The alcohol form, panthenol, is available as a stable liquid but is not widely used in foods. The three commercial forms, calcium and sodium D-pantothenate and D-pantothenol, are moderately stable to atmospheric oxygen and light when protected from moisture. All three compounds are hygroscopic, with sodium pantothenate being the worst.

Aqueous solutions of both the salts and the alcohol form are thermolabile and will undergo hydrolytic cleavage, particularly at high or low pH. The compounds are unstable in both acid and alkaline solutions and maximum stability is in the pH range of 6 to 7. Aqueous solutions of D-pantothenol are more stable than the salts, particularly in the pH range 3 to 5.

### 6.5.5 Folic acid/folates

Folic acid (pteroylglutamic acid) does not occur in nature but can be produced commercially. The naturally occurring forms are a number of derivatives collectively known as folates or folacin, which contain one or more linked molecules of glutamic acid. Polyglutamates predominate in fresh food, but on storage these can slowly break down to monoglutamates and oxidise to less biologically available folates. The folic acid synthesised for food fortification has only one glutamic group.

For many years folic acid has been the only source of this vitamin for food fortification and supplementation. An isolated folate, 5-methyltetrahydrofolic acid, became available in 1999. By 2005, following official safety reviews and approvals, it was considered suitable for use in foods and supplements.11

Most of the stability studies have been carried out with the commercially available folic acid, which has been found to be moderately stable to heat and atmospheric oxygen. In solution, it is stable at around pH 7 but becomes increasingly unstable in acid or alkali media, particularly at pH less than 5. Folic acid is decomposed by oxidising and reducing agents. Sunlight, and particularly ultraviolet radiation, has a serious effect on the stability of folic acid. Cleavage by light is more rapid in the presence of riboflavin. This reaction can be retarded by the addition of the antioxidant BHA to solutions containing folic acid and riboflavin.12

The stability of the folates in foods during processing and storage is variable. Folic acid loss during the pasteurisation of milk is normally less than 5%. Losses in the region of 20% can occur during UHT treatment and about 30% loss is found after sterilisation. UHT milk stored for three months can lose over 50% of its folic acid. The extra heat treatment involved in boiling pasteurised milk can decrease the folic acid content by 20%.

Stability studies carried out on 5-methyltetrahydrofolic acid showed that its degradation in all the model systems could be described by first-order reaction kinetics.13 The thermostability of the folate was enhanced at a pH
of 7. The study also investigated the pressure stability of the folate in fruit and vegetable juices subjected to high pressure processing and at different temperature/pressure combinations. It was found that it was relatively pressure stable at temperatures lower than 40°C and that both the temperature and pressure stabilities were enhanced in the presence of ascorbic acid.

6.5.6 Vitamin B₆ (pyridoxine)

Vitamin B₆ activity is shown by three compounds, pyridoxol, pyridoxal and pyridoxamine. These are often considered together as pyridoxine. Vitamin B₆ is found in red meat, liver, cod roe and liver, milk and green vegetables. The commercial form normally used for food fortification is the salt, pyridoxine hydrochloride.

Pyridoxine is normally stable to atmospheric oxygen and heat. Decomposition is catalysed by metal ions. Pyridoxine is sensitive to light, particularly in neutral and alkaline solutions. One of the main causes of loss of this vitamin in milk is sunlight, with a 21% loss being reported after eight hours exposure.⁷

Pyridoxine is stable in milk during pasteurisation but about 20% can be lost during sterilisation. Losses during UHT processing are around 27%,¹⁴ but UHT milk stored for three months can lose 35% of this vitamin. Average losses as a result of roasting or grilling of meat are 20%, with higher losses (30 to 60%) in stewed and boiled meat. Cooking or canning of vegetables results in losses of 20 to 40%.

6.5.7 Vitamin B₁₂

The most important compound with vitamin B₁₂ activity is cyanocobalamin. This has a complicated chemical structure and occurs only in animal tissue and as a metabolite of certain micro-organisms. The other compounds showing this vitamin activity differ only slightly from the cyanocobalamin structure. The central ring structure of the molecule is a ‘corrin’ ring with a central cobalt atom. In its natural form, vitamin B₁₂ is probably bound to peptides or protein.

Vitamin B₁₂ is commercially available as crystalline cyanocobalamin, which is a dark red powder. As human requirements of vitamin B₁₂ are very low (about 1–2 µg a day), it is often supplied as a standardised dilution on a carrier.

Cyanocobalamin is decomposed by both oxidising and reducing agents. In neutral and weakly acid solutions it is relatively stable to both atmospheric oxygen and heat. It is not particularly stable in alkaline solutions and strong acids. It is sensitive to light and ultraviolet radiation, and controlled studies on the effect of light on cyanocobalamin in neutral aqueous solutions showed that sunlight at a brightness of 8000 foot candles caused a 10% loss for each thirty minutes of exposure, but exposure to levels of brightness below 300 foot candles had little effect.⁵
Vitamin B$_{12}$ is normally stable during pasteurisation of milk but up to 20% can be lost during sterilisation and losses of 20 to 35% can occur during spray drying of milk.

The stability of vitamin B$_{12}$ is significantly influenced by the presence of other vitamins.

### 6.5.8 Biotin

The chemical structure of biotin is such that eight different isomers are possible and, of these, only the dextro-rotatory or D-biotin possesses vitamin activity. D-Biotin is widely distributed, but in small concentrations, in animal and plant tissues. It can occur both in the free state (milk, fruit and some vegetables) and in a form bound to protein (animal tissues and yeast). It is commercially available as a white crystalline powder.

Biotin is generally regarded as having a good stability, being fairly stable in air, heat and daylight. It can, however, gradually be decomposed by ultraviolet radiation.

Biotin in aqueous solution is relatively stable if the solution is either weakly acid or weakly alkaline. In strong acid or alkaline solution the biological activity can be destroyed by heating.

Avidin, a protein complex which is found in raw egg white, can react with biotin and bind it in such a way that the biotin is inactivated. Avidin is denatured by heat and biotin inactivation does not occur with cooked eggs or egg products.

### 6.5.9 Vitamin C

Although a number of compounds possess vitamin C activity, the most important is L-ascorbic acid. Vitamin C is widely distributed in nature, can occur at relatively high levels in some fruits and vegetables, and is also found in animal organs such as liver and kidney. Small amounts can be found in milk and other meats.

Ascorbic acid is the enolic form of 3-keto-1-gulofuranolactone. The endiol groups at C-2 and C-3 are sensitive to oxidation and can easily convert into a diketo group. The resultant compound, dehydro-L-ascorbic acid, also has vitamin C activity. The D-isomers do not have vitamin activity.

L-Ascorbic acid in foods is easily oxidised to the dehydro-L-ascorbic acid. In fresh foods the reduced form normally predominates, but processing, storage and cooking increase the proportions of the dehydro form. Commercially, vitamin C is available as L-ascorbic acid and its calcium, sodium and magnesium salts (the ascorbates). It is also available as ascorbyl palmitate and can be used in this form as an antioxidant in processed foods. Ascorbic acid and the ascorbates are relatively stable in dry air but are unstable in the presence of moisture.

Ascorbic acid is readily oxidised in aqueous solutions, first forming dehydro-
L-ascorbic acid which is then further and rapidly oxidised. Conversion to dehydroascorbic acid is reversible but the products of the latter stages of oxidation are irreversible.

Ascorbic acid is widely used in soft drinks and to restore manufacturing losses in fruit juices, particularly citrus juices. Research has shown that its stability in these products varies widely according to the composition and oxygen content of the solution. It is very unstable in apple juice but stability in blackcurrant juice is good, possibly as a result of the protective effects of phenolic substances with antioxidant properties.

The effect of dissolved oxygen is very significant. As 11.2 mg of ascorbic acid is oxidised by 1.0 mg of oxygen, 75 to 100 mg of ascorbic acid can be destroyed by one litre of juice. Vacuum treatment stages are normally added to the process to deaerate the solution and reduce the problem. It is also important to avoid significant head-spaces in containers of liquids with added ascorbic acid as 3.3 mg of ascorbic acid can be destroyed by the oxygen in 1 cm$^3$ of air.$^{15}$ Different production and filling processes can have a significant effect on the retention of vitamin C in drinks. For example, the ascorbic acid loss in a drink packed in a 0.7 litre glass bottle with a partial deaeration of the water and vacuum deaeration of the drink immediately before filling was 16% of the same product filled without any deaeration.

Traces of heavy metal ions act as catalysts to the degradation of ascorbic acid. Studies on the stability of pharmaceutical solutions of ascorbic acid showed that the order of the effectiveness of the metallic ions was $Cu^{+2} > Fe^{+2} > Zn^{+2}$. A $Cu^{+2}$-ascorbate complex has been identified as being intermediate in the oxidation of the ascorbic acid in the presence of $Cu^{+2}$ ions. Other work on model systems has shown that copper ion levels as low as 0.85 ppm was sufficient to catalyse oxidation, and that the reaction rate was approximately proportional to the square root of the copper concentration. Cu and Fe ions play such a significant part in metal catalysed oxidation of ascorbic acid that the selection of process equipment can have a marked effect on the stability of vitamin C in food and drink products. Contact of product with bronze, brass, cold rolled steel or black iron surfaces or equipment should be avoided and only stainless steel, aluminium or plastic should be used.

Work with sequestrants has shown that ethylenediamine tetra-acetate (EDTA) has a significant effect on the reduction of ascorbic acid oxidation, with the optimal level of EDTA required to inhibit the oxidation of vitamin C in blackcurrant juice being a mole ratio of EDTA to $[Cu + Fe]$ of approximately 2.3.$^{16,17}$ Unfortunately, EDTA is not a permitted sequestrant for fruit juices in many countries. The amino acid cysteine has also been found to effectively inhibit ascorbic acid oxidation.

The rate of ascorbic acid degradation in aqueous solutions is pH dependent, with the maximum rate at about pH 4. Vitamin C losses can occur during the frozen storage of foods, and work has shown that oxidation of ascorbic acid is faster in ice than in liquid water. Frozen orange concentrates can lose
about 10% of their vitamin C content during twelve months’ storage at
−23°C (−10°F).\textsuperscript{18}

Light, either in the form of sunlight or white fluorescent light, can have an
effect on the stability of vitamin C in milk, with the extent of the losses being
dependent on the translucency and permeability of the container and the
length and conditions of exposure. Bottled orange drinks exposed to light
have been found to lose up to 35% vitamin C in three months.\textsuperscript{19}

The destruction of vitamin C during processing or cooking of foods can
be quite considerable, with losses during pasteurisation being around 25%,
sterilisation about 60% and up to 100% in UHT milk stored for three months.
Milk boiled from pasteurised can show losses of between 30 and 70%.

6.6 Vitamin–vitamin interactions

One of the least expected and less understood aspects of maintaining the
stability of vitamins in foods is the detrimental interaction between vitamins.
This can lead to the more rapid degradation of one or more of the vitamins
in a food or beverage. These interactions should be taken into consideration
when vitamins are used to restore or fortify products presented in the liquid
(aqueous) phase, such as soft drinks or fruit juices. Most of the work in the
area of vitamin–vitamin interactions has been carried out by the pharmaceutical
industry in relation to the development of liquid multivitamin preparations.

Four of the 13 vitamins have been identified as having interactions with
each other with deleterious effects. These are ascorbic acid (vitamin C),
thiamin (vitamin B\textsubscript{1}), riboflavin (vitamin B\textsubscript{2}) and vitamin B\textsubscript{12}. The principal
interactions are given in Table 6.3.

Other interactions have been identified that can be advantageous, particularly
in increasing the solubility of the less soluble vitamins in aqueous solutions.
For example, niacinamide has been shown to act as a solubiliser for riboflavin
and folic acid.

6.7 Effect of irradiation on vitamin stability in foods

The use of ionising radiation (irradiation) as a sterilisation technique for
certain foods and ingredients has been accepted in a number of countries,
including the European Union. In many countries, the foods and ingredients
that are allowed to be irradiated are restricted by law and the process is
normally used only for foods at risk of high levels of microbiological
contamination.

It has been shown that vitamin levels in a food can be affected by irradiation
and the losses can, in general, be related to the dose. At low doses (e.g. up
to 1 kilogrey), the losses for most vitamins are not significant. At higher
Food fortification and supplementation

At the highest permitted radiation doses, care has to be taken to protect the food by using packaging that excludes air and by carrying out the irradiation process at a low temperature.

There is evidence that the fat-soluble vitamins A, E and K and the water-soluble thiamin are the most sensitive to irradiation, whereas niacin, riboflavin and vitamin D are relatively stable. There is conflicting evidence for vitamins in some foods showing significant radiation losses and others almost none.

If it is intended that nutrition claims are to be made for irradiated foods, it is essential that studies are carried out on the content and stability of the vitamins after the treatment with ionising radiation.

6.8 Food product shelf-life and its determination

As the tendency to include nutritional information on the labels of food products has increased, so have the liabilities of the manufacturers. For many, if not most, foods the inclusion of nutrition information is optional but any statements made on the label come under the force of law. A company making an inaccurate voluntary nutritional declaration can be subject to prosecution. Within a nutritional information statement, vitamins are the main category of declared nutrients where the quantities can significantly decrease during the shelf-life of the food. The vitamin content of processed foods can decrease during storage and, surprisingly, it has been demonstrated that losses of vitamin C can occur in frozen vegetables stored at temperatures as low as –23°C (Section 6.5.9).

If declarations of vitamin levels are required on the label, whether voluntary or statutory, the manufacturer needs to carry out suitable stability trials to determine the stability of each vitamin claimed on the label, over the duration of the declared shelf-life. The actual procedures used for the study will depend on the composition of the food, the processing and the form in which it is presented and stored. The type of packaging can have a significant

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**Table 6.3** Principal vitamin–vitamin interactions

<table>
<thead>
<tr>
<th>Activator</th>
<th>Increased instability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid</td>
<td>Folic acid</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>Vitamin B₁₂</td>
</tr>
<tr>
<td>Thiamin</td>
<td>Folic acid</td>
</tr>
<tr>
<td>Thiamin</td>
<td>Vitamin B₁₂</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>Thiamin</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>Folic acid</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>Ascorbic acid</td>
</tr>
</tbody>
</table>

(Adapted from Berry Ottaway 1993[19])
effect on vitamin stability and the quality of the barriers to oxygen, moisture and light is very important. A requirement for label claims for vitamins can influence the selection of the form of packaging. The need to retain the vitamins often means that a compromise has to be achieved between the length of required shelf-life and the barrier quality of the packaging. Due to the wide variety of products, processes and packaging, it is not possible to give specific procedures for the determination of the shelf-life of vitamins in fortified foods and supplements. However, guidelines have been established for the determination and prediction of shelf-life. The determination of the vitamin levels at each stage of the shelf-life study should be built into the protocol. The International Committee for Harmonisation (ICH) has published requirements and procedures for the stability testing of pharmaceutical products and it is recommended that these are followed for food supplements and modified where necessary for other food products.

As the degradation of most of the vitamins follows ‘first order’ or ‘zero order’ kinetics, it is possible for shelf-life predictions to be made using a classical Arrhenius model on the assumptions that the model holds for all the reactions being studied; that the same reaction mechanism occurs throughout the temperature range of the study; that the energy of activation is between 10 and 20 kcal/mole; and that the effects of moisture at ambient temperature are equivalent to maintaining the same relative humidity at the higher temperatures.

6.8.1 Vitamin overages

As no two vitamins will degrade at the same rate in a food at any set of conditions, the food technologist has to determine the rates of deterioration of each vitamin and then increase the amount added to the product during manufacture to ensure that the label claim is met throughout the life of the product. The difference between the formulated and declared levels is known as the ‘overage’. The amount of overage will vary according to the inherent stability of the vitamin, the conditions under which the food is processed and packed, the packaging materials selected and the anticipated shelf-life of the product.

Overages are normally expressed as a percentage of the declared value, so that an input of 45 mg of vitamin C and a declared amount of 30 mg would give an overage of 50%. For food supplements where the added vitamins are the only significant source of these nutrients in the food, the overages are usually calculated as a percentage of the amount required in the product at the end of its shelf-life. When determining the overage for a vitamin in a product, consideration must also be given to the total amount of the vitamin in the product, particularly in the case of vitamins A and D where there may be safety concerns. As vitamins A and D are often the most unstable vitamins in a product, overages tend to be higher than those of the other vitamins. The consequences of large overages must be considered, and at all times the
amount of overage added must be the minimum necessary and well within any safety levels for the vitamin.

The shelf-life of a product is often dictated by commercial pressures, which must take into account the time taken for the product to reach the consumer and the range of temperatures that it may be subjected to during the time between its manufacture and sale to the consumer. Once this information has been established, the vitamin overages to achieve the required shelf-life have to be assessed. The only realistic estimations of the shelf-life and required overages are those obtained by stability trials on the product carried out in the packaging to be used and under the anticipated storage conditions.

A methodology using the Arrhenius model has been developed which allows predictions to be made for both shelf-life and overages. As already stated, this is based on the assumption that the degradation of most of the vitamins follows ‘first order’ or ‘zero order’ kinetics. The precision of the technique has been found to be related to the number of storage temperatures that can be used and the number of samples that can be taken from each temperature. Typical storage temperatures used for stability studies are 0°C, 25°C, 35°C, 40 or 45°C and 50 or 55°C. Ideally, at least three temperatures should be used and the selection of the higher temperatures depends on the composition of the product under test, as phase changes (e.g. solid to liquid) during storage should be avoided. The tests should normally run for at least 24 weeks, with samples from each temperature being removed at predetermined intervals and stored at 0°C. All samples are stored at 0°C until the final samples are taken and all are assayed at the same time. The data are analysed using the Arrhenius equations. The data obtained enable estimates to be made of overage amounts for each vitamin to meet a given shelf-life; it assists in the comparison of different packaging materials and also helps to identify potential stability problems. Whilst it can be demonstrated that the technique has some limitations, work has shown that, if all the experimental controls are maintained, useful predictions of a product’s stability can be obtained. A comparison of the predicted and actual vitamin losses in a multivitamin tablet is given in Table 6.4.

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Predicted loss (%)</th>
<th>Actual loss (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin A</td>
<td>43.0</td>
<td>44.0</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>24.0</td>
<td>23.0</td>
</tr>
<tr>
<td>Vitamin B_{12}</td>
<td>9.2</td>
<td>7.7</td>
</tr>
<tr>
<td>Folic acid</td>
<td>12.0</td>
<td>10.5</td>
</tr>
</tbody>
</table>
6.8.2 Stability studies

The objective of an accelerated stability study is to subject the product to stress in the form of high temperatures and humidity (moisture), to induce potential reactions and interactions. In all cases the product being stored should be in the packaging in which it will be marketed. In cases where the product is likely to be sold in more than one pack size, all potential pack sizes should be tested. This is important as the larger pack sizes could have a greater relative head space or packaging surface area, which could affect the stability.

Real time stability studies should be set up to run concurrently with the accelerated studies and be continued for at least the anticipated shelf-life of the product.

When determining the storage conditions to be used for stability studies, they need to reflect the temperature and humidity conditions in the intended markets. For the Northern European market, the accepted room (ambient) temperature is $25^\circ C$ and the ambient relative humidity recommended by the IHC for this temperature is 60%rh. For markets with a tropical climate, and particularly those where a high proportion of retail outlets do not have air-conditioned storage, the temperature and humidity baselines have to be increased to represent the increased temperatures. As a general rule, the higher the ambient temperature to which the product is exposed, the shorter the shelf-life. However, it cannot be assumed that there is a linear relationship between the temperature and product shelf-life, particularly with multivitamin products.

Stability trials conducted to an internationally accepted protocol are costly. For many new products, particularly food supplement products, it may be possible to extrapolate data or apply previous experience from existing products, provided that the form and size of the packaging and the intended market are the same. For entirely new products or ingredient combinations, it is advisable that accelerated stability studies are instigated 3–6 months before the launch of the product. This is necessary to provide some confidence in the stability of the product, as there have been a number of cases of products being withdrawn from the market due to serious stability problems overlooked during development.

Storage facilities can be of various types, depending upon availability. These can include purpose-built cabinets, incubators and rooms, both with or without humidity control, but in all cases with accurate temperature control. Whichever storage system is used, it should have the means to monitor both the temperature and the humidity. Ideally, the recording of both these parameters should be continuous, but if this is not possible, frequent readings should be taken.

Products will be removed from storage at predetermined intervals for physical examination and chemical assay. Depending on the product, it may be necessary to conduct microbiological tests at the periodic sampling. Notwithstanding the possible periodic tests, microbiological testing should
be carried out on samples at the beginning and end of the study. The removal/assay periods should reflect the intended shelf-life of the product. For example, a food supplement with a proposed shelf-life of three years should have initial assays at the start of the real-time study to establish a base-line, and then at 1, 3, 6, 12, 18, 24 and 36 month intervals at storage conditions of 25°C/60%rh. For shorter proposed shelf-lives, more frequent assay periods would be required in order to generate the same quantity of data. For accelerated studies, the assay periods will be much shorter and relate to weeks, not months, of storage. Fortified foods tend to be assigned shorter shelf-lives.

It is essential when planning stability studies for a product that the ‘in use’ period is also taken into consideration. This should simulate the period of time the product is likely to be used between opening the pack and using all the contents. For example, if the amount of product in the pack provides for three months supply at recommended consumption levels, the ‘in use’ stability study should have a minimum of three months duration. In such studies the recommended daily consumption of product should be removed each day to replicate the gradual loss of product from the pack and its replacement by air with ambient oxygen and humidity levels.

### 6.9 Protection of vitamins in foods

With all products for which claims for vitamins are intended, it is essential that all stages of the processing, handling and storage of the product are evaluated to minimise the degradation of the vitamins. This can be accomplished by keeping residence times at high temperatures to a minimum and reducing or eliminating exposure to light and oxygen. For example, during the processing of fruit juices, fruit squashes and fruit drinks, the de-aeration of the solution can have a protective effect on the vitamin C levels in the product, by reducing or eliminating oxygen.

Commercial sources of vitamins for addition to foods can be obtained in forms that have been encapsulated or coated to improve their stability. In some cases, particularly with certain supplement formulations, it may be necessary to use coated forms of the mineral sources, such as those providing iron, copper and zinc. A good example is an iron and vitamin C tablet, where it is necessary to use the coated forms of either the ascorbic acid or the iron source to prevent a reaction between the two, resulting in black spots in the product.

### 6.10 References

The stability of vitamins in fortified foods and supplements


7.1 Introduction

New scientific research is increasingly supporting the notion that foods, food ingredients and ingredient components play a major role in reducing the risk of disease and in addressing specific conditions such as high cholesterol, obesity and low immunity. In fact, organisations such as the World Health Organization (WHO) and the Global Alliance for Improved Nutrition (GAIN) are launching programmes worldwide to fortify basic foods in order to address diseases and conditions caused by malnutrition. Similarly, a growing number of manufacturers are responding to consumer interest by turning to fortification, specifically combining multiple ingredients, to capitalise on scientific advances revealing how certain food components can delay the onset of diseases such as cancer, osteoporosis and diabetes, and assist particular demographic groups with ailments and lifestyle issues.

7.1.1 Differences between functional and medical foods

The food industry makes wide marketing use of the concept of functional food. Functional food is typically defined as any food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains. The term ‘traditional nutrients’ typically refers only to vitamins and minerals, which can be problematic because functional foods are also considered essential to the diet and may correct diseases resulting from classical nutrient deficiency. For example, vitamin C, which prevents scurvy, and the vitamin D found in sardines, which alleviates rickets, are not examples of functional food, while soy, which contains soy protein associated with a reduction in cardiovascular
disease, is defined as a functional food because soy protein is not considered to be essential for human nutrition. Examples of other functional foods include red grapes, cranberry juice and oat bran (for the fibre content), all of which have health benefits that are attributed to ‘non-nutrient’ compounds as classified by standard agreement of the term functional food. The so called super-fortified foods, which are foods fortified with more than 100% of the recommended daily intake, or contain added botanicals or other supplements, also fall into the category of functional foods. Those foods include products such as orange juice with Echinacea and salad dressing with omega-3 polyunsaturated fatty acids.

Another category of added value foods is medical foods. Compared to functional foods, medical foods are formulated to assist in the dietary management of a disease or condition that has established nutritional requirements. Medical foods are commonly used to treat diabetes, obesity or heart disease. These products are sold and administered under the supervision of a physician, not through conventional retail outlets. Depending upon the regulations of individual countries, functional and medical foods may carry health or structure/function claims provided that adequate evidence supports the claim. The current law in the European Union (EU), for example, says that any health claim must be scientifically substantiated and labels are prohibited from making a claim that the food can treat, prevent or cure any diseases or adverse conditions.

7.1.2 Public health benefits of food fortification
Governments have policies concerning the addition of nutrients to foods to help maintain and improve the overall nutritional quality of the national food supply and address the nutritional needs of specific groups. Under the Codex General Principles for the Addition of Essential Nutrients to Foods, the addition of vitamins and minerals is related to specific public health benefits:

- Correcting or preventing deficiencies
- Restoring nutrients lost in processing
- Standardising the nutrient content of specific products
- Achieving nutritional equivalence of substitute foods
- Ensuring appropriate nutrient content of special-purpose foods

To meet the challenges posed by the widening definition of nutrient requirements that now includes specific indicators related to reducing chronic disorders, additional approaches to food fortification policies need to:

- Improve quality to achieve nutrient adequacy, rather than just prevent deficiency
- Increase nutrient density of foods targeted to specific groups
- Increase number of food products that are a good source of specific nutrients
There are other important reasons for fortifying foods in addition to satisfying government public health requirements: to provide alternative forms of vitamin and mineral supplements, to respond to consumer demands for specific nutrients, to satisfy product ‘health claims’, and many more.

7.2 Preparatory requirements

7.2.1 Setting accurate specifications in nutrient premixes
Achieving homogeneity in nutrient systems so that they provide the vitamins, minerals, amino acids and other micronutrients needed to meet the customer’s specifications or finished product label claims can be one of the most challenging aspects of the product development process.

Every premix can provide a variety of different nutrient levels, depending on the specific application or final product label claims, and it will have variable chemical and physical properties. Because of the unique requirements of any particular application, and the many variables involved, specifications must be drawn up to satisfy very specific finished product attributes. Based on the answers to these important questions/variables, scientists work to set specified minimum levels of nutrients as absolute figures, with the appropriate overages added to them in order to compensate for such variables as shelf-life losses, processing degradation and analytical variations.

7.2.2 Understanding the fortification policies
Fortification involves adding specific micronutrients to process staple foods such as vitamin A to sugar, wheat and corn flour, vegetable oil and margarine, and iron and iodine to salt. Successful food fortification requires appropriate regulatory instruments, effective public/private partnerships, and functioning quality assurance and monitoring systems. The United States has been fortifying its milk with vitamins A and D since the 1930s.

Fortification of foods can greatly contribute to reducing micronutrient deficiencies because it is generally socially acceptable, may not require changes in food habits and can be introduced quickly. Food-based strategies, diversifying diets and fortification of certain commonly consumed foods increases the amount of micronutrients that people get each day.

Each country, and sometimes states within countries, has its own set of regulations for use of nutrients and functional components in foods, beverages and supplements due to its own dietary practices. Likewise, product labelling and health claim regulations also vary between countries. These regulations may also depend on the type of food products developed for different demographic segments or nutritional needs. Therefore, manufacturers must be prepared to formulate and label products in accordance with the laws of the lands in which their products are sold. For example, in the United States, the Food and Drug Administration (FDA) fortification policy guidelines...
state that the words ‘enriched,’ ‘fortified,’ ‘added’ and similar terms may be used interchangeably to indicate the addition of one or more vitamins, minerals or proteins to a food unless an applicable regulation requires the use of a specific term. When the United States Nutrition Labelling and Education Act (NLEA) of 1990 was signed into law, the state pre-emption provision of the Act in part declares ‘that no state or political subdivision of a state directly or indirectly establish or continue any requirement for any food in interstate commerce having a federal standards of identity (SI) that is not identical to federal standards.’ Therefore, a state can no longer have its own enrichment standards for products in interstate commerce that are subject to federal SI. Enrichment products from all states in interstate commerce must meet Federal Enrichment Standards for cereal grain products.

7.3 Preserving good taste while adding in-demand nutrients

With regard to functional food and beverage products, response by mass markets demonstrates that, despite interest in eating for better health, the discriminating palate determines repeat purchase. Consumer interpretation of good taste involves many attributes including mouthfeel as well as the experience of bitter, sweet, salt, sour, umami (savoury) – and smell. This poses many challenges for food formulation worldwide. Not only must manufacturers attend to geo-cultural sensory preferences, but the integration of functional ingredients itself creates consumer acceptance issues by virtue of the nutrients’ individual and interactive flavour notes. Low-calorie foods, fortified and functional foods, reduced fat or reduced sugar foods all have considerable taste, texture and stability challenges that affect the overall consumer taste experience. A primary solution to optimising taste in healthful foods and functional beverage applications is selection of the appropriate ingredients that can modify or minimise unpleasant taste attributes which often occur when formulating these products.

7.3.1 The functional ingredient taste challenge

Historically, manufacturers started differentiating healthier products with the addition of one or two ingredients that allowed them to promote a simple consumer message such as ‘now with added vitamin C.’ They worked with ingredients that could be relatively easily incorporated in their product line and avoided working with ingredients that offered benefit but were difficult to process or were known for bad taste and aroma. Polyunsaturated fatty acids (PUFAs) and choline are prime examples of ‘problem ingredients’ that offer nutrient value but whose smell and taste are undesirable to many consumers. Some vitamins, such as B1, can impart a bitter taste and sulphurous
Food fortification and supplementation

Egg aroma, while minerals such as zinc, copper and iron can present a lingering metallic taste. Within the past few years, consumer interest in promoting health has forced manufacturers to revisit challenging ingredients and figure out how to make them work. Moreover, consumers today are not simply looking for one or two added beneficial ingredients, they are looking for more complex products that are formulated to deliver a health benefit to their demographic (gender, age) or to address specific health conditions (immunity, bone, cardiovascular and gut health). This means not just overcoming the challenges of single ingredients, but overcoming the issues of combining and processing multiple ingredients – and it means finding creative ways to make the functional ingredients work in the type of products consumers are demanding, pushing nutrient integration into some environments that are not an easy fit.

When working with multiple functional ingredients, the core challenge facing product developers is the complexity of the food matrix. A food product is comprised of many different ingredients that together form a complete, uniformly balanced physical and chemical nutritional system. Many of these ingredients are multifunctional, so removing or adding new functional food ingredients may disrupt the total balance of the product. Adding in-demand nutrients together may affect taste (flavour), appearance, texture or all three of these important parameters that together constitute perceived product quality.

Undesirable interactions between the various components of complex foods and beverages increase the risk of quality-deterioration in a product. Of particular concern is moisture transfer between components with different water activity. Other interactions that can affect the sensory quality of a product include the migration of colouring, fats, oxygen and other flavouring substances: the migration of vitamins and minerals is also a concern. These changes present further challenge to taste and limit a product’s shelf-life.

7.3.2 Solutions for taste modification and minimisation

Today, there are several options in ingredient selection that can help product developers preserve good taste: flavouring, texturants, market form and encapsulation:

Flavouring

Selection of flavour ingredients not only offers consumers taste options but may also help mask undesirable flavour notes imparted by functional ingredients. Some flavours are more transparent than others, so manufacturers should consider matching stronger flavours with nutrient profiles that present stronger off-notes. Chocolate, for example, is a relatively stronger masker of taste than vanilla.
Texturants
Texture has a direct impact on mouthfeel. It concerns those properties of finished foods apprehended by the eyes and by the skin and muscle senses of the mouth, including the roughness, smoothness and graininess. The overall textural experience is derived from the sensations of the skin in the mouth after ingestion of the food or beverage. It relates to density, viscosity, surface tension and other physical properties of the material being selected or sampled.

Market form
Choosing the right market form is of critical importance when formulating for specific applications. For ready-to-drink beverage products, nutrients such as calcium, iron and copper would use a different market form from than that for powdered beverages. The same is true for other nutrients relating to solubility. When a nutrient has a high solubility level, it will ionise, altering the taste as a result of interactions.

Encapsulated ingredients
Selection of the right kind of coating can preserve taste by limiting migration and preventing premature release of ingredients that can negatively affect taste and limit shelf-life. Beyond ingredient selection, processing methods may also play a role in delivering flavour characteristics to please consumers.

7.3.3 Overcoming challenges
Overcoming taste ‘off-notes’ when formulating a fish oil-based beverage is difficult. The following examples of challenges illustrate current approaches to overcoming taste issues with functional ingredients:

- Taste
- Aroma
- Stability
- Solubility
- Bioavailability

Minimising oxidation of long chain PUFAs – long chain polyunsaturated fatty acids with chains of 18, 20, or 22 carbon atoms having 2–6 double bonds in their composition – will prevent them becoming rancid at room temperature. Oxidation generates unpalatable peroxides which result in objectionable odour and taste. Solutions to this problem include the addition of antioxidants and/or metal chelates; and modification of processes such as the use of split-stream processing, nitrogen blanketing and low-shear mixing.

If off-flavours cannot be avoided, they can sometimes be masked by flavour modifiers or flavour maskers; or by the use of emulsification or encapsulation techniques.

To overcome taste off-notes when formulating, for example, nutrition bars, the areas of taste, aroma, and stability must be considered. To ensure
good taste and enhance the shelf-life of nutrition bars, selection of the proper market forms of ingredients that are encapsulated is a key step. Edible barriers and coatings are used to prevent migration of macro- and micronutrients along with moisture and oxygen transfers. Preventing migration will virtually guarantee a high quality, acceptable product with extended shelf-life and sensory properties. These ingredients provide stability during storage and minimise interactions during processing. They also block bad-tasting derivatives, or by-products that could negatively impact the taste of the finished product.

7.4 What product quality are consumers looking for?

A number of research cycles between nutritional development and product development at the bench must take place before a product is deemed acceptable for the final test which is consumer acceptance. In many companies, the sensory group is an essential part of the screening process and its members can help determine when to expand the screening – first through available employees, then finally to the consumer.

Companies are aware of the importance of consumer acceptance of their products and, therefore, are investing money and resources on consumer research. There is considerable pressure on companies to learn more about the consumer response pattern, to be able to develop rapid and reliable measures of consumer response behaviour relating to competitive products and pressure to understand what consumers mean by the word ‘quality.’

Researchers have obtained results from consumer tests which show very good correlation between overall preference and preference for the individual sensory attributes. With those results, one would imagine that everything is acceptable and the product will be a success, but sometimes when the product is introduced on the market – it ultimately results in failure. So while it is known that a product will not sell in mainstream markets unless its taste is acceptable, there are still other variables that affect ultimate success. The fact here is that, while it is possible to detect the number of units sold in different stores and it is possible to measure consumer preferences, the actual reasons behind a consumer’s choice and preference needs to be better understood.

Ingredients have not changed in name over the last several years; however many other factors have changed. For example, consumer awareness has changed, the regulatory landscape has changed and the product developers and suppliers have since learned to use available technology more effectively. At the same time, various new ingredients have recently emerged which may prove useful in applications that previously had little or no hope of efficacy because of their potential off-notes. Innovation in ingredient options and expertise in application of these ingredients now makes it possible for
manufacturers to turn formerly problematic ingredients into consumer opportunities.

7.5  The science behind sensory and textural issues

In today’s very competitive and dynamic marketplace, thousands of new products and services are being offered to consumers on a regular basis. Consumers are faced with a variety of alternatives from which to choose and are forced to assess the product’s factors (i.e. quality, cost) when making their product selection. Some consumers consider quality the most important factor while for others it is cost or other parameters, such as availability, convenience etc. Quality can be thought of as the degree of, or grade of, excellence. The problem with this definition is that different people look for different things. Even so, there is some consensus among consumers as to what constitutes a high-quality product. All consumers expect a safe and wholesome product and they are greatly interested in nutrition. They also look for convenience, acceptable taste and value. Taken together, these characteristics largely determine the quality of food products and act as a useful yardstick for comparing one product with another.

7.5.1  Texture of a product

As previously stated, those properties of a food/beverage product apprehended by the eyes and by the skin and muscle senses of the mouth, including the roughness, smoothness and graininess after ingestion of a food or beverage are collectively called texture or mouthfeel. This relates to density, viscosity, surface tension and other physical properties of the material being sampled. Stabilisers, (e.g. carboxymethyl cellulose (CMC), gelatin, xanathan gums, alginates) and emulsifiers (polysorbate and mono- and diglycerides) would have a pronounced effect in improving the smoothness of body and texture. All stabilisers have a high water holding capacity, effective in smothering the texture and giving body to the finished product. Their most important function is to prevent the coarsening of texture due to temperature fluctuations in the supply chain.

Texture of a product is dependent upon the number, size, shape and arrangement of the particles in a food/beverage matrix. The body and texture characteristics are closely associated and are important in influencing consumer acceptance of food products. Depending upon the finished product, acceptance criteria in general texture should be smooth, uniform and present a pleasing reaction when it is consumed. Body defects are commonly described as crumbly, soggy, etc., while the common texture defects are coarse, sandy, gritty, etc. Sources of common body and texture defects are:

- Improper composition of formulated product
Improper processing method
Improper packaging, storage and handling

In addition to the usual ingredients, particle size of specific fortificants, for example minerals, are of the utmost importance. While minerals such as calcium, magnesium and potassium offer significant nutritive value, they are characterised by large particle size that can impart an unwanted gritty, sandy or chalky sensation. These undesirable attributes may be overcome through choice of market form. While there are a number of market forms of mineral salts that can boost a product’s mineral content, the challenge for formulators is to select the right form to enable optimised bioavailability, flavour, solubility, sensory properties and mouthfeel of the finished product. Calcium carbonate, for example, is an effective source of calcium. However, it has a tendency to provide a chalky taste and have a gritty mouthfeel, as does dicalcium phosphate. Using a blend of calcium sources instead of a single source can prevent some processing issues of these calcium salts. Expert formulation is what is crucial to finding the right balance.

In addition to selection of market form, the negative effects of particle size may also be mitigated through milling and micro pulverising. The physical increase of surface area reduces the sensation of grittiness (but also the surface area available for negative interactions). As much as large particle size can be negative in some products (e.g. sandiness in ice-cream), it can be positive in others – for instance where crunchiness is desirable.

7.5.2 Sensory attribution of a product

Table 7.1 lists the most common sensory attributes of food products. Over the last 10 years, significant progress has been made in the physical determination of such textural parameters. Dynamometers, with some
adaptation, are very useful tools for the determination of texture profiles. For some applications, results from physical testing have been found to correlate very well with results from taste panel evaluations based on the same parameters. There is a significant variation and great potential for variation in textural parameters. The choice of an appropriate functional ingredient in the proper formulation and at the correct concentration can greatly influence and alter the texture of food products.

Sensory properties of food products are very important for consumer preference. This fact is obvious to all consumers, food producers and retailers: also to researchers and technologists in development of new products. The relationship between sensory properties and preference for food products is therefore investigated by researchers in the food industry all over the world.

As sensory science is a relatively young science with a lack of formal training, there is a great potential for the misuse of sensory methods. The misuse can easily cause wrong decisions that might be fatal for success in the launching of new products, and, furthermore, discredit the validity of product development activities.

7.6 Preserving nutrient stability and in-demand ingredients

Food and beverage manufacturers worldwide recognise that delivering nutrient value can open market opportunities and drive sales. Competitive edge can be won through factors including functional ingredient load, health claims and product customisation for specific demographics and health conditions. The key to manufacturers’ ability to meet the promise of their nutritional labels and health claims is the stability of the nutrients in their food or beverage matrix. There are, however, many variables internal and external to the products’ environment that affect nutrient integrity, potentially limiting their potency, efficacy and shelf-life. It stands to reason, too, that the effects of these variables are compounded as the number of functional ingredients being integrated increases.

When designing a food or beverage, it is essential to consider five basic factors as a foundation for optimising nutrient stability:

- Nutrient activity
- Composition of the finished food
- Manner of addition
- Processing condition and procedures
- Storage and other conditions prior to consumption

7.6.1 Nutrient activity

First, the product developer must think about the physical properties and vulnerabilities of individual ingredients being integrated. Following are some
general facts regarding vitamins, minerals, and amino acids, but similar activity assessment must also be done for other ingredient categories such as nucleotides and botanicals.

7.6.2 Popular vitamins associated with food fortification
The following vitamins are commonly associated with food fortification:

- Vitamin A and Beta Carotene
- Vitamin D
- Vitamin E
- Vitamin K
- Vitamin C (Ascorbic acid)
- Vitamin B₁ (Thiamin)
- Vitamin B₂ (Riboflavin)
- Niacin
- Vitamin B₆
- Vitamin B₁₂ (Cyanocobalamin)
- Folic acid (Folacin)
- Biotin
- Pantothenic acid

The properties (solubility stability, etc.) of these vitamins are detailed in Chapter 6 (Sections 6.4 and 6.5) and Chapter 8 (Sections 8.2.1 and 8.2.2).

7.6.3 Minerals
Minerals are all quite stable in and of themselves. However, chelated forms of some minerals, including calcium and zinc for example, may provide enhanced stability and bioavailability. Another consideration is interactivity of minerals and vitamins. As noted with regard to some of the aforementioned vitamins, the metallic ions present in some minerals may cause depletion of vitamin nutrient stability. Additionally, the interaction of vitamin and mineral additives may impact stability of other food quality factors – such as colour. A common example of such an undesirable outcome is the brown spotting that occurs when combining vitamin C and iron. Here, too, consideration of market form – for example using an encapsulated ferrous sulphate – may greatly diminish or eliminate the risk of spots.

7.6.4 Amino acids
Degradation of one or more of the essential amino acids will result in an imbalance and subsequent loss in quality. Probably the foremost effect is the Maillard reaction, whereby amino acids react with reducing sugars to produce pigments that are similar to caramel colour. The amino acids, specifically lysine and threonine, are made unavailable and overall protein quality suffers.
This reaction can be avoided or prevented by low-temperature storage, by avoidance of appreciable amounts of reducing sugars in formulated foods and by reducing water activity. Fat oxidation can produce levels of peroxides and aldehydes that also may react with certain amino acids and deplete or destroy their activity. Amino acids, especially free sulphur-containing amino acids, can also be destroyed directly by heat; therefore processes must be developed and monitored carefully.

### 7.6.5 Nutraceuticals

Nutraceutical is a comprehensive term which includes substances that are considered as foods, parts of foods or dietary supplements that may claim to provide medical or health benefits – this includes the treatment of or prevention of disease. In addition to vitamins, they contain naturally occurring ingredients such as garlic, ginseng and other herbal products such as phytochemicals extracted from plants. Soluble and insoluble fibres are also included. The market for nutraceuticals has steadily been growing and the trend is expected to continue as aggressive marketing campaigns are launched.

The self-medication trend is important to nutraceutical products. The consumer’s attitude toward healthcare is changing and more people are taking responsibility for their own health rather than passively accepting medical decisions. This desire to exercise individual decision means many consumers are looking outside the traditional dependencies on pharmaceuticals, to herbal remedies and supplements. This self-medication trend will continue to boost the nutraceutical food category. The opportunities will range from hangover remedies and energy boosters to products that ward-off future ailments.

The nutraceutical market offers the potential for growth, but the issues of consumer education and legislation surrounding claims are still a challenge. Trust in a specific brand is important and the entrance of large, multinational companies with healthy images will help to grow the market.

### 7.6.6 Nucleotides

Nucleotides are bio-chemicals found in mothers’ milk consisting of one molecule of phosphoric acid, one molecule of sugar (ribose or dextrose) and one molecule of a purine or pyrimidine. In all, there are five nucleotides produced enzymatically. Nucleotides have been added to some infant formulations to simulate mothers’ milk. The addition of these nucleotides may enhance immune function and gastrointestinal development, and promote the development of a less pathogenic intestinal flora. Certain nucleotides also have specific flavour enhancing qualities – specifically dealing with bitterness, sweetness and enhancement of meat flavours.
7.6.7 Botanicals
Herbs have been used in phyto-medicines (herbal) for centuries. A survey of the historical development of herbal extracts clearly shows that plants have been traditionally used as therapeutic agents for various disorders due to their apparent or evident medical properties. Medicinal products of herbal origin are gradually gaining in importance due to clinically proven therapeutic efficacy. These products also are typically very tolerable, with high compliance and relatively minimal side-effects. The use of dietary supplements containing botanical products is rapidly expanding – the public is using these products for a wide range of health-related problems. It is obvious that herbal medicine will play a major role in the medical/health care industries in the future.

7.6.8 Composition of the finished food
As manufacturers deal with a widening array and longer lists of nutrients for addition to a single product, they commonly find that a single variable can present multiple, and sometimes opposing, effects upon the product. Components of finished foods that have the most profound effect on stability of added nutrients are fats, proteins, carbohydrates, minerals, water and antioxidants. Whether naturally occurring or added, fat has a positive effect in protecting fat soluble vitamins (A, D, E and K) from oxidative deterioration. The peroxides formed during fat breakdown, however, can be quite detrimental to other vitamins, particularly vitamin C. Lower levels of moisture in finished foods can result in decreased susceptibility of vitamins to heat and/or light degradation. On the other hand, soluble reactants generated during the processing phase can concentrate in the water phase of low and intermediate moisture products to a point where reactions are triggered. Iron and copper catalyse many oxidative reactions and must be taken into consideration. Antioxidants such as ascorbic acid and alpha-tocopherols can be advantageous in some products, not only to provide nutritional benefits but also to prevent oxidation of fats and other vitamins. Other components of food that affect stability are buffers, chelating agents, entrapped air and preservatives.

7.6.9 Manner of addition
As manufacturers often attempt to add nutrients to food and beverage compositions that have no natural precedent for these ingredients, food scientists must take special care to anticipate and circumvent potential damage to nutrient integrity. The manner of addition, essentially the market form and timing of integration of a nutrient or nutrients, can have great impact on stability. Ideally, the chemical form and timing of integration should provide uniformity of distribution and maximum stability in the product. Choice should include consideration of factors such as chemical and physical properties of the nutrients, the nature of the food environment – e.g. dry or moist – and how the food is to be handled following addition. The choice of the chemical
form of the nutrient is critical. Certain salts or esters are more stable than others. It may be necessary in some food systems to add the nutrient in a protective carrier or coating in order to prevent destruction or losses during processing or storage. Of course, such protective mechanisms must not interfere with the availability of the nutrient upon ingestion. It is important that the consideration of particular market forms be specific to each given premix or specific application, since data cannot be extrapolated from one system to another.

Time of addition is also significant, since it is best to subject some nutrients to the least amount of heat or the least exposure to air. For example, certain nutrients that are known to degrade under heat (vitamin C, D, thiamin) may be sprayed onto the finished product rather than integrated during processing. Or nutrients that quickly degrade in moist or wet environments (vitamin B₁₂, thiamin, pantothenic acid) may be prepared to be added as mix-ins to a liquid substrate just before consumption.

Thermal processing has to be optimised in order to obtain its desirable values, yet minimise nutrient losses by leaching or destructive reactions. Some of the most significant losses of vitamins and minerals occur during washing and cooking of food products. Water soluble vitamins and certain trace minerals will be lost during this process. If possible, any additions of fortifying nutrients would be made after these steps. Most difficult to deal with are the destructive effects of retort sterilisation. Vitamin B₁, panthothenic acid and several other vitamins are quite heat labile. Substantial overages have conventionally been utilised to compensate for these losses, though coated market forms may provide an alternate to some overages. Lysine, threonine and other sulphur amino acids are particularly reactive to heat as are proteins containing these amino acids. If reducing sugars are present or if they are formed during processing, proteins may be degraded by the Maillard or other reactions.

To pre-empt costly errors in development and production, consideration of all the variables affecting nutrient stability should start at the inception of product development or improvement. The rewards of this due diligence are many, including: improved stability, uniformity and consistency; fulfilment of the desired nutrient profile; and up to as much as 60% reduction in development time – altogether producing not only a better product, but a healthier financial reward to the manufacturer.

7.7 From production to final product

7.7.1 Dehydration and baking

Many of the previously mentioned reactions also occur during drying of foods and, in some cases, at a more rapid rate. Careful control of drying conditions, use of antioxidants, and encapsulation methods of addition can
be helpful, but if possible, any additions of heat labile nutrients should be made after drying has been accomplished.

7.7.2 Blending and processing

Blending and processing techniques can make the difference between producing a reliable, high quality, homogeneous, shelf-stable product and an inferior one that may cause poor consumer confidence, potential regulatory issues or recall situations. Particle size, blending equipment and the type of ingredients used are primary blending and processing considerations, as are potential ingredient interactions (Fig. 7.1).

The challenge in blending ingredients with different particle sizes is that bulk density and the variable particle sizes can lead to segregation. Therefore, minor nutrients should be diluted with another carrier to get the different materials to blend well in order to make a homogeneous product. In the nutraceutical/functional food industries, combination products are the norm and the most common nutrients are vitamins, minerals, amino acids, nucleotides and other functional food ingredients, offered in a single serving of powdered

Fig. 7.1 Vitamin tablets.
products – tablet or capsule (Fig. 7.2). The average premix formulation contains 10 to 14 active nutrients and 3 to 6 functional ingredients, or carriers (excipients). Some formulations contain more than 30 active nutrients and carriers. To comprehend the challenges when producing a homogeneous, correctly-proportioned blend of these active ingredients, imagine trying to create a uniform blend of 1 spoonful of granular sugar, 3 spoonfuls of flour and 5 spoonfuls of rice – then add to that blend a one-half teaspoon of salt and a quarter spoonful of colour. Presuming success in combining these ingredients into a homogeneous blend, the next challenge is compressing small amounts of the blend into a capsule/tablet or serving of a nutritional product. Each serving, tablet or capsule must contain each ingredient in the same proportion as the blend. Making a uniform blend is one of the most complicated steps in manufacturing premixes that contain multiple nutrients. While there are many possible reasons for these deficiencies, inadequate blending is often the source of variations or absence of nutrients.

There are basic steps to follow when dry-blending a multiple-ingredient formula to make a homogeneous premix:

- Test all active ingredients, identify any potency limits. If raw materials are not tested prior to use, it may be difficult to determine whether a problem with the final product is related to blending or to the ingredients.
- If possible, render all ingredients free-flowing. This can be done with milling, particle coating, granulation, making pre-blends, tituration, spray drying and other techniques.
- Purchase ingredients that have consistent particle size distribution or that have a narrow range of variation.
- Screen lumpy or cohesive ingredients before they are added to the blender. It will reduce agglomeration during mixing.
- Always add a portion of the largest quantity ingredient to the blender.

Fig. 7.2 Mixing and blending process.
first. It will coat the blender and prevent lesser ingredients from sticking to the walls.

- Before adding small-quantity active nutrients to the blend, ensure that each one is geometrically diluted to assist with adequate blending. That helps prevent loss from ingredients adhering to the blender wall or because the material had not been dispersed enough for uniform blending. Never add ingredients that account for less than one percent of the total blend into an empty blender.

- When using a V-type blender, divide the ingredients into equal parts, and then add one portion to one side and the other portion to the other side. This improves distribution. Blending time and the level of fill in the vessel play a critical role in determining the adequacy of blending. These parameters should be established during the product development stages.

- Finally, take adequate samples from the top, bottom and centre of the blender. List at least three of the lower potency ingredients to determine the adequacy of the blend. Take samples again after discharge to identify any segregation that may have occurred during material transfer.

Most experts on formulation agree that there is no science to blending powders that are part of the finished product that will work for every product. However, blending powders is very different from blending liquids. Where over-blending is almost impossible, powder-to-powder blends can be ‘un-mixed’ when particles segregate.

There are two common blending processes employed in the nutraceutical/dietary-supplement industry to achieve a homogeneous product: dry-blending and wet-granulation. Dry blending is the most common method used to manufacture premixes. The physical property of powders is a critical aspect of dry blending. Before blending starts, first consider the properties of the ingredient powders including flowability, particle size, shape and density.

### 7.7.3 Storage, shipping, retail sale and cooking

Losses of nutrients can occur during storage, shipping and sale, but generally these losses are minimal and can be avoided. Particular attention must be focused on time/temperature relationships and protection from light and oxygen. Proper packaging and inventory controls are effective means of avoiding problems.

### 7.8 Conclusions

Food fortification is an essential element in nutrition strategies to alleviate micronutrient deficiencies. It is a dynamic area developing in response to the needs of population groups and global industries. Efforts should continue to develop new systems of delivering micronutrients to target populations through
appropriate fortification procedures. To facilitate this, those involved in the establishment of food fortification programmes must have ready access to information concerning fortification techniques and procedures being used all over the world. A multi-disciplinary approach is essential for successful fortification, with active collaboration with all parties involved. Adequate monitoring of food fortification is essential and should include both monitoring of critical control points in the production and distribution of fortified foods and the strict monitoring of micronutrient status for target populations.

From a business perspective, fortification can also make for more marketable products. However, blending nutrients is a science involving consideration of many factors. Good practice calls for high quality nutritional blends that address these issues and gain consumer confidence in the product and in the role that nutrition can play in improving health and fighting disease.

7.9 Reference

International Food and Information Council.
126  Food fortification and supplementation
Part II

Analysis, regulation and safety
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8
Vitamin analysis in fortified foods and supplements
S. Ötleş, Ege University, Turkey

8.1 Introduction
The concept of a vitamin was developed in the early part of last century. The development of chemical and instrumental methods for the separation, identification and quantitative analysis of vitamins has become extremely important to the food industry and to academic and governmental institutions, to access the nutritional value of food and food products for human health and nutritional labelling. Vitamins are organic compounds that promote and regulate essential biochemical reactions within the human body, which is generally unable to synthesise these compounds so that they have to be obtained from food in trace amounts for growth, health and reproduction. If vitamins are not present in the diet there can be symptoms of deficiency. Vitamins play an important role in metabolism, such as helping to convert fat and carbohydrates to energy, whilst not actually providing energy themselves. They also assist the processes involved in the formation of bone and tissue.¹ The vitamins are classified as vitamin D, vitamin E, vitamin A, and vitamin K, or the fat-soluble vitamins; and as thiamin (vitamin B₁), riboflavin (vitamin B₂), pantothenic acid, folate (folic acid), vitamin B₁₂ (cyanocobalamine), biotin, vitamin B₆, niacin and vitamin C (ascorbic acid), or the water-soluble vitamins.²

Recommended Dietary Allowances (RDAs), prepared by the Food and Nutrition Board of the National Academy of Sciences in the United States of America, have been around for over 70 years, with periodic updates. The RDA is the average daily dietary intake level that would adequately meet the nutritional needs of nearly all (98 percent) healthy persons. RDAs include nutrients for which there is sufficient scientific evidence that they are required
Food fortification and supplementation

for good health. Their intention has always been to establish ‘standards to serve as a goal for good nutrition.’ RDAs provide the basis for evaluating the adequacy of diets of population groups. They are set at a level that includes a safety factor appropriate to each nutrient; so this level actually exceeds the requirement for most individuals. Currently, the Food and Nutrition Board is establishing Dietary Reference Intakes (DRIs). In addition to RDAs, DRIs will include recommendations for food components for which RDAs cannot be established. Some of these include fat, carbohydrate, vitamins, minerals, fibre and plant estrogens. The RDAs are categorised by age, weight, height, sex, pregnancy and lactation. The DRI of fat-soluble vitamins is shown in Table 8.1, the DRI of water-soluble vitamins in Table 8.2, as a standard for daily amounts of vitamins needed by a healthy person.3–4

Food fortification encompasses a broader concept, and might be done for several reasons: restoration, enrichment, standardisation and supplementation. Restoration is the addition of a nutrient to a food in order to restore the original nutrient content. Enrichment is the addition of nutrients to foods in accordance with a standard of identity as defined by food regulations. Both restoration and enrichment programmes usually involve the addition of nutrients that are naturally available or present in the food product. Standardisation is the addition of nutrients to foods to compensate for natural variation, so that a standard level is achieved. Standardisation is an important step to ensure a consistent standardised quality of the final product. Supplementation is the addition of nutrients that are not normally present or are present in only minute quantities in the food. More than one nutrient may be added and they may be added in high quantities. As compared with restoration and standardisation, fortification has a special meaning: the nutrient added and the food chosen as a carrier have met certain criteria, so that the fortified product will become a good source of the nutrient for a targeted population.

Table 8.1 Dietary Reference Intake (DRI) values of fat-soluble vitamins3

<table>
<thead>
<tr>
<th>Age</th>
<th>Energy (k. cal)</th>
<th>Protein (g)</th>
<th>Vitamin A (µg RE)</th>
<th>Vitamin D (µg)</th>
<th>Vitamin E (mg TE)</th>
<th>Vitamin K (µg)</th>
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<tbody>
<tr>
<td>Children</td>
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<tr>
<td>4–6</td>
<td>1800</td>
<td>30/24</td>
<td>500</td>
<td>5</td>
<td>7</td>
<td>–/20</td>
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<tr>
<td>7–10</td>
<td>2400/2000</td>
<td>36/28</td>
<td>500</td>
<td>5</td>
<td>7</td>
<td>–/30</td>
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<tr>
<td>Males</td>
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<tr>
<td>15–18</td>
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<td>1000</td>
<td>5</td>
<td>10</td>
<td>–/65</td>
</tr>
<tr>
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<td>54/58</td>
<td>1000</td>
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<tr>
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<td></td>
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<tr>
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<td>48/44</td>
<td>800</td>
<td>5</td>
<td>8</td>
<td>–/55</td>
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<td>8</td>
<td>–/65</td>
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<tr>
<td>Age</td>
<td>Ascorbic acid (mg)</td>
<td>Folacin/Folate (µg)</td>
<td>Niacin (mg)</td>
<td>Riboflavin (mg)</td>
<td>Thiamine (mg)</td>
<td>Vitamin B&lt;sub&gt;6&lt;/sub&gt; (mg)</td>
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<tr>
<td>15–18</td>
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<td>180</td>
<td>13</td>
<td>1.2</td>
<td>1.0</td>
<td>1.6</td>
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</table>
Nutrients added for food fortification may or may not have been originally present in the food carrier. Therefore, depending on the reasons for adding nutrients, the objectives may be:

- to maintain the nutritional quality of foods, keeping nutrient levels adequate to correct or prevent specific nutritional deficiencies in the population at large or in groups at risk of certain deficiencies (i.e. the elderly, vegetarians, pregnant women, etc.);
- to increase the added nutritional value of a product (commercial view);
- and to provide certain technological functions in food processing.5

8.2 Chemistry of vitamins

Vitamins are a group of organic substances that are required in the diet of humans and animals for normal growth, maintenance of life and normal reproduction. Vitamins act as catalysts; very often either the vitamins themselves are coenzymes, or they form integral parts of coenzymes. A substance that functions as a vitamin for one species does not necessarily function as a vitamin for another species. The vitamins differ in structure, and there is no chemical grouping common to them all. The chemical structures of the vitamins are all known, and all of them have been synthesised. Vitamins were originally classified according to their solubility in water or fats, and, as more and more were discovered they were also classified alphabetically. The fat-soluble vitamins are A, D, E and K; the B complex and C vitamins are water-soluble. As more is learned about the vitamins, it is becoming apparent that the differences between these two groups concern more than just solubility. Although the modes of action for the fat-soluble vitamins are not nearly as clear as those for the water-soluble vitamins, it is evident that their activities are different from the coenzyme activities of water-soluble vitamins. The proper balance of vitamins is also important for the proper functioning of all vitamins (Table 8.3). Scientific research has proved that an excess of an isolated vitamin can produce the same symptoms as a deficiency. The water-soluble vitamins are not significantly stored by the body and need to be replaced daily by food or supplement to maintain adequate levels. These vitamins can be rapidly depleted in conditions interfering with intake or absorption. On the other hand, the fat-soluble vitamins are better stored in the body, and, if not excreted, toxic levels can occur. In Table 8.3, the structures, functions in the body, good dietary sources and deficiency signs of fat- and water-soluble vitamins are summarised.6–11

8.2.1 Water-soluble vitamins

Many vitamins work together to regulate several processes within the body. A lack of vitamins or a diet that does not provide adequate amounts of
<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Synthetic forms</th>
<th>Some naturally occurring forms</th>
<th>Structure</th>
<th>Functions in body</th>
<th>Good dietary sources</th>
<th>Deficiency signs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B₁</strong> (Thiamin)</td>
<td>Thiamine hydrochloride</td>
<td>Mainly thiamine diphosphate (cocarboxylase)</td>
<td><img src="image" alt="Thiamine Structure" /></td>
<td>Used in energy metabolism (the conversion of protein, carbohydrates and fat into energy). Supports normal appetite and needed for brain and nervous system function. A role in detoxification and heart functions.</td>
<td>Bread, brewer’s yeast, blackstrap molasses, brown rice, cereals, cereal products, egg yolk, peas, potatoes, milk and meat.</td>
<td>Beriberi, a disease resulting in atrophy, weakness of the legs, nerve damage and heart failure.</td>
</tr>
<tr>
<td><strong>B₂</strong> (Riboflavin)</td>
<td>Riboflavin phosphate (FMN) and FAD</td>
<td><img src="image" alt="Riboflavin Structure" /></td>
<td>Essential for cellular energy metabolism. Supports hormone production, neurotransmitter function, healthy eyes and skin and the production of red blood cells.</td>
<td>Brewer’s yeast, milk, meat, cereals, eggs, yoghurt, blackstrap molasses, brown rice, egg yolk, peas.</td>
<td>May result in itching and burning eyes; cracks and sores in the mouth and lips; bloodshot eyes; purplish tongue; dermatitis; retarded growth; digestive disturbances; trembling; sluggishness; oily skin.</td>
<td></td>
</tr>
<tr>
<td>Vitamin</td>
<td>Synthetic form</td>
<td>Some naturally occurring forms</td>
<td>Structure</td>
<td>Functions in body</td>
<td>Good dietary sources</td>
<td>Deficiency signs</td>
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</tr>
<tr>
<td>B3 (Niacin/ Niacinamide)</td>
<td>Nicotinamide, nicotinic acid</td>
<td>Pyridine nucleotide coenzymes: NAD, NADP</td>
<td><img src="image" alt="Structure" /></td>
<td>Used in the release of energy from carbohydrates. Health of skin, nervous system, digestive system. Essential for normal growth.</td>
<td>Milk, brewer’s yeast, eggs, meat, fish, peanuts, potatoes, poultry, rice and all protein-containing foods.</td>
<td>Pellagra, gastrointestinal disturbance, nervousness, headaches, fatigue, mental depression, vague aches and pains, irritability, loss of appetite, insomnia, skin disorders, muscular weakness, indigestion, bad breath, canker sores.</td>
</tr>
<tr>
<td>B5 (Pantothenic acid)</td>
<td>Pantothenic acid</td>
<td>CoA</td>
<td><img src="image" alt="Structure" /></td>
<td>Used in the release of energy from carbohydrates. Part of a coenzyme vital for energy release. Important for the health of the adrenal gland. Formation of antibodies.</td>
<td>Liver, brewer’s yeast, legumes, salmon, chicken, porridge, meat, nuts, cereals, eggs, dairy products.</td>
<td>Painful and burning feet, skin abnormalities, retarded growth, dizzy spells, digestive disturbances, vomiting, restlessness, stomach stress, muscle cramps.</td>
</tr>
<tr>
<td>B6 (Pyridoxine)</td>
<td>Pyridoxine hydrochloride</td>
<td>Pyridoxal 5’-phosphate, pyridoxal, pyridoxamine 5’-phosphate</td>
<td><img src="image" alt="Structure" /></td>
<td>Important in protein synthesis and the manufacture of hormones, red blood cells and enzymes. Vital for maintaining a healthy nervous system.</td>
<td>Liver, wholegrain foods, brewer’s yeast, meat/poultry, peanuts, walnuts, bananas, fish, broccoli, potatoes, soybeans, milk,</td>
<td>Nervousness, insomnia, skin eruptions, loss of muscular control, anaemia, mouth disorders, muscular weakness, dermatitis, arm and leg cramps,</td>
</tr>
</tbody>
</table>


B₁₂  Cyanocobalamin  Hydroxo and methyl cobalamins

System, skin, muscles and blood and crucial for a healthy immune system. Involved with conversion of the omega 6 essential fatty acids which play a role in hormone health.

Involved with red blood cell formation and health of nervous system. Absorption requires intrinsic factor which is secreted by the stomach. Aids in the replication of the genetic code within each cell and plays a role in the processing of carbohydrates, protein and fats in the body.

Meat, pork, liver, fish, eggs, dairy products, also fortified soya products, yeast extract, miso, bananas, kelp, peanuts.

Pernicious anaemia, poor appetite, growth failure in children, tiredness, brain damage, nervousness, neuritis, degeneration of spinal cord, depression, lack of balance.

Loss of hair, slow learning, and water retention.
<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Synthetic form</th>
<th>Some naturally occurring forms</th>
<th>Structure</th>
<th>Functions in body</th>
<th>Good dietary sources</th>
<th>Deficiency signs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folic acid</td>
<td>Pteroylglutamic acid</td>
<td>5-methyl and 5-formyl tetrahydrofolates (polyglutamate forms inside cells)</td>
<td><img src="image" alt="Folic acid Structure" /></td>
<td>Regulates cell division and the transfer of inherited traits from one cell to another. Supports the health of gums, red blood cells, skin, the gastrointestinal tract and the immune system. Reduces risk of neural tube birth defects. Heart health – helps regulate blood levels of homocysteine.</td>
<td>Wheatgerm, nuts, green leafy vegetables (broccoli, Brussels sprouts), peas, chickpeas, brewer’s yeast, brown rice, oranges, bananas, liver, fish, eggs.</td>
<td>Gastrointestinal disorders, anaemia, Vitamin B12 deficiency, pre-mature grey hair.</td>
</tr>
<tr>
<td>Biotin</td>
<td>D-Biotin</td>
<td>Biotin, biocytin, biotin-containing enzymes</td>
<td><img src="image" alt="Biotin Structure" /></td>
<td>Part of coenzyme used in energy metabolism and fat synthesis. Supports healthy skin, hair and mucous membranes.</td>
<td>Organ meats (kidney, liver), eggs, yeast extract, cereals, beer, some fruit and vegetables.</td>
<td>Extreme exhaustion, drowsiness, muscle pain, loss of appetite, depression, greyish skin colour.</td>
</tr>
</tbody>
</table>
L-ascorbic and dehydroascorbic acids

An important antioxidant that helps protect cells against damage caused by free radicals. Immune system (antibodies and white blood cells), strengthening resistance to infection. Collagen formation. Iron

Liver, fruit and vegetables, esp. bell peppers (capsicum), blackcurrants, Brussels sprouts, fresh oranges, strawberries, melons, asparagus, rosehips, broccoli and watercress.

Scurvy; soft and bleeding gums, swollen or painful joints, slow-healing wounds and fractures, bruising, nosebleeds, tooth decay, loss of appetite, muscular weakness, skin haemorrhages, capillary weakness, anaemia, impaired digestion.

Choline and Inositol

Lipotropic factors helping to prevent fat accumulations in the liver. Choline involved in formation of the brain chemical acetylcholine. Inositol functions in nerve transmission and the regulation of certain enzymes.

Liver, meat, nuts, brewer’s yeast, pulses, bread. Components of lecithin.

Cirrhosis and fatty degeneration of the liver, hardening of the arteries, heart problems, high blood pressure, haemorrhaging kidneys in choline deficiency; high blood cholesterol, constipation, eczema, hair loss in inositol deficiency.

PABA

Involved in vitamin metabolism as an enzyme cofactor, amino acid metabolism and health of red blood cells.

Liver, yoghurt, green leafy vegetables, eggs, wheatgerm and molasses.

Extreme fatigue, eczema, irritability, depressions, nervousness, constipation, headaches, digestive disorders, hair turning prematurely grey.

C

1-ascorbic acid 1-ascorbic and dehydroascorbic acids

An important antioxidant that helps protect cells against damage caused by free radicals. Immune system (antibodies and white blood cells), strengthening resistance to infection. Collagen formation. Iron

Fresh fruit and vegetables, esp. bell peppers (capsicum), blackcurrants, Brussels sprouts, fresh oranges, strawberries, melons, asparagus, rosehips, broccoli and watercress.

Scurvy; soft and bleeding gums, swollen or painful joints, slow-healing wounds and fractures, bruising, nosebleeds, tooth decay, loss of appetite, muscular weakness, skin haemorrhages, capillary weakness, anaemia, impaired digestion.
Table 8.3  Continued

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Synthetic form</th>
<th>Some naturally occurring forms</th>
<th>Structure</th>
<th>Functions in body</th>
<th>Good dietary sources</th>
<th>Deficiency signs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bioflavonoids</td>
<td></td>
<td></td>
<td></td>
<td>absorption. Formation of corticosteroid hormones in the adrenal gland. Mild anti-histamine. Plays a role in healthy gums, skin and vision.</td>
<td>A complex closely associated with vitamin C. There are many compounds in this group, including rutin and quercitin. Bioflavonoids can increase the absorption of vitamin C. They are protective antioxidants; some have anti-inflammatory properties.</td>
<td>Citrus fruits, blackcurrants, fruits, buckwheat.</td>
</tr>
</tbody>
</table>
Fat-soluble vitamins

A (Retinol)  Retinol acetate
Retinol
Retinyl esters
Retinaldehyde (Retinal)
Retinoic Acid


Milk, cream, cheese, butter, eggs, liver, cod liver oil, mackerel, herring.

Night blindness; increased susceptibility to infections; rough, dry, scaly skin; loss of smell and appetite; frequent fatigue; lack of tearing; defective teeth and gums’ retarded growth.

Beta carotene

Precursor of vitamin A; converted to vitamin A only when body needs it. Functions as for vitamin A, but also a significant antioxidant. One of the carotenoid compounds – important antioxidant plant pigments.

Carrots, spinach, watercress, sweet potato, broccoli, apricots, tomatoes. (Beta carotene provides orange pigment in fruit and vegetables)

As for vitamin A

D  Vitamin D₃ (cholecalciferol)  Vitamins D₂ and D₃ and hydroxylated forms, especially 25(OH)– and 1,25(OH)₂–vitamin D

Converted from cholesterol by action of sunlight on the skin. Bone and teeth strength. Vitamin D is required for correct calcium absorption as it is changed to a calcium-controlling hormone in the body. Healthy immune function.

Milk, cheese, butter, margarine, eggs, liver, fish, cod liver oil.

Osteoporosis (brittle bone disease), rickets (in children) and osteomalacia (bone thinning in adults).
Table 8.3 Continued

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Synthetic form</th>
<th>Some naturally occurring forms</th>
<th>Structure</th>
<th>Functions in body</th>
<th>Good dietary sources</th>
<th>Deficiency signs</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>α-Tocopherol acetate</td>
<td>α-, β-, γ- and δ-tocopherols and tocotrienols</td>
<td><img src="image" alt="Structure" /></td>
<td>Fat-soluble antioxidant. Protects the fatty parts of cell walls; prevents oxidation of polyunsaturated fatty acids. Reduces oxygen requirement of muscles. Prevents degeneration of nerves and muscles.</td>
<td>Wheatgerm oil, other vegetable/plant oils, green leafy vegetables, wholegrains.</td>
<td>A rupture of red blood cells, loss of reproductive powers, lack of sexual vitality, abnormal fat deposits in muscles, degenerative changes in the heart and other muscles; dry skin.</td>
</tr>
<tr>
<td>K</td>
<td>Menadione</td>
<td>Vitamin K₁ (phyllloquinone) and bacterial menaquinones</td>
<td><img src="image" alt="Structure" /></td>
<td>Synthesis of blood-clotting proteins. Involved in bone formation and the regulation of blood calcium levels.</td>
<td>Manufactured by colon bacteria. Meat, liver, green leafy vegetables, soya margarine, tea. Occurs widely in other foods.</td>
<td>Spontaneous bleeding.</td>
</tr>
</tbody>
</table>
certain vitamins can upset the body’s internal balance, or block one or more metabolic reactions.

Thiamin is a water-soluble B-complex vitamin, previously known as vitamin B₁ or aneurine. Thiamin occurs in the human body as free thiamin and as its phosphorylated forms: thiamin monophosphate (TMP), thiamin triphosphate (TTP), and thiamin pyrophosphate (TPP), which is also known as thiamin diphosphate. Thiamin deficiency results in beriberi, a disease resulting in atrophy, weakness of the legs, nerve damage and heart failure.

Vitamin C is also known as L-ascorbic acid. Vitamin C deficiency results in scurvy, a disease that involves bleeding.

Riboflavin is a B-complex vitamin, also known as vitamin B₂. In the body, riboflavin is primarily found as an integral component of the coenzymes flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). Coenzymes derived from riboflavin are also called flavins. Enzymes that use a flavin coenzyme are called flavoproteins.

There are six forms of vitamin B₆: pyridoxal (PL), pyridoxine (PN), pyridoxamine (PM) and their phosphate derivatives: pyridoxal 5’-phosphate (PLP), pyridoxine 5’-phosphate (PNP) and pridoxamine 5’-phosphate (PMP). PLP is the active coenzyme form, which is the most important in human metabolism.

Pantothenic acid, also known as vitamin B₅, is essential to all forms of life and is found throughout living cells in the form of coenzyme A (CoA), a vital coenzyme in numerous chemical reactions.

Specific diseases uniquely associated with deficiencies in vitamin B₆, riboflavin or pantothenic acid have not been found in humans, though persons who have been starving, or consuming poor diets for several months, might be expected to be deficient in most of the nutrients, including vitamins B₂, B₆ and pantothenic acid. Niacin is known as vitamin B₃. The term niacin refers to nicotinic acid and nicotinamide, which are both used by the body to form the coenzymes nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP).

Niacin deficiency results in pellagra. Pellagra involves skin rashes and scabs, diarrhoea and mental depression.

Vitamin B₁₂ is the largest and most complex of all the vitamins. It is unique among vitamins in that it contains a metal ion, cobalt. For this reason, cobalamin is the term used to refer to compounds having vitamin B₁₂ activity. Methylcobalamin and 5-deoxyadenosyl cobalamin are the forms of vitamin B₁₂ used in the human body. The form of cobalamin used in most supplements, cyanocobalamin, is readily converted to 5-deoxyadenosyl and methylcobalamin. Vitamin B₁₂ deficiency occurs with the failure to consume meat, milk or other dairy products. Vitamin B₁₂ deficiency causes megaloblastic anaemia and, if severe enough, can result in irreversible nerve damage.

The terms folic acid and folate are often used interchangeably for this water-soluble B-complex vitamin, but in fact there are differences between the two forms. Folic acid, the most stable form, occurs rarely in foods or the
human body, but is the synthesised form most often used in vitamin supplements and fortified foods. Naturally occurring folates exist in many chemical forms. Folates are found in foods as well as in metabolically active forms in the human body. Mild or moderate folate deficiency is common throughout the world, and can result from the failure to eat green, leafy vegetables or fruits and fruit juices. Folate deficiency causes megaloblastic anaemia, which is characterised by the presence of large abnormal cells called megaloblasts in the circulating blood. The symptoms of megaloblastic anaemia are tiredness and weakness.6–12

**8.2.2 Fat-soluble vitamins**

Vitamin A is a generic term for a large number of related compounds. Retinol (an alcohol) and retinal (an aldehyde) are often referred to as preformed vitamin A. Retinal can be converted by the body to retinoic acid, the form of vitamin A known to affect gene transcription. Retinol, retinal, retinoic acid and related compounds are known as retinoids. Beta-carotene and other carotenoids that can be converted by the body into retinol are referred to as provitamin A carotenoids. Hundreds of different carotenoids are synthesised by plants, but only about 10% of them are provitamin A carotenoids. Vitamin A deficiency is common throughout the poorer parts of the world and causes night blindness. Severe vitamin A deficiency can result in xerophthalmia, a disease which, if left untreated, results in total blindness.

Vitamin D is essential for maintaining normal calcium metabolism. Vitamin D3 (cholecalciferol) can be synthesised by humans in the skin upon exposure to ultraviolet-B (UVB) radiation from sunlight, or it can be obtained from the diet. Plants synthesise vitamin D2 (ergocalciferol), which also has vitamin D activity in humans. Vitamin D deficiency causes rickets, a disease of the bones.

The term vitamin E describes a family of eight antioxidants: four tocopherols, alpha-, beta-, gamma- and delta-, and four tocotrienols (also alpha-, beta-, gamma- and delta-). Vitamin E deficiency occurs only very rarely, and causes nerve damage.

There are two naturally occurring forms of vitamin K. Plants synthesise phylloquinone, also known as vitamin K1. Bacteria synthesise a range of vitamin K forms, using repeating 5-carbon units in the side chain of the molecule. These forms of vitamin K are designated menaquinone-n (MK-n), where n stands for the number of 5-carbon units. MK-n are collectively referred to as vitamin K2. MK-4 is not produced in significant amounts by bacteria, but appears to be synthesised by animals (including humans) from phylloquinone. MK-4 is found in a number of organs other than the liver at higher concentrations than phylloquinone. Vitamin K deficiency results in spontaneous bleeding (Table 8.3).

Although a great deal is known about the vitamins, investigations continue to determine chemical structures and functions.6–12
8.3 Extraction and purification methods

Extraction and purification of vitamins in foods, whether they are inherently present or added during food manufacture, is a challenging task due to the sensitivity of these compounds towards light, oxygen, heat and pH, and has become extremely important to the food industry to assess the nutritional quality of foodstuffs and to the medical and pharmaceutical industries for protection or control of human health. Some authors have simply recommended chemical methods. Other authors have preferred to perform these procedures by means of an acid phosphatase or by means of a takadiastase, sometimes combined with a β-glucosidase treatment. In some cases, the enzymatic treatments are preceded by mineral acid hydrolysis. The first step in the analysis is extraction from the food sample, which is done with a combination of either strong acid, or alkali, or solvent, with heating. This is necessary because the measurement techniques cannot be used for solid food substances. The next step is enzyme treatment to release any of the water-soluble vitamins bound to proteins and other components in the food matrix. The conventional method for the isolation of fat-soluble vitamins from a simple food matrix includes solvent extraction, using solvents such as hexane, ethanol, methanol, tetrahydrafuran and petroleum ether. Complex food matrices are usually saponified prior to extraction in order to disrupt the matrix and degrade triacylglycerols to glycerol and produce soaps of the free fatty acids. Solid phase extraction (SPE) techniques have been developed to replace many traditional liquid–liquid extraction methods for the determination of vitamins in aqueous samples. Solid-phase extraction is applied as an isolation method of the two fractions of the vitamins (water-soluble and fat-soluble), in case both of them exist in the same sample. Modern supercritical fluid extraction (SFE) offers shorter extraction times, potentially higher selectivity and increased sample throughput (due to available automated instruments) compared to conventional solvent extraction techniques.13–23

8.4 Methods of analysis of vitamins

The standard and official analytical methods, which are tedious, sometimes non-specific and time-consuming, involve pre-treatment of the sample through complex chemical, physical or biological reactions to eliminate the interferences commonly found, followed by individual methods for each different vitamin. These methods include spectrophotometric, polarographic, fluorimetric, enzymatic and microbiological procedures. Besides colorimetric methods, there are numerous non-spectrophotometric methods and their number and kind are increasing rapidly. These include titrimetry, voltammetry, fluorometry, potentiometry, radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), kinetic-based chemiluminescence (CL), flow injection analyses and chromatography.24
8.4.1 High performance liquid chromatography (HPLC)

High performance liquid chromatography (HPLC) is a form of column chromatography used frequently in biochemistry and analytical chemistry. It is sometimes referred to as high-pressure liquid chromatography, although this terminology is considered out-of-date and disapproved by many. HPLC is a popular method of analysis because it is easy to learn and use and is not limited by the volatility or stability of the sample compound. Where resources are available, more sophisticated methods such as HPLC, which separates the fat- and/or water-soluble vitamins in a pre-treated food sample, followed by spectrophotometric or fluorometric methods, can also be used. High performance liquid chromatography combined with a UV–Vis detector is the most common method for identification and quantification of fat- and water-soluble vitamins in foods. Several HPLC methods have been presented for the determination of vitamins in fortified food products and supplements. In the past, reversed-phase columns that were capable of tolerating high organic mobile phases, needed for the analysis of fat-soluble vitamins, could not tolerate the high aqueous conditions needed for retention of water-soluble vitamins. At the same time, reversed-phase columns that could tolerate high aqueous mobile phases lacked the hydrophobicity needed to adequately retain and separate fat-soluble vitamins. Therefore, a highly versatile column capable of tolerating a wide range of mobile phase conditions is needed to simultaneously analyse both types of vitamins. For the vitamin analysis there is wide range of HPLC techniques, such as reversed phase (RP-HPLC), normal phase (NP-HPLC), ion-exchange (IEC), paired ion (PIC) with different columns (C_{18}, NH_{2}) and detections (UV, fluorescence, coulochemical). Most of the published methods involve the use of complex buffered mobile phases. Several bonded and stationary phases or column packing materials are developed, and several detection methods can be applied: UV–Vis absorbance with a single or variable wavelength, or photodiode array, fluorimetric or electrochemical. The high performance liquid chromatography method used to determine vitamins and their isomers has high sensitivity, good selectivity and the ability of simultaneous multicomponent determination. Therefore, HPLC methods have been widely used in the study and application of vitamin analyses, particularly when applied to the separation and determination of the vitamins in supplements and fortified foods.\textsuperscript{25–53}

8.4.2 Gas chromatography

Gas chromatography (GC) procedures were developed for water- and fat-soluble vitamins before the advent of HPLC practices. GC can be used to determine the homologs of some vitamins and their isomers. Trimethyl-silyl (TMS) or other derivatives of vitamins are suitable for flame ionisation detector (FID) detection on capillary columns. Application of GC to the determination of vitamin C has been on a limited scale, and only a few papers have appeared which deal with its application to functional foods and
supplements. Utilisation of GC creates a high risk of thermal degradation of the vitamins, even when derivatisation is done prior to GC separation, e.g. in GC-based vitamin K applications. Due to high retention times and possible on-column degradation, vitamin K compounds are not very well suited for GC analysis. GC, however, results in some interesting features when used in conjunction with specific and sensitive detectors, such as gas chromatography-mass spectrometry (GC-MS), gas chromatography-electron capture detector (GC-ECD) and gas chromatography-selected ion monitoring (GC-SIM).54–63

8.4.3 Capillary electrophoresis
Capillary electrophoresis (CE) is a fairly recent analytical technique that allows the rapid and efficient separation of sample components based on differences in their electrophoretic abilities as they migrate or move through narrow bore capillary tubes. In spite of considerable developments, CE still suffers from problems of poor peak shape, lack of sensitivity and poor robustness. It has not yet been accepted in the food industry due to the absence of well-validated analytical procedures applicable to a broad range of food products. In recent reports, a number of capillary electrophoresis (CE) techniques such as capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC), microemulsion electrokinetic chromatography (MEEKC) and in-capillary enzyme reaction methods have emerged as potential alternative techniques for the determination and monitoring of vitamins in samples. A careful examination of these reports has revealed that the CE-based methods for vitamin analysis utilised to date relate solely to the vitamin forms in foods, biological samples and pharmaceuticals in conjunction with different vitamins. To our knowledge, no extensive CZE methods have come forth for the simultaneous separation and quantification of vitamin esters in samples. Separation of L-ascorbic acid (L-AA) and D-isoascorbic acid (D-IAA) in a model system by capillary zone electrophoresis (CZE) has been recently described.

High-performance capillary electrophoresis (HPCE) is a relatively new separation technique which has several advantages compared to RP-HPLC. These advantages comprise possibilities to obtain higher efficiency, higher resolution, and method simplification, in addition to the availability of various techniques developed for charged as well as uncharged analytes in free zone capillary electrophoresis (FZCE) and micellar electrokinetic capillary chromatography (MECC). Vitamins have been determined in different kinds of pharmaceutical formulations, such as tablets, injections, syrups and gelatine capsules, using MEKC or CZE methods.

Over the last decade, capillary zone electrophoresis with laser induced detection (CE-LIF) has gained much in popularity. While high resolutions can be obtained in the separation of both ionogenic and neutral compounds, LIF detection is recognised to be an extremely sensitive detection method.
More recently, the experimental conditions in CE with LIF detection have been optimised and successfully applied to the analysis of milk and wine samples.

The widespread use of multi-vitamin preparations has stimulated research on accurate, efficient and easy methods for quality control. Use of CE for the purpose of simultaneous determination of fat- and water-soluble vitamins in preparations has been undertaken with CZE or MEKC. Methods based on CE have the capability of rapid, high-resolution separation of analytes from extremely low sample volumes and are suitable for simultaneous determination.64–74

8.4.4 Spectroscopic methods
Although the literature is replete with the different types of methods for the analysis of such diversified products, efforts continue in the search of better methods. Such attempts to quantify fat- and water-soluble vitamins in fortified foods and supplements have resulted in a large number of methods. At present, the most common methods used are fluorimetry, spectrophotometry and other spectroscopic methods (Fourier transform infrared-photoacoustic (FTIR-PAS) and FT-Raman spectroscopy). Fluorimetry is particularly useful in pharmaceutical analysis, and the lack of fluorescence of many compounds has led to the development of reagents which aid the formation of fluorescent derivatives.

The most widely used method for thiamin determination is the so-called thiochrome method, which involves its oxidation in alkaline solution and extraction of the thiochrome formed from the aqueous phase into an organic phase, which is then measured fluorimetrically.

Riboflavin is usually assayed fluorimetrically by measurement of the characteristic yellowish green fluorescence.

Niacin is determined by first hydrolysing the samples with sulphuric acid to liberate nicotinic acid from combined forms. The pyridine ring of the nicotinic acid is opened with cyanogen bromide, and the fission product is coupled with sulphanilic acid to yield a yellow dye at 470 nm.

For routine analysis of fat-soluble vitamins in various samples, the first procedure is to liberate the vitamins from the sample by saponification, but this is not needed when extracting them from biological fluids. It is followed by extraction and evaporation and, for accurate quantitation, by fluorimetry or spectrophotometry.75–84

8.4.5 Microbiological methods
Microbiological assay is applicable only to the B vitamins. The rate of growth of a species of microorganism that requires a vitamin is measured in growth media that contain various known quantities of a foodstuff preparation containing unknown amounts of the vitamin. Microbiological methods use
test microorganisms such as bacteria, protozoa or yeast, where growth is proportional to the presence of a specific vitamin. These microorganisms are cultivated in the presence of known quantities of the studied vitamin and in the presence of a food extract where the vitamin needs to be evaluated. The turbidity measurement and other parameters such as gravimetry, acid production or gas production generally monitor the growth. An interesting related technique is the radiometric-microbiological assay based on the measurement of a $^{14}$C-labelled metabolite, normally $^{14}$CO$_2$, formed by the test microorganism from a $^{14}$C-labelled substrate. Microbiological assays have the advantage of specificity and sensitivity, but they are highly time-consuming if compared with the physicochemical methods, and the analytical protocol must be strictly followed due to the variability in microbiological techniques. There are no microbiological methods for fat-soluble vitamins. The first assays developed for the vitamin B group were animal assays, followed by microbiological methods. Vitamin B$_6$ (S. carlsbergensis as the test organism), folic acid (Lactobacillus casei), vitamin B$_{12}$ (Lactobacillus leichmannii), pantothenic acid (Lactobacillus plantarum) and biotin (Allescheria boydii) are usually assayed microbiologically. Novel microbiological assays for vitamins in test kit format have been marketed by different companies. These kits can be used in place of traditional microbiological determinations and have a much shorter application time. The microtitre plates for these microbiological determinations are coated with specific microorganisms, which grow according to the presence/absence of vitamins in the sample. After placing sample, standard and assay medium into the wells, the plates are incubated and subsequently evaluated using a microtitre plate reader. These test kits provide a fast and easy method for determining the content of vitamins in food products, feed, and pharmaceutical products.

8.4.6 Other methods
The standard and official analytical methods, which are tedious, sometimes non-specific, and time-consuming, involve pre-treatment of the sample through complex chemical, physical or biological reactions to eliminate the interferences commonly found, followed by individual methods for each different vitamin. The use of mass spectrometry (MS) is gaining ground and can be combined with several other separation techniques, such as GLC, HPLC and CE. The coupled technique liquid chromatography/mass spectrometry (LC–MS) enables the separation of these non-volatile, thermally labile substances before introduction into the mass spectrometer for reliable identification. Although the assay of vitamins is important in food analysis due to their important biological activity in humans, few papers dealing with the LC–MS analysis of fat-soluble and water-soluble vitamins have been published recently. Nevertheless, the few studies have revealed that this coupled technique has considerable potential in their characterisation and determination. Other methods include titrimetry, voltammetry, potentiometry, radioimmunoassay
(RIA), enzyme-linked immunosorbent assay (ELISA), kinetic-based chemiluminescence (CL), flow injection analyses, competitive protein binding assays (CPBA) and radioreceptor assay (RRA).

### 8.5 Future trends

The literature contains numerous references to the determination of fat and water soluble vitamins in fortified foods and supplements by a wide variety of techniques. Many of these methods, however, suffer from lack of selectivity, are complicated and tedious procedures, and they require the use of either expensive instrumentation or dangerous reagents. Therefore, we need the improvements of sensitivity and selectivity of standardised international methods and combined techniques.

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9

Analysis of fatty acids in fortified foods
C. Crews, Central Science Laboratory, UK

9.1 Introduction

Fatty acids have long been known as major components of edible fats and oils and methods for their analysis are well established. It is comparatively only recently that the importance of the inclusion in the diet of a variety of fatty acids has been recognised and, with the passing of this information to the public, a market for foods containing physiologically active components has grown. This chapter will describe the important fatty acids and their occurrence in oils and fats derived from plants, and in other whole and processed foods used for their functional characteristics. The role and use of fatty acids in fortified and functional foods in terms of their nutritional properties will be discussed.

The sale of any foodstuff has associated legal requirements and there is an obvious need for reliable, precise and sensitive methods for the determination of the composition of the functional compounds in particular, in terms of providing proof of composition and quality to meet technical, manufacturing and commercial needs, and legal, marketing and consumer requirements.

The major part of this chapter will concentrate on describing the application of both established and novel methods of fatty acid analysis to functional foods. Fatty acids were among the earliest organic compounds to be separated by chromatographic techniques and the methods, now well established, to determine them (principally by gas chromatography) will be described in detail. These procedures have recently been supplemented by newer techniques including high performance liquid chromatography, mass spectrometry and other spectroscopic methods, the advantages and disadvantages of which will be described.
Finally, some likely future trends in fatty acid analysis techniques will be considered, along with potential changes in quality assurance requirements, and the need to provide analytical data for functional food authenticity and traceability.

9.2 Structure and occurrence

9.2.1 Structure
Despite their complex and biological functions, the basic structures of fatty acids are relatively simple, being in almost all cases a saturated or partially unsaturated alkyl chain of even carbon number, usually unbranched and containing a terminal carboxyl group. However, minor modifications to this skeleton are responsible for many biological functions and require careful characterisation. The alkyl chain length can vary from 2 to over 80 carbons, although in food plants and edible oils those acids having between 12 and 24 carbon atoms are of primary concern. The structures of the important omega-6 linoleic acid and omega-3 linolenic acid are shown in Fig. 9.1.

9.2.2 Nomenclature
The nomenclature of a fatty acid is rather convoluted considering the simplicity of the skeleton. This is due primarily to the failure of newer naming systems to supplant older ones and the need to provide detail regarding, for example, the numerous possibilities for unsaturation. In practice, the preservation of older names such as palmitic acid and linolenic acid has proved convenient, and somewhat surprisingly some of the newer names have been adopted into functional food advertising and have become familiar to the public.

Systemic naming systems identify fatty acids based on the carbon number, such as octadecanoic acid (stearic acid) or 9-octadecenoic acid (oleic acid, with a double bond between C9 and C10). The systemic names are often abbreviated to the carbon number with the number of double bonds indicated.

![Linoleic acid](Linoleic acid.png)

![Linolenic acid](Linolenic acid.png)

**Fig. 9.1** Structure of omega-6 linoleic acid (top) and omega-3 linolenic acid (alpha-linolenic acid, bottom).
after a colon, such as C18:2 for linoleic acid (9, 12-octadecadienoic acid). The positions of unsaturation are usually measured from the carboxyl carbon atom as indicated by a preceding $\Delta$ symbol and depicted by reference to the relevant carbon atom (9, 12), frequently with the cis configuration indicated as cis or Z. More recent naming systems are, however, based on numbering double bond positions from the terminal alkyl group and use the symbols $\omega$ (omega) or n- to indicate the position of the first carbon containing a double bond. The ‘alpha’ carbon is the carbon closest to the carboxyl group (carbon number 2), and the ‘omega’ is the last carbon of the chain.

Monounsaturation is most often found at $\Delta$ 9 and polyunsaturation at positions separated by a methylene group giving a 1,4 pattern. Typical fatty acid structures are shown in Fig. 9.1 and some fatty acids important in functional foods are listed in Table 9.1 with their common and scientific names where these are often used.

9.2.3 Occurrence

The major fatty acids that have been shown to have significant physiological activity are described in this chapter. For the most part, the physiological effects of fatty acids are beneficial; however, some do have deleterious effects, such as erucic acid found in various Brassica species and now effectively removed from edible varieties by selective breeding.

<table>
<thead>
<tr>
<th>Common name</th>
<th>Scientific name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butyric</td>
<td>Butanoic</td>
</tr>
<tr>
<td>Caproic</td>
<td>Hexanoic</td>
</tr>
<tr>
<td>Caprylic</td>
<td>Octanoic</td>
</tr>
<tr>
<td>Capric</td>
<td>Decanoic</td>
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<tr>
<td>Lauric</td>
<td>Dodecanoic</td>
</tr>
<tr>
<td>Myristic</td>
<td>Tetradecanoic</td>
</tr>
<tr>
<td>Palmitic</td>
<td>Hexadecanoic</td>
</tr>
<tr>
<td>Palmitoleic</td>
<td>9-Hexadecenoic</td>
</tr>
<tr>
<td>Stearic</td>
<td>Octadecanoic</td>
</tr>
<tr>
<td>Oleic</td>
<td>9-Octadecenoic</td>
</tr>
<tr>
<td>Ricinoleic</td>
<td>12-Hydroxy-9-octadecenoic</td>
</tr>
<tr>
<td>Linoleic</td>
<td>9,12-Octadecadienoic</td>
</tr>
<tr>
<td>Alpha-linolenic (ALA)</td>
<td>9,12,15-Octadecatrienoic</td>
</tr>
<tr>
<td>Gamma-linolenic (GLA)</td>
<td>6,9,12-Octadecatrienoic</td>
</tr>
<tr>
<td>Arachidic</td>
<td>Eicosanoic</td>
</tr>
<tr>
<td>Arachidonic (AA)</td>
<td>5,8,11,14-Eicosatetraenoic</td>
</tr>
<tr>
<td>Eicosapentaenoic (EPA)</td>
<td>5,8,11,14,17-Eicosapentaenoic</td>
</tr>
<tr>
<td>Behenic</td>
<td>Docosanoic</td>
</tr>
<tr>
<td>Erucic</td>
<td>13-Docosenoic</td>
</tr>
<tr>
<td>Docosahexaenoic DHA</td>
<td>4,7,10,13,16,19-Docosahexaenoic</td>
</tr>
<tr>
<td>Lignoceric</td>
<td>Tetracosanoic</td>
</tr>
</tbody>
</table>
Fatty acids are found in all living tissues. Dietary sources used as functional foods include vegetables and vegetable oils, fish and fish oils, and eggs. The major dietary sources of high levels of fatty acids that can be considered to have functional food activity are fish oils and those from emerging seed oil plants such as flaxseed, borage and evening primrose. Olive oil is held in high esteem on account of its major role in the healthy Mediterranean diet and its established contribution to good health. This is due, in part, to its relatively high proportion of monounsaturated fatty acids, but many other components, such as polyphenols, are involved. Rapeseed oil is similar in that it has a high monounsaturated fatty acid content.

Linoleic acid (LA) is a major component of all of the major seed plant lipids, is usually the most abundant di- or polyenoic fatty acid in mammals, and is a significant component of fish oils. Conjugated linoleic acids (CLA) are a group of isomerized C18:2 acids differing in geometric and positional isomerisation, with methylene bridges and unsaturation possible between C6 and C13. A variety of cis and trans isomerisation is possible. The mixture occurs naturally in several foods and is also produced commercially by the isomerisation of linoleic acid present in seed oils with naturally high levels, such as sunflower and safflower.

Some fatty acids are not produced in the human body and must be obtained from the diet. For this reason they are known as essential fatty acids. They can be categorised into two groups, \( \omega-3 \) (or omega-3 or n-3), found in high levels in fish oils, and \( \omega-6 \) (omega-6, or n-6) fatty acids found primarily in plant oils.

The major omega-3 fatty acids are alpha-linolenic acid, 9Z,12Z,15Z-octadecatrienoic acid (ALA); eicosapentaenoic acid, eicosa-5Z,8Z,11Z,14Z,17Z-pentaenoic acid (EPA); and docosahexaenoic acid, 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoic acid (DHA).

The most important omega-6 fatty acid is gamma-linolenic acid, 6Z,9Z,12Z-octadecatrienoic acid (GLA). The polyunsaturated fatty acids as a group are known as PUFAs and the omega-3 and omega-6 polyunsaturated fatty acids are frequently named n-3 PUFAs and n-6 PUFAs. Fish oils are high in PUFAs, in particular the oils found in fatty fish such as tuna, salmon and halibut (Henninger and Ulberth, 1997; Kris-Etherton et al., 2000), which contain omega-3 PUFAs and are used as functional food components. GLA is found in some seed oils, particularly those of evening primrose, borage and blackcurrant, which are grown commercially for their oil. It is found only at small levels in animal tissues as it is rapidly converted to higher metabolites. The compositions of some vegetable oils rich in GLA have been specifically described (Uzzan et al., 1992) and detailed reviews are available describing the composition and analysis of borage oil (Senanayake and Shahidi, 2000; Eskin, 2002) and evening primrose oil (Christie, 1999; Eskin, 2002).

The PUFAs most commonly associated with functional foods, and which have become familiar to the public through marketing information, are conjugated linoleic acid (CLA) and gamma linolenic acid (GLA).
9.3 Role and use in functional foods

Polyunsaturated fatty acids are considered essential to human health. The form of the fatty acids affects subsequent fat digestion and subsequent absorption and bioavailability (Amate et al., 2001a). LA and ALA are available in relatively high levels in certain foods. Most oilseed plants, particularly those of rapeseed, flax, borage and evening primrose, contain linoleic acid, but the more important sources of ALA are fish oils, in particular those of fatty fish including salmon, trout, mackerel, sardines and herring.

The importance of LA and ALA is related to their biological function as precursors of longer chain polyunsaturated acids, which are components of cell membranes and nervous tissues. The principal long-chain polyunsaturated fatty acids (LCPUFA) are arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), the last being essential for the development and function of the retina and the brain (Clandinin et al., 1980; Bazan et al., 1986; Martinez, 1992). PUFAs have been shown to help prevent heart disease, hypertension, type 2 diabetes, arthritis and autoimmune diseases, and to restrict tumour growth (Simopoulos, 1999). EPA and AA are precursors of prostaglandins, leukotrienes and thromboxanes (the eicosanoids), which have competing and complementary thrombotic and inflammatory effects (Dobson, 2002). The associations between LCPUFAs and human health have been reviewed (Horrocks and Yeo, 1999; Simopoulos, 1994; Uauy-Dagach and Valenzuela, 2000). On account of these functions, expert committees have suggested that infant formulae be supplemented with ALA and DHA to match the elevated levels found in human milk (Amate et al., 2001b).

Functional foods can be prepared by enriching the fatty acid composition of frequently consumed food products by incorporation of the oilseed tissue as the intact seed or as a milled flour, or by addition of the oil. Fish oils are usually refined by bleaching and deodorisation to remove unacceptable odour and flavour, prior to direct consumption or incorporation into other food products. Liquid and encapsulated fish oil preparations have been included in instant powder-milk-based formulae concentrates, oils, fats and products in which the fishy taste and odour could be masked by the high sweetness and/or flavour of the food (Kolanowski, 1999).

An indirect technology has developed to modify the fatty acid composition of a number of foods (Warude et al., 2006). The major technique used is to feed oilseed rich in the desired acids to food-producing animals such as cattle and hens. The fatty acid profile of egg yolks responds to changes in the hen’s diet (Lewis et al., 2000). Laying hens fed fish meal or flaxseed can convert the alpha linolenic acid present to long chain omega-3 acids with considerable efficiency, and also accumulate them in the yolk (Cherian and Sim, 1991; Nash et al., 1995). The practice is not yet widespread but could lead to an important role for eggs in improving human nutrition (Surai and Sparks, 2001). Feeding dairy cattle, with oils rich in linoleic and linolenic acids has been shown to provide some enhancement of the levels of CLA in WPNL1103
the milk produced (Bu et al., 2007). Feeding diets rich in ALA to pigs, dairy and beef cattle, and broiler and layer chickens enhanced the levels of the acid available in the flesh, eggs and milk (De Henauw et al., 2007). The fatty acid profile, notably the Ω3 acid content, of fish oil and flesh can similarly be enriched to produce functional food (Chen et al., 2006).

9.4 Analytical requirements

The requirement for analytical methods for fatty acids is derived from technical and manufacturing needs in order to ensure that the raw materials for producing commercial products are of appropriate content and suitable quality. Following from this, analyses of the product are required to ensure that it meets commercial needs, also in terms of composition and quality. Of equal importance is the requirement to meet the demands of legislation, particularly with regard to proving safety, and labelling and marketing claims.

9.5 Established methods of analysis

Fatty acids are present in the lipid fraction of foods. The bulk of the fatty acid material is invariably present as the tri-esters of glycerol which are known as triacylglycerols, a term which is gradually replacing ‘triglyceride’. Much lower proportions are usually present as di- and mono-acylglycerols, esters of sterols and other bound forms including phospholipids, as well as the free acid.

Fatty acids can be analysed by a number of chromatographic and spectroscopic methods including those outlined below, with gas chromatography plus flame ionisation detection (GCFID) of the fatty acid methyl esters (FAME) being by far the most frequently applied. The application and use of gas chromatography (GC) based fatty acid determinations in health-focused research has been reviewed by Seppänen-Laakso et al., (2002), and a convenient compilation of official methods for the analysis of the active ingredients in functional foods assembled by Buchgraber and Karaali (2005) includes the important methods for fatty acids.

9.5.1 Extraction

In order to determine the fatty acid composition of a foodstuff, classical practice has been to extract the lipid fraction with an organic solvent and release the acids from their bound forms in a manner that is as quantitative as the purpose demands. More advanced techniques are required to obtain a detailed picture of the composition, especially with regard to the fatty acid positional arrangement of individual triacylglycerols, where the possible
combinations are today still very challenging in number. For simple practical purposes the lipid content of most food samples can be extracted with solvents of low polarity, such as hexane. This has the advantage of being safer to use than the more polar alternatives such as diethyl ether, dichloromethane and chloroform at the expense of possibly failing to extract certain bound acids. Digestion with dilute acid has been used prior to solvent extraction to hydrolyse bound forms, and alkaline hydrolysis, which is claimed to give improved recoveries, can be used in similar fashion (Dobson, 2002).

The solvent extraction has traditionally been carried out in a Soxhlet apparatus in which the sample is held in a porous cellulose thimble while solvent is evaporated and then condensed to soak the sample before being siphoned back to the evaporator in a cyclical process. This still remains an invaluable technique today, but with moves to reduce sample sizes and solvent use, in many instances similar results may be achieved using smaller quantities of sample and solvent with extraction accelerated by the use of microwaves or ultrasonic vibration. It is, however, useful to keep the sample chilled to reduce oxidation and enzyme activity that might alter the nature of the acids. Approaches to solvent extraction specifically for fatty acid determination have been critically reviewed by Palmquist and Jenkins (2003).

Fatty acid complexes with starch in relatively low fat foods such as bread can be hydrolysed with a mixture of ethanol and sulphuric acid and the resulting fatty acid ethyl esters determined by GCFID without further derivatisation (Jochum et al., 2001).

9.5.2 Methylation
To permit satisfactory gas chromatography, the fatty acids must be cleaved from the glycerol and converted to volatile derivatives, and for the majority of purposes the methyl esters are produced. Procedures for determination of FAME by GCFID are well established and many have been accepted as standard procedures by official bodies.

The conversion of triacylglycerols to methyl esters is known as methanolysis and involves the substitution of a methyl group for the glycerol backbone of the triacylglycerol in reactions which take place under acidic or basic conditions. The reactions are reversible and therefore require a large excess of methanol, which, under most circumstances, presents no difficulty.

The reagents most commonly used for acidic methanolysis are sulphuric acid, hydrogen chloride and boron trifluoride, whilst sodium methoxide is the preferred reagent for the basic process. In both approaches, a solution of the oils is heated with the reagent for a prescribed period of time and temperature. When the reaction is complete, washing with water is used to remove the reagent, and the organic solution of methyl esters is dried and analysed directly by GCFID. The addition of an antioxidant such as butylated hydroxytoluene (BHT) to the sample is recommended, and fresh reagents must be prepared frequently.
Methanol containing dissolved hydrogen chloride (HCl) at about five percent is the preferred reagent as it is mild and can be removed by evaporation. Its preparation in bulk from gaseous HCl is rather cumbersome but it can be purchased ready made in small quantities or prepared by the careful addition of acetyl chloride to cold methanol. Alternatively, sulphuric acid can be used as a solution of about one to two percent in methanol, which is added in excess to the oils dissolved in toluene. Esterification with acid is known to cause migration of conjugated double bonds (Kramer et al., 1997).

Methanolysis with methanol/boron trifluoride is quite successful in producing good yields of FAME with many oils (Craske, 1993), including those from fish (Ackman, 1998). The reagent is readily available commercially and is convenient to use. However, adverse effects have been reported (Christie, 1993; Kramer et al., 1997; Rosenfeld, 2002), particularly when the reagent is old or used in high concentration.

Base catalysed transesterification is often carried out with methanolic potassium hydroxide. However, this reagent does not react with free fatty acids but actually tends to saponify the methyl esters produced, resulting in the production of free acids which are not subsequently methylated with sufficiently high efficiency (Ackman, 1998). The effect can be diminished by using short reaction times of only a few minutes. Official procedures, such as Method 969.33 of the Association of Official Analytical Chemists (AOAC, 2006) and Method Ce 2-66 of the American Oil Chemists’ Society (AOCS, 1998) use a combination of methanolic KOH transesterification and methylation with methanolic boron trifluoride.

Carvalho and Malcata (2005) have shown that the polarity of the reaction medium is important in that intermediate polarity solvents, such as an equivolume mixture of methanol with diethyl ether or toluene, provide statistically higher amounts of extracted polyunsaturated fatty acids than those obtained via standard methods. Direct transesterification with sodium methoxide is preferred when the free fatty acid content of the food is low (Christie, 1999).

An alternative approach which has been applied to many foods is to carry out the methylation without prior extraction of the lipid fraction (Ulberth and Henninger, 1992), in which case the methyl esters are formed in the sample and subsequently extracted.

### 9.5.3 Gas chromatography

Fatty acid methyl esters can be determined using a range of different gas chromatography (GC) column lengths and phases, depending on the target acids and the resolution required. For general analyses of fatty acid profiles, suitable results can be obtained using chemically bonded Carbowax-based stationary phases with a relatively thin film thickness (0.1 to 0.2 µM) and a length of 50 to 60 metres. Hydrogen carrier gas provides better resolution than helium or nitrogen, and unless sample quantity is severely restricted,
solutions can be sufficiently concentrated for splitless injection, although on-column injection is preferred. The oven temperature programme is normally increased slowly from about 150°C to 210/220°C.

Full chromatographic resolution of cis and trans isomers of CLA and of trans monoenoic acids is challenging. The more polar CP-Sil 88, BPX-70 or SP-2560 columns, in lengths of up to 120 metres, are required for baseline resolution, with hydrogen as carrier gas. The polarity of these columns varies with temperature, allowing fine adjustment of resolution. This is the basis of official methods for determining cis-, trans- and unsaturated fatty acids, such as the AOCS method Ce 1h-05 (AOCS, 1998), for which specific operating conditions have been recommended in view of the large number of closely eluting fatty acids (Ratnayake et al., 2006).

Identification of fatty acids from gas chromatograms of their methyl esters can be challenging where many peaks are observed. The identification of a FAME from its retention time is difficult as the elution order changes with different stationary phases, and unsaturated acids and isomers are not eluted in predictable patterns. The methyl esters can best be identified by analysis of mixtures of standards that are readily available commercially. As the number of acids can be substantial, it is best to use a small number of mixtures covering different ranges depending on the range of fatty acids to be determined. Also available are standards of named vegetable oils, including several reference materials of certified composition. In the case of some of the less common acids, oils known to contain elevated levels of the target analytes can provide a guide.

Confirmation of the identity of fatty acids from gas chromatograms of their methyl esters is most easily obtained by carrying out a second analysis on a column phase of different polarity. Mass spectrometry can be used to a limited extent but as the molecular ions of FAME are of relatively low intensity after electron impact ionisation, this approach is usually restricted to more specialised initial identification of structural composition, often using other esters and derivatives. This will be discussed later in the Chapter.

The determination of short-chain fatty acids presents a special problem due to their volatility and relative polarity. Most attention has been focused on methods for the determination of butanoic acid because of its presence in milk fat. Short-chain fatty acids can be measured by GCFID as the free acids or the methyl or higher esters, and also, due to their volatility, by injecting a sample taken from the vapour (headspace) above a warmed food sample. A comparison by Ulberth (1997) of the most important methods for determining butanoic acid demonstrates these approaches. The volatility of short chain fatty acids can be reduced by esterifying with ethanol or propanol in place of methanol, at the risk of producing excessive retention of the higher acids.

Measurement of fatty acids is frequently performed to produce qualitative data in the form of the percentage composition of the constituent acids. This commonly used procedure is risky in that an error in the measurement of one fatty acid, such as a misplaced baseline, affects the result for all of the other
acids (Ulberth, 2003). Semi-quantitative data can be obtained by adding a gravimetrically prepared internal standard, and comparing the peak area of this with those of the target fatty acids. Due to their low level of natural occurrence, odd-numbered fatty acids (C15, C17, C21 or C23) are most often used, added as the acid prior to derivatisation or subsequently as the methyl ester. Adding the internal standard as a triacylglycerol can correct for losses during the methanolysis stages. The accuracy of this measurement can be improved by calculation of response factors for the target acids and internal standard, and by making the necessary correction. Quantitative results showing the concentration by weight of the individual acids in the sample can be enhanced by measurement against gravimetrically prepared individual standards. A standard procedure for fish oils, AOCS Ce 1b-89, provides percentage composition data for the major acids with quantitative measurement of EPA and DHA (AOCS, 1998).

9.5.4 Gas chromatography mass spectrometry

The quantification of the chemical composition of foods is, in most fields, based on the use of mass spectrometric detectors, on account of their high sensitivity and specificity. Proposals have been made to apply this approach to fatty acids and a detailed comparison of the performance of gas chromatography with mass spectrometry (GCMS) with GCFID applied to marine-relevant FAME standards and a fish tissue reference material has been reported (Dodds et al., 2005).

Scanning mass spectrometry provides a total ion current (TIC) signal which can be used to quantify FAMEs in the same manner as an FID response. It is non-specific but spectra from it can be examined to confirm the peak identity. Alternatively, signals from a few individual ions specific to certain FAME can be extracted from the TIC response to reduce or eliminate interferences. Selective monitoring of these ions (Selected Ion Monitoring, SIM) during acquisition provides considerably enhanced sensitivity whilst retaining the ability for identity confirmation by comparison of relative response ratios.

Dodds et al. (2005) showed that roughly comparable results were obtained by GCFID and a range of GCMS methods. Linearity was poorer for GCMS methods but equivalent results were obtained when this was compensated for by use of a C21:0 internal standard. Relative responses to a wide number of fatty acids were similar for GCFID but varied with acid for the GCMS methods. The best overall GCMS performance was obtained with SIM with a quadrupole detector for quantitative measurements; based on full scanning, the ion trap detector was better than the quadrupole. Sensitivity was not improved by use of the GCMS but the high level of spectral information and confidence in peak identity mean the technique will continue to find application.

The dietary importance of omega-3 and CLA has led to dedicated studies into their determination, such as those reviewed by Kramer et al. (2001),

Determination of the degree of saturation of fatty acids has long been of importance due to the adverse effects of high levels of *trans* fatty acids which are produced during hydrogenation. The detailed composition of *trans* acids in oils and fats may be determined by means of fatty acid profiling using the methods described above, provided a suitably polar column phase (typically cyanopropylpolysiloxane) is used which can separate *cis-* and *trans-*monoenoic acids. The separation is improved by the use of long (100 m) capillary columns or by using silver ion thin layer chromatography (TLC) or silver ion high performance liquid chromatography (HPLC) prior to GCFID (Delmonte et al., 2004; Ratnayake, 2004).

The resolution and elution order of fatty acids of C18 isomers are affected by the temperature programme and best results are obtained with long columns operated isothermally (Ratnayake et al., 2002). The separation achieved is illustrated in Fig. 9.2, which shows the C18 region of FAMEs from margarines, separated on an SP-2560 capillary column.

For the determination of the fine structures of fatty acids, the established approach is to study the mass spectra obtained after electron impact

![Figure 9.2](image-url)

**Fig. 9.2** The C18 region of FAMEs from margarines separated on an SP-2560 capillary column (100 m × 0.25 mm id × 20 µm film thickness. The first peak is C18:0 and the last C18:3n-3. The large peak after 25 minutes is C18:2n-6. (From Ratnayake et al., 2002.)
Food fortification and supplementation

fragmentation. The mass spectra of FAME do not provide much evidence of the double bond positions or branching and so more exotic derivatives are employed to induce the production of more diagnostic ions. The best approach is to use a reagent containing a nitrogen atom. In this case, ionisation in the mass spectrometer produces an ion with a charge carried on the nitrogen atom and not the alkyl chain. This ion undergoes cleavage at each C–C bond to form a series of relatively abundant ions, which are diagnostic of the double bond positions. Picolinyl esters (Destaillets and Angers, 2002), made from the acid chloride and picolinyl alcohol, or 4,4-dimethyloxazoline esters, made from the free fatty acid and 2-amino-2-methylpropanol (Christie et al., 2000), are most often used. They provide complementary information. Useful rules for the interpretation of the mass spectra obtained from these derivatives have been published (Christie et al., 1987; Christie et al., 2000) and their mass spectra have been catalogued in an on-line Lipid Library available at http://www.lipidlibrary.co.uk/massspec.html.

Fatty acid determination is usually performed on the whole sample. In some cases, however, it is necessary that the fatty acid composition of components of the lipid fraction, such as the mono- or diglycerides or the phospholipids, are known. To do this, the components of interest can be isolated by TLC, HPLC or by solid phase extraction (SPE), the latter using pre-packed columns containing a range of sorbent fillings. The techniques are more often applied to tissue samples (Giacometti et al., 2002).

9.5.5 Thin-layer chromatography

Thin-layer chromatography (TLC) is still of considerable value in the preliminary separation of lipid classes but has few applications in the determination of fatty acids. The technique can rapidly and inexpensively separate mono-, di- and tri-acylglycerols, for example, using a wide range of mobile phases. Plates can be developed more than once to improve resolution. Fatty acids in the separated fractions can be extracted from the excised silica with solvent or derivatised without extraction, although losses of unsaturated acids have been reported (Sowa and Subbaiah, 2004).

Unsaturated compounds form complexes with silver ions which can be separated efficiently by chromatography, and this phenomenon has proved extremely useful in fatty acid determination. The technique has long been exploited in TLC, where the adsorbent layer can relatively easily be impregnated with silver salt by spraying the plate with a solution of silver nitrate, by dipping or developing the plate in such a solution, or by preparing the plate from silica gel slurried with the salt solution. A silver content of 5 to 20% in the adsorbent layer is now most often used. Regardless of its usefulness, the practice requires care and experience and the conditions of use (preparation method, silver content, developing solvent, temperature, etc.) have not been fully elucidated. An overview of the technique has been provided by Nikolova-Damyanova and Momchilova (2001).
9.5.6 High performance liquid chromatography

High Performance Liquid Chromatography (HPLC) has surpassed TLC in most applications due to its speed and ease of use, and this is true for fatty acid analysis. Again, fractionation of lipid classes prior to GC is its major role, applied to glycerides or groups of isomers (such as CLA). HPLC has not competed with GCFID for the end determination of fatty acids owing to the ease of use of GC when analysing relatively small and volatile molecules such as FAMEs.

Esterified fatty acids can be separated by HPLC using standard reverse-phase column techniques (bonded octadecylsilane stationary phases and acetonitrile or methanol based mobile phases) with UV detection. Ultraviolet spectrophotometric detectors are the most widely used in HPLC, but as fatty acids absorb poorly in the UV range they are usually reacted to form specific derivatives of lipids that absorb above 235 nm. For example, fatty acids can be converted to $p$-bromophenacyl esters instead of methyl esters, and diacylglycerols, derived from phospholipids, can be analysed as benzoates rather than acetates. Much higher sensitivity can be achieved by using derivatives amenable to fluorescence detection.

Evaporative light-scattering detectors are probably the best choice for monitoring fatty acids by HPLC. They can be used with a range of volatile solvents and are unaffected by solvent gradients or flow-rate variations.

Silver ion based separations based on HPLC have been used in combination with GC for the separation of positional and geometrical isomers of unsaturated fatty acids, including cis and trans isomers (Adlof, 2003; Guil-Guerrero et al., 2003; Fournier et al., 2006). In this case, the method is easier to use than silver ion TLC but the columns are relatively short-lived. The technique is particularly useful in studies of fish oils, where the necessary refining processes lead to isomerisation of the long-chain polyunsaturated acids. Silver treated columns, where the silver is bound to silica gel with chemically-bonded phenylsulphonic acid groups, are commercially available but can also be prepared relatively easily. Low pressure liquid chromatographic systems have also been employed in which the silver is bound to macroreticular sulphonic acid resins or to pre-packed benzenesulphonic acid cation-exchange columns designed for solid-phase extraction.

Procedures, including the above, for the separation and identification of molecular species of triacylglycerols have been reviewed by Christie et al. (1987), Nickolova-Damyanova (1997) and Nikolova-Damyanova and Momchilova (2001).

9.5.7 Analysis of positional distribution triacylglycerols

The fatty acid in the $sn$-2 of triacylglycerols is often more characteristic of individual oils and its identification is therefore of particular interest. The composition of position 2 of triacylglycerols can be determined by incubating them with the enzyme pancreatic lipase in an appropriate buffer. The fatty
acids are partially hydrolysed from the primary positions leaving a 2-monoacyl-sn-glycerol, which can be isolated by TLC or HPLC for determination of its fatty acid composition by GCFID. This method has been adopted as an official procedure (AOCS Ch 3-91).

The identification of which fatty acids are present in all particular positions of triacylglycerols presents considerable challenges in view of the very high number of possible configurations. The general approach to this is to fractionate the triacylglycerols and subsequently selectively remove the fatty acid from the terminal positions using enzymes such as pancreatic lipase and phospholipase (Schreiner et al., 2006). Pancreatic lipase cleaves the acid on the outer (α-) positions of triacylglycerols and the resulting 2-acylglycerol can be separated by TLC or HPLC. Phospholipase A2 can be used to hydrolyse phospholipids such as lecithin in eggs (Amate et al., 1999).

Hydrolysis must be generated randomly to provide diacylglycerols showing the fatty acids in each position. Hydrolysis with pancreatic lipase has mostly been replaced by reaction with the Grignard reagent ethyl magnesium bromide, which has a non-specific action. The diacylglycerols formed must be isolated immediately and preserved as phosphatidylcholines for subsequent analysis (1,2-, 2,3- and 1,3-diacylglycerols). The procedure, which is rather lengthy and complex, has been described by Christie (1986).

If the partial hydrolysis products are reacted with a chiral derivatising agent such as (S)-(+)1-(1-naphthyl) ethyl isocyanate, diacyl-sn-glycerol urethane derivatives are formed that can be isolated by chromatography on C18 (octadecylsilyl) solid-phase extraction columns and subsequently separated by normal phase HPLC with UV detection. The 1,3-diacylglycerol urethanes readily undergo acyl migration and cannot be characterised, but the chiral derivatives of 1,2- and 2,3-diacylglycerol are now diastereomers and can be separated. If 3,5-dinitrophenyl urethane (DNPU) derivatives are made from the partial glycerides they can be resolved by HPLC using chiral stationary phases.

Triacylglycerols can be fractionated according to the type and degree of unsaturation using silver-ion chromatography applied as TLC or by HPLC. The fractions obtained in this way can be fractionated further, for example by reversed-phase HPLC (Kallio et al., 2006). The resulting samples can be analysed by GCFID and/or GCMS in order to identify the constituent acids.

9.5.8 Other methods
The percentages of conjugated diene, triene, tetraene and pentaene acids and of linoleic, linolenic, arachidonic and pentaenoic acids can be calculated from measurements of the ultraviolet absorption of the conjugated bonds of acids that contain one single bond between two double bonds and where the non-conjugated constituents contain two or more single bonds between two double bonds. This method has been adopted by the AOCS (Method Cd7-58, AOCS, 1998).

Other spectroscopic methods, particularly infrared spectroscopy in
reflectance or transmittance modes, can be used to determine a limited number of unsaturated fatty acids with rapidity after calibration (Azizian and Kramer, 2005; Christy and Egeberg, 2006). The technique, which is rapid and non-destructive, has been applied to the determination of trans fatty acid content (Van de Voort et al., 1995) and also to the analysis of single seeds (Velasco et al., 1999).

9.6 Newer methods of analysis

Nuclear magnetic resonance spectroscopy (NMR) can rapidly provide both qualitative and quantitative data on fatty acid composition and can be applied to the major unsaturated fatty acids. Techniques are based on the chemical shift of a proton (H-1 NMR) or a carbon atom (13C-NMR), the shift depending on the range of atoms in the subject atom’s vicinity. Applications of NMR are growing rapidly in number as much more sensitive instruments become commercially available. The technique is attractive due to the relatively small amount of sample preparation required. NMR techniques can be used with minimal sample preparation for the determination of the fatty acid composition and distribution in the triacylglycerol molecule and in the measurement of free fatty acids (Hidalgo and Zamora, 2003).

In proton NMR (H-1 NMR), the signal obtained from the terminal methyl group of linolenic acid is well separated from those of other acids, allowing it to be quantified using equations derived from integration of the signal to give a peak area (Knothe and Kenar, 2004; Miyake et al., 1998). Terminal methyl protons, divinyl protons and allyl protons are useful to calculate linolenic acid, linoleic acid and oleic acid, respectively. This method uses the area per proton (determined by integration) and gives equations for determining the amounts of the unsaturated fatty acids.

13C-NMR can also be used for the quantitative analysis of fatty acids (Gunstone, 1994; Hutton et al., 1999). In this case the omega-2 carbons, divinyl carbons and allylic carbons are used for determination of the fatty acids. Given sufficient sample size, 13C-NMR can be used for the identification and quantification of all of the positional and geometrical CLA isomers in complex mixtures (Davis et al., 1999; Christie et al., 2001) and has been applied to oils (evening primrose, borage, blackcurrant) containing gamma linolenic acid (Gunstone, 1990).

Both H-1 and 13C-NMR give data with reasonable agreement with those obtained from GCFID (Sacchi et al., 1993).

9.7 Future trends

For routine analysis of fatty acid profiles, as would be conducted by food industry laboratories, it is likely that GCFID will continue to have a prominent
place. The major development in GC which might be applied to fatty acid analysis is the use of two-dimensional gas chromatography (GC × GC), which provides rapid analysis times for complex samples. The disadvantage is that such methods require rapid peak detection and this can be provided realistically only by mass spectrometers with a Time of Flight (TOF) analyser system. The resurrection of TOF mass spectrometry as a viable detection tool has yet to make an impact on fatty acid analysis, but it offers considerable potential and has already been used to determine fatty acids along with alcohols and other components (Jover et al., 2005).

The most likely replacement for GCFID is benchtop NMR, which will be used increasingly as the capital cost, analysis time and required sample size decrease. Complementary structural information can be provided by linking GC to Fourier-transform infra-red detectors, but the interfaces are, so far, rather complex and some degree of chromatographic resolution is lost.

For research purposes, studies will most likely be focused on the available form of the fatty acids; that is, the positional structure of the parent triacylglycerols. Techniques based on HPLC with mass spectrometric detection have so far found limited application but HPLC/MS is rapidly overtaking GCMS in many fields following the development of turbomolecular pumping systems, improved reliability, and ease of use, coupled with the high processing power of personal computers. In particular, tandem mass spectrometry (MS/MS) systems of decreasing cost, and the selection of specific ions for further fragmentation, give very high sensitivity and specificity because of the reduced background signal. Tandem techniques can also give insights into structure.

The use of TOF analysers linked to HPLC can provide data from complex samples in a very short time and the technique is rapidly gaining a high profile. Applications to fatty acid analysis are so far limited but there is certain to be an expansion of applications in the near future. For example, electrospray ionisation in the negative mode with MS/MS and TOF MS has been used to analyse fatty acyl compositional profiles from anions derived from free fatty acids and complex lipids (Esch et al., 2007).

Fatty acid analysis will continue to be affected by a trend towards enhancing analytical quality assurance procedures. Laboratories now need to demonstrate that the results they produce have sufficient accuracy and precision, that the methods used have been suitably validated, that the equipment used is properly functioning and has been maintained, that staff have been trained, and that full records are kept to ensure that all steps in a procedure can be checked. Quality assurance measures include the use of standard reference materials and the testing of both methods and laboratory performance by means of collaborative trials. In the case of fatty acid determination, as the common methods are long established and based on a technique (GCFID) that has changed little in recent years, comprehensive evaluations of the methods have been limited. Two examples are provided, Buchgraber and Ulberth (2002) and Campo et al. (2006).
Newer applications of fatty acid analysis will arise from the modification of oil-producing plants and animals to produce functional foods through selective feeding and genetic modification processes. Genetically modified crop production can be adapted to produce high yields of PUFA, including the long-chain unsaturated acids, in oil seeds such as sunflower, safflower and flax. Genes responsible for the production of PUFA have recently been identified, with the potential for the production of plants modified to produce oil with high yields of these desirable acids (Warude et al., 2006).

Finally, the growing fortified and functional food market will require ever more comprehensive testing of the authenticity of its products and their traceability from production to consumption. The analysis of fatty acids is certain to have a prominent role in providing this assurance to the consumer.

### 9.8 Sources of further information and advice

A large number of textbooks are available covering the area of fatty acid analysis. Some of the most useful and comprehensive texts are those published by The Oily Press Lipid Library. These include the overview *Lipid Analysis* (Christie, 2003), which is dedicated largely to fatty acids, and the six books in the ‘Advances in Lipid Methodology’ series of which the last was published in 2003 (Adlof, 2003).

Journals specialising in lipid chemistry regularly include updates on fatty acid analysis. The most prominent publications are the *Journal of the American Oil Chemists Society*, *Lipids* and the *European Journal of Lipid Science and Technology*.

Internet resources on lipid analysis are now well established and have been reviewed (Adlof, 2004). Copious valuable information is freely available from the Lipid Library website at http://www.lipidlibrary.co.uk, including practical guides on fatty acid analysis and a large databank of mass spectra of derivatives useful for confirmation of structure. Similar and related information is also available free of charge from the Cyberlipid Center at http://www.cyberlipid.org.

The major professional bodies concerned with analysis of oils and fats are the American Oil Chemists’ Society (AOCS, http://www.aocs.org) and the European Federation for the Science and Technology of Lipids (EuroFedLipid). The International Society for Fat Research (ISF, http://www.isfnet.org) is a federation of national and regional scientific associations active in the areas of fats, oils and lipids.

Several prestigious bodies issue standard methods for the analysis of fatty acids; these include the AOCS, the Association of Official Analytical Chemists International (AOAC International), the International Organization for Standardization (ISO, http://www.iso.org), and the International Union of Pure and Applied Chemistry (IUPAC). The majority of these methods are based upon qualitative determination (percentage composition) achieved by
GCFID procedures. As well as fatty acid profiling, the official methods cover a good range of targeted analytes such as trans acid and CLA. Many are offered in several different formats, including printed documents, compact discs and on-line formats.

9.9 References


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Analysis of polyphenol antioxidants in fortified foods and supplements

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10.1 Introduction to polyphenols

Polyphenols are a ubiquitous class of non-volatile secondary plant metabolites, characterised by the possession of one or more hydroxyl groups attached to an aromatic ring. Their structures range from the simplicity of catechol (1,2-dihydroxybenzene) up to the complexity of the oligomeric procyanidins (poly-hydroxylated C15 units linked by carbon–carbon bonds to provide oligomers comprising five, ten or more units joined together). Other closely related components such as vanillin and eugenol (with one free and one methylated hydroxyl) also occur in plants. There are probably upwards of 5000 total plant polyphenols known, with more being described on a regular basis. Many polyphenols fall into more-or-less defined categories, such as the following examples:

*Hydroxycinnamic acids*, e.g. chlorogenic, caffeoyl tartaric, chicoric, echinacoside
*Substituted benzoic acids*, e.g. gallic acid, ellagic acids, ‘gallotannins’ (e.g. pentagalloyl glucose)
*Flavonoids*, e.g. sub-classes such as chalcones, flavans (catechins), flavones (and flavonols), anthocyanins, procyanidins.

The structures of individual compounds differ primarily in terms of substitution patterns, such as the number and position of hydroxyl or methoxyl groups on a basic skeleton. In nature, many of these structures are present in glycosylated or conjugated forms, which further increases their complexity (Macheix et al., 1990). For instance, the widely-distributed flavonol quercetin (named for its original isolation from oak bark) is rarely, if ever, found in the free
state but is found e.g. in buckwheat as rutin (quercetin rhamno-glucoside), and in apple peel as a mixture of the galactoside, arabinoside, xyloside, glucoside and rhamnoside glycosides. The confusion between the aglycone and glycosylated forms of polyphenols is frequently a source of misunderstanding when assays are requested and reported, not least since the mass of material calculated as glycosides is often double or more the amount when calculated as aglycone.

10.1.1 Polyphenol complexity
Generally when polyphenols are extracted from plants as dietary supplements, their complexity is retained and few of them are ever isolated in a pure state. For example, the extract of milk thistle popularly known as ‘silymarin’ is, in practice, a mixture of at least 6 individual major flavanones. Before the days of High Performance Liquid Chromatography (HPLC) separation, when only gross colorimetric assays were available, it was possible only to assay the silymarin mixture as a whole – nowadays it is possible to assay each individual component accurately, but their sum does not add up to the total value expressed by the older methods. For this reason, if no other, certificates of analysis for polyphenol extracts should always state the nature of the assay that has been used, and needs to be mutually agreed in advance between buyer and seller. Many trade misunderstandings have arisen through failure to address this point.

Many polyphenols, especially those containing adjacent dihydroxy groups (e.g. catechins and procyanidins) are especially prone to polymerisation and loss through oxidation. Sometimes (e.g. in the case of catechins during conversion of green tea to black) this may be due to enzymic processes in the original plant material. Otherwise, oxidative polymerisation and browning takes place simply through repeated handling and exposure to air and moisture, and many polyphenols are especially unstable in air at pH values much above 6. Generally, polyphenols are better protected at lower pH values, although some hydrolysis and polymerisation reactions (e.g. cleavage and re-forming of the C–C bond in procyanidins) are encouraged at pH < 2.

The analysis of individual named phenolic compounds, such as echinacoside or chicoric acid in *Echinacea*, is frequently possible by HPLC using UV detection, so long as appropriate standards or surrogates are available. But because of the diversity of polyphenol structures and the arbitrary nature of many of the broader classifications, there are few specific assays for many of the popularly used collective terms such as ‘flavonoids’ and ‘catechins’, and those assays that do exist are likely to be very antiquated. Hence a request for analysis of ‘total flavonoids in propolis’ or ‘total catechins in tea’ may be almost impossible to fulfil without going back to century-old methods such as colorimetric reactions with aluminium chloride or vanillin. Such methods are non-specific, lack defined standards and generally do not satisfy the modern requirements of world trade in terms of product definition and
authenticity. Conversely, however, it is often impossible to provide an exact mass-balance of the many hundreds of components that may be present in an extract, even by modern techniques such as HPLC. In part this is due to lack of pure and verifiable individual standard components, in part due to lack of resolving power and in part due to issues around oxidation and degradation in the extracts. In some cases these problems are more easily solved than others. Thus, in the examples given, the analysis of all flavonoids in propolis is not practicable by any current HPLC methodology since there are too many of them and standards are not available – but it is quite feasible to offer the analysis of a limited set of marker compounds. In green tea, by contrast, the pattern of major catechins is rather simple and it is possible to analyse and sum these individual components by HPLC to provide a ‘total catechin’ value.

A current confusion, which is unlikely to be resolved in the short term, is the relationship between ‘polyphenol’ and ‘antioxidant’, and much marketing of dietary supplements blurs the distinction. It is true that many polyphenols are antioxidants, although not all (e.g. quercetin) are truly antioxidants in the chemical sense, even though they may display free-radical scavenging capacity in vitro. Many dietary antioxidants, e.g. ascorbic acid, beta-carotene or tocopherol (vitamin E) are certainly not polyphenols. In any case, the concept of ‘antioxidant’ as deployed here is in terms of in vivo antioxidant capacity as related to the ‘oxidative free radical theory’ of chronic diseases such as CVD or cancer. Whether this hypothesis itself will stand the test of time or whether the beneficial effect of these components is more truly related to their impact on cell signalling processes is a moot point, but is not a topic for this chapter (Halliwell, 2006; Serafini, 2006). However, a request for analysis of ‘total antioxidants’ is always one that the analyst must refer back and discuss with his client so that there is no confusion. For instance, the Folin-Ciocalteu ‘total polyphenol’ redox method may be appropriate in some cases whereas the Oxygen Radical Absorbance Capacity (ORAC) or Trolox Equivalent Antioxidative Capacity (TEAC) free-radical scavenging assay methods may be more appropriate in others. Often the judgement will depend on whether the data is intended to be comparative and is to be used with reference to existing analytical data obtained elsewhere.

10.1.2 Sample extraction

Before any analysis can take place, the materials of interest must be extracted from the sample. In the case of dietary supplements, we may distinguish three cases:

(i) the original plant material
(ii) a solvent extract made from it, often dried on a carrier
(iii) a formulated and finished product.

Original plant material is not so frequently encountered by the analyst since
it is primarily (ii) and (iii) that are traded. Fresh plant material is typically macerated or homogenised in aqueous ethanol or methanol solutions (ca 70%). The alcohol acts as a solvent for most polyphenols and the presence of water prevents cell structures from shrinking or drying out. Dried plant material may need some re-hydration before extraction, or the solvent may not penetrate the shrunken cells. Dried extract material of type (ii), as supplied in 25 kg drums, is often the easiest material to prepare for analysis. However, if alcohol-soluble materials have been spray-dried onto malto-dextrin or other polar carrier, the lack of wettability may give surprisingly poor recoveries. Usually it is necessary to homogenise the powder with water to fully re-hydrate it, before adding alcohol or other solvent to complete the final extraction. Finished product samples (iii) can be found in a bewildering variety of forms and often present the analyst with the greatest difficulty. Simple powder formulations in two part capsules may be treated as for (ii). Supplement formulations in oils or other unctuous matrices such as creams and ointments are often a considerable challenge. In these cases, sample contents can be pooled (after ensuring homogeneous mixing of contents) and defatted by extraction with hexane or dichloromethane (in which polyphenols are insoluble), after addition of water to ensure a two-phase system. Hexane/methanol is a useful immiscible solvent system although it often forms an emulsion when blended with finished product. However, the drop-wise addition of water can ensure the separation into two discrete phases from which the lower layer is taken for analysis. It is important to ensure complete phase separation since otherwise polyphenol material may be removed in emulsified form with the lipid layer. An alternative procedure, if the subsequent HPLC system can tolerate it, is to deliberately establish a single homogeneous phase by the addition of tetrahydrofuran (THF). This solvent has both lipophilic and hydrophilic properties and dissolves phenolics well in the presence of 20% water. It can often transform an intractable ‘gloop’ into a usable analytical proposition. (The THF is itself prone to peroxide formation and must be used in a stabilised form with the prior addition of an antioxidant such as BHT.)

For fortified foodstuffs there can be no specific extraction rules. The analyst must use his/her own judgement, bearing in mind the nature of the food to be extracted and the nature of the fortificant. The same general principles apply as discussed above, especially as concerns hydration of carbohydrate material and removal of fat. An additional complication may be the presence of protein, since many polyphenols will bind to protein in a ‘tanning-like’ reaction. This is especially the case for catechins, procyanidins and gallo-tannins. Protein disrupting agents such as SDS or 8M urea may be considered. In some cases the practicality will depend on how much the client is prepared to spend to have the analysis and its associated development work carried out, and it may be important to recognise that some analyses of polyphenols in foods simply cannot be done at a realistic cost.

In all these complex cases, recovery trials are essential if any credence is
to be put on the results. For instance, in our laboratory, we could obtain reasonable HPLC recovery data on a green tea extract added (before baking) to white bread. The addition of a similar extract to biscuits (cookies) gave very poor recovery, and the recovery from fortified dog food after retorting was essentially nil – oxidative and protein binding reactions led to the catechins being essentially unrecoverable and unanalysable. By comparison, in the case of colorimetric assays, if the food matrix already contains materials likely to react in the assay, then the assay is worthless. For instance, although total tea polyphenols may be reliably assayed by the Folin-Ciocalteu procedure, this is quite unusable in an iced tea beverage containing ascorbic acid, since the ascorbate will also react to give a false positive in this assay. Here, only HPLC of individual catechins is of value.

Clients who request analysis, especially those lacking a technical background, frequently do not understand the limitations imposed on any given assay by differing analytical matrices. It is important for the analyst to stress that data can be guaranteed only if recoveries have been properly validated in the same or a similar matrix. Where fortification of complex foodstuffs is concerned this is rarely the case, and the analyst must be appropriately cautious when offering a service on an untried matrix.

10.1.3 Sources of methods
There are no one-stop ‘handbook’ sources of methods for polyphenol assays in foods and supplements, nor are there any generally agreed methods across the whole industry, and the analyst must be prepared to search various primary literature sources via online databases to find or to develop suitable methods. In recent years, however, there has been a certain amount of convergence, at least where HPLC methodology of raw materials is concerned. For instance, the American Herbal Pharmacopoeia monographs describe analytical methods for a number of botanicals in which phenolics are key components. A number of methods for dietary supplements (including phenolics) have been published by the US-based Institute for Nutraceutical Advancement (INA) and are now freely available on the internet (Institute for Nutraceutical Advancement, 2007). AOAC International launched a dietary supplement method validation programme in 2001, and the US National Institutes of Health in the following year (Saldanha et al., 2004). The United States Pharmacopoeia (USP) now describes analytical methods for a number of dietary supplements using HPLC techniques; amongst those included in the 2006 edition are the phenolic-containing Echinacea (various parts and species), Gingko and Milk Thistle. For the most part, all these methods are validated only for the dried plant material or simple compressed tablets, and not for complex multi-component formulations nor for fortified foods.

Foodstuffs that are naturally high in phenolics and antioxidant properties are well covered in the current food science literature. Amongst those we may count grapes, blueberries, raspberries, apples, pomegranate, cherries,
chocolate and many more. Unfortunately, even a cursory inspection of the literature will reveal many dozens of papers using such a wide range of methods that in most cases the data from one study cannot be compared with that from another, unless performed by the same workers in the same institute. There has justifiably been concern about the robustness and validity of these data from such diverse sources. In the USA, the United States Department of Agriculture (USDA) nutrient database (United States Department of Agriculture, 2007) has attempted to address this issue by setting, for example, a standard HPLC method (developed by Masterfoods from a French wine analysis method) for the analysis of catechins and procyanidins in foods. However, such methodologies are complex, purified standards are generally not available, and in the author’s opinion it will be many years, if ever, before consensus as to the most appropriate methodologies for specific phenolic components in foodstuffs is obtained. A similar debate is ongoing for the ‘total phenolic’ assays, e.g. Folin and ‘total antioxidant’ assays, e.g. ORAC. Here agreement may be easier to reach, in due course, if sufficient ring trials and cross-validation exercises are conducted across various laboratories (Prior et al., 2005).

10.2 Methods for total polyphenols

Methods for total polyphenols generally fall into two groups, those that rely on bulk properties of phenolic groupings and those that rely on other features of polyphenolic molecules. Probably the simplest ‘bulk property’ method is the use of direct UV spectroscopy at 280 nm, relying on the light absorption of phenolic groups. Although this method is sometimes specified, e.g. in the assay of milk thistle extracts, it is subject to considerable potential interference from non-phenolic components that are also UV-active (e.g. some alkaloids), and must be used with defined reference materials or pre-defined calibration curves to provide quantitative data. A drawback to any simple UV or colorimetric method is its potential for adulteration of the product by the addition of a UV-active filler, e.g. benzoic acid, which raises the apparent content of the product.

One of the oldest and once widespread methods for determining phenolic groups is the Lowenthal permanganate titration (Kirk and Sawyer, 1991), which dates from Victorian times and is essentially a redox titration using permanganate as an oxidant in the presence of indigo carmine as indicator. Due to the difficulty of determining the endpoint and its relative lack of sensitivity, this method has gradually fallen into disuse. Its replacement in many fields has undoubtedly been the Folin–Ciocalteu colorimetric method. Although dating from the early years of the twentieth century and introduced originally for the assay of proteins by the determination of their phenolic tyrosine groups, its application was fostered in the 1970s by the detailed work of Singleton et al. for the Californian wine industry (Singleton and
Rossi, 1965; Singleton et al., 1999). It is now pretty much standard in industries such as wine, tea and beer. It is a colorimetric method in which a phosphomolybdoo-tungstic acid complex reagent (now commercially available) is reduced by phenols to give a stable blue complex in moderately alkaline solution. Key features for robustness, as optimised by Singleton, are the correct alkalinity, the order of addition of reagents, and the timing of the colour development. A set of calibration standards is carried through the assay. Although any appropriate phenolic may be used as a standard, gallic acid is most generally used, since it has the advantage (as the monohydrate) of being available as a pure ACS reagent, readily soluble and not easily prone to autoxidation. Data is then expressed (after correction for typically 9% moisture) as GAE (gallic acid equivalents) even though gallic acid may not be a component of the mixture under investigation. Since the Folin and Lowenthal methods are essentially redox assays, other natural reducing agents that are present in plant extracts may also interfere at high levels. Amongst these the most commonly encountered are ascorbic acid and reducing sugars such as glucose and fructose, which may be present in relatively large amounts.

Methods discussed so far are, by and large, responsive to all phenolic groupings, but some assays are more specific to particular orientations on the molecules. For instance, the reaction of aluminium chloride with flavones or flavonols causes a bathochromic shift of typically 30 nanometres, shifting the visual colour from a barely perceptible yellow at the edge of human colour sensitivity to a defined canary yellow that may be measured on a simple colorimeter or spectrophotometer (Ribereau-Gayon, 1972). This method has been used for the assay of flavones in propolis but also allows for an easy adulteration with the cheap flavonol quercetin, which reacts in the same way and indeed is usually the external standard for the assay. Similarly, the reaction of flavanols such as the catechins with complex aldehydes such as vanillin or dimethylaminocinnammaldehyde in strong acid solution gives defined adducts (red or blue respectively) which are moderately specific and well suited to colorimetry. However, when applied to longer chain flavanols such as the procyanidins, the response is not linear and decreases with increasing molecular size, making a calibration with simple catechins inappropriate and a cause of underestimation for the procyanidins. When used with grape seed extracts, these methods are over-responsive to the monomeric catechins and less responsive to the procyanidins. This provides a fruitful area for the generation of dubious certificates of analysis claiming elevated levels of procyanidins when, in reality, it is catechins that are being reported.

‘Total polyphenol’ methods certainly have a place in the analytical armoury. They do not require expensive HPLC equipment nor exotic unobtainable standards, and they can also provide comparative data across a wide range of sample types. For samples of trusted provenance, where the costs of a more detailed analysis could not be justified, they may be invaluable. The Folin method, at least, estimates all forms of polyphenols even when polymeric and otherwise intractable. However, their strength is also their Achilles’ heel
since they are indiscriminate assays, prone to matrix interference, and can be manipulated by unscrupulous suppliers to give erroneously high values by comparison with more specific methods. The remainder of this section will look at specific assays for a number of individual phenolic-containing supplements.

10.3 Specific analysis of individual plant materials

10.3.1 Echinacea

Various parts of the plant genus *Echinacea*, a North American herb, are traded as immune system stimulants. They contain alkylamides, a range of caffeic acid derivatives and (in common with most of the *Asteraceae*) fructose in the oligomeric form as inulin. Although it is more likely that their therapeutic action is due to alkylamides than to polyphenols, many methods for *Echinacea* analysis rely on HPLC of their caffeic acid derivatives. The primary analytes are caftaric acid, echinacoside, chlorogenic acid, cynarin and chicoric acid. The parent caffeic acid is not present at significant levels. Purified standards of these may be purchased, although they are expensive, and are useful for primary confirmation of peak identity and for the setting of relative response factors and retention times with respect to a cheaper surrogate standard such as chlorogenic acid. This is the approach adopted in the INA and USP methodologies. The HPLC system is typically reverse phase C18 running in a 10–40% gradient of acidified water (e.g. 0.1% phosphoric acid) to acetonitrile. UV monitoring is carried out at 330 nm, the $\lambda_{\text{max}}$ of caffeic acid. Since all the components of interest bear this chromophore they cannot be distinguished individually by diode-array spectral monitoring, only by retention time. Dried plant material is typically dissolved in 70% alcohol prior to HPLC. As indicated previously, commercial extracts that have been dried onto maltodextrin may require a specific hydration step before the use of the alcoholic solvent.

*Echinacea* preparations are unusual in that any one of three species (*E. angustifolia*, *E. pallida* and *E. purpurea*) are used, mostly roots but sometimes the aerial parts. The three species differ in their phenolic patterns. For instance, *E. angustifolia* and *E. pallida* both display a large peak for echinacoside but much smaller peaks for caftaric and chicoric acids. *E. angustifolia* contains cynarin but *E. pallida* does not. *E. purpurea* contains very little echinacoside but significant amounts of chicoric acid and caftaric acids. The aerial parts of *E. purpurea* display a simpler HPLC pattern, more dominated by chicoric acid, than do the roots. Hence, HPLC analysis can be used as a tool to investigate the authenticity of the named material. Often, an *Echinacea* specification will be set in terms of total phenols after summing the individual identifiable peaks by HPLC (United States Pharmacopoeia, 2007; Perry *et al.*, 2001). There is some indication that the process of drying *Echinacea* can affect the proportion of the various caffeic acid derivatives, so the difference between fresh and dried material may be of significance.
Caffeic acid groupings are prone to oxidation and this can also lead to losses during preparation or in liquid form (Bergeron et al., 2002).

10.3.2 Milk thistle (Silymarin)
Milk thistle comprises the dried fruit (seeds) of the thistle *Silybum marianum*, another plant of the *Asteraceae* family, and is sold primarily for its action on the liver. The dried solvent extract is known as silymarin, and, until the advent of HPLC, was assayed in its entirety by UV spectroscopy directly or by colorimetric techniques after reaction with dinitrophenylhydrazine. In fact this extract contains a number of closely related flavanonols, principally silychristin, silydianin, silybin A and B, and isosilybin A and B, all of which are nowadays readily separated by HPLC and detected by UV at 288 nm (INA, USP). Reverse phase HPLC is usual, in a solvent gradient from about 10–40% acetonitrile in acidified water (0.5% phosphoric). Dissolution of the sample extract (and of the standards) requires methanol as solvent and a significant period of ultrasonic treatment to ensure complete solubility of the flavones. This is noted in the USP and has also been observed in our laboratory.

Standards of the individual components can be purchased but are expensive; hence, as with *Echinacea*, these can be used on a one-time basis as primary standards to establish relative retention times. Thereafter, a cheaper surrogate standard can be used. In our laboratory, and in the USP method, Silybin A+B is used for this purpose, and it is usual to report all the components as silybin equivalents using the same response factor throughout. The sum of the components may then be reported as ‘silymarin’. In practice, even the best commercial ‘silymarin’ standards contain only 60% of total flavanones, which gives significant discrepancies where suppliers are using older colorimetric methods; the summed HPLC data tends to give a much lower value. To get around this, we also analyse a commercial ‘silymarin’ mix (Aldrich) for total silymarins by HPLC, and then apply a correction factor to the client data to convert back to the apparent results which would be obtained using the older methods. Although this procedure is scientifically dubious, we have found it to be commercially expedient!

10.3.3 Gingko biloba
Extracts from the leaf of *Gingko biloba* are sold primarily to alleviate circulatory and cerebrovascular symptoms. The primary active constituents are believed to be terpene lactones (gingkolides) and a range of flavonol glycosides (gingkoflavones). The latter are based on the three common aglycones quercetin, kaempferol and iso-rhamnetin. The glycosidic pattern is enormously complex and direct HPLC of these components shows up to a couple of dozen individual components. These would be impossible to assay reliably in the free state given the absence of commercial standards, the complexity
of the chromatograms, and the virtual superimposibility of their UV spectra. Fortunately the flavonol aglycones are quite stable to acid hydrolysis even in the presence of air, and by taking advantage of this, the complex mixture may be reduced to just three components. The total flavonol glycoside content is then computed by applying nominal correction factors which give the mass of the putative glycosides in the mixture (Institute for Nutraceutical Advancement, 2007; United States Pharmacopoeia, 2007). The hydrolysis is carried out according to the USP procedure in ethanol:water:hydrochloric acid (50:20:8 v:v:v), refluxed for 135 minutes. In our laboratory we have preferred to replace the hydrochloric acid with its equivalent in perchloric acid, bearing in mind the corrosive effect of hydrochloric acid in HPLC injector and column systems constructed of stainless steel.

The HPLC of the liberated aglycones in the hydrolysate is straightforward and typically takes place on a C18 reverse-phase column in an acidified water–methanol gradient in the order quercetin, kaempferol and iso-rhamnetin (the USP procedure is isocratic in 50% water methanol containing 0.5% phosphoric acid). Methanol is a preferable solvent to acetonitrile for this application since the flavonol aglycones exhibit poor and tailing peak shapes otherwise (presumably due to the aprotic nature of acetonitrile). Detection is in the UV, preferably at 350 nm, which is selective for the flavonols amongst other potentially interfering phenolics (although the USP uses 270 nm). Standards of all three aglycones may be purchased, although quercetin is cheaper and probably available in greater purity. The USP procedure therefore uses quercetin as a surrogate for all the aglycones and a blanket mass conversion factor of 2.51 to express results as glycosides; the INA procedure uses individual standards and specific conversion factors for each component. Since many commercial gingko extracts are standardised to a known level of flavonol glycosides, this analysis can be used to verify the label claim on supplements where such an extract has been used.

Because of the hydrolytic procedure and the relative stability of the flavonol aglycones, it is possible to use this procedure to determine levels of gingko addition to foodstuffs if a reference extract or specification is also available: In our laboratories we have done this successfully on pet food for example. However, due to the ubiquitous nature of flavonol glycosides (especially quercetin derivatives) in plant material, the method would not be reliable if other fruits and vegetables were also present. In this case a careful note should be taken of the relative proportions of the three aglycones – in gingko, the quercetin and kaempferol are of roughly similar amounts while the iso-rhamnetin is about one half of either – and any distortion of this expected pattern should be treated with caution.

10.3.4 Tea catechins
The catechins are the characteristic polyphenols of green tea, forming up to 30% of the dry weight of the leaf, and in which epigallocatechin gallate
(EGCG) and epicatechin gallate (ECG) predominate. Lesser amounts of epigallocatechin (EGC), epicatechin and catechin are also found. During the oxidative processing of green to black tea, most of the catechins are lost to more complex forms such as the theaflavins and thearubigins. Analysis of the individual catechins is well suited to HPLC procedures and has, in recent years, been the subject of a rigorous worldwide collaborative trial which resulted in the publication of an International Standards Organisation (ISO) standard in 2004 (International Standards Organisation, 2004). The catechins are extracted from tea leaf with hot 70% methanol, or in the case of instant teas by dissolution in hot water and acetonitrile. Due to their propensity to oxidation, the diluted catechin solutions need stabilising with EDTA and ascorbic acid. The extract is chromatographed on a reverse-phase column in an aqueous acetonitrile/acetic acid gradient containing EDTA to minimise the effect of stray metal ions on catechin oxidation, and peaks are quantified at 278 nm. The column suggested in the ISO standard is an unusual phenyl-hexyl phase which gives greater selectivity for catechins, but if this is not available other reverse phase columns may be used and the solvent gradient adjusted to suit. The use of diode array detection is valuable to ascertain if any co-elution is taking place with other tea components such as cinnamic acids or flavonol glycosides: although not described in the ISO standard, this is a known issue to analysts who use the method regularly.

Since it is virtually impossible to obtain standard tea catechins of adequate purity and known moisture content at reasonable cost, and because they are inherently unstable, the ISO method offers a set of response factors for catechins relative to caffeine as a surrogate standard (these were determined empirically during the collaborative trials, using high purity catechins isolated by one of the partners, Unilever Tea Research Laboratories). Caffeine, although not structurally related to the catechins, is stable and obtainable in high purity and has similar chromatographic properties to the catechins but does not co-elute with them. This method, although designed for tea leaf and instant teas, can equally well be applied to tea beverages or those that incorporate green tea extracts. As remarked earlier, it is less well suited to solid foods incorporating green tea extracts (especially those that are cooked) since the catechins are prone to loss by oxidation and also by binding to protein. However, it is probably a fair assumption that if the catechins cannot be extracted from the matrix by hot 70% methanol, then they will not be bioavailable either and so their loss from the system is a genuine one.

10.3.5 Isoflavones

The isoflavones of soya are known to act as phytoestrogens in mammals and are claimed to be beneficial for menopausal symptoms and for cardiovascular protection. They are unusual amongst the compounds considered in this chapter since they are marketed in extracted form as dietary supplements and food fortificants, as well as occurring naturally in mainstream soya
products which are widely consumed in the normal diet over large parts of Asia and increasingly in the Western world. The main isoflavone aglycones of soya are daidzein, genistein and glycitein. In the native state, as found in soya, these aglycones are glucosylated at the 7-position, to give the compounds daidzin, genistin and glycitin respectively. The glucose moiety may then be further acylated with malonic or acetic acids, thus making for a very complex analytical pattern. Partial de-acylation can occur during normal soya bean processing, and some soya extracts also undergo further deliberate degradation to the parent aglycones.

It is possible to analyse the full range of twelve soya isoflavones in soya extracts by reverse-phase HPLC in acidified water–acetonitrile gradients, using UV detection typically at 260 nm (Wang and Murphy, 1994). However, most of the standards required are not commercially available and have to be specially prepared, so this technique is more applicable to research laboratories than to routine analytical use. The pattern can be simplified by hydrolysis of the acyl groups using dilute alkali (saponification), which collapses the complex and labile pattern to essentially the three glucosides. Aglycones (if present) may also be separated in the same analytical run, giving no more than six individual peaks to be quantified. This is the basis of the INA method. Isoflavones are extracted into 80% methanol at 65 °C for 2 hours (the amount of sample taken depends upon the likely concentration of isoflavones present), followed by a 10 minute room temperature hydrolysis in 2M sodium hydroxide, which is then quenched with acid prior to HPLC analysis. Individual isoflavones are quantified and then expressed as aglucone equivalents using calculated conversion factors. The author has found the hydrolysis to be a somewhat erratic step, and that some loss of glucosides can occur; it has therefore become our customary practice to measure (in duplicate) the isoflavones both before and after hydrolysis, to provide some indication of possible losses during this step and to repeat the procedure if necessary.

Although the required standards are commercially available, they are expensive and the glucosides at least are not stable when stored in solution, even deep frozen. However, the aglycones are more stable and so they can be used as surrogate standards once the appropriate conversion factors have been calculated.

10.3.6 Anthocyanins
The anthocyanins are a group of flavonoids, widespread in flower petals and fruits such as grapes, blackcurrants, blueberries and cranberries. Although long believed to have specific beneficial effects on functions in the retina, they have more recently become of interest because of their high antioxidant values and their wider potential health benefits (Wu et al., 2006). Their core structures are unusual, being based (at low pH) on a charged flavylum salt aglycone which loses its colour as the pH rises, and reversible hydration takes place to the carbinol base and chalcone forms. There are six common
aglycones (cyanidin, delphinidin, petunidin, peonidin, pelargonidin and malvidin) which differ in their hydroxylation/methoxylation patterns. The aglycones are not stable in the free state and so the variety of anthocyanins is hugely extended by a range of glycosylation patterns – hence hundreds of anthocyanins are, in practice, known. In real-life extracts, degradation and polymerisation of monomeric anthocyanins also takes place to a considerable degree, making their analysis a potentially complex task (Henry, 1996).

In cases where only the monomeric forms are of interest, gradient Reverse Phase HPLC (RP-HPLC) in water–acetonitrile and visible detection at 520 nm has been the technique of choice for more than 20 years (Lea, 1988). Diode-array detection is helpful to distinguish between some forms of aglycone and glycosylation pattern. To maintain the anthocyanins in their coloured (flavylium salt) form, it is necessary to work at low pH (<2) by addition of acid, e.g. 1% perchloric or phosphoric to the mobile phase. Although the effective extraction of anthocyanins from supplements and foodstuffs does not, strictly speaking, require the addition of acid to the water–alcohol mixtures which are typically used, it is helpful to do so because the anthocyanins then remain coloured and the extraction efficiency may be estimated by eye.

For extracts of defined provenance, distinctive and recognisable patterns are obtained such as the ‘double doublet’ 3-glucoside and 3-rutinoside of cyanidin and delphinidin in blackcurrant or the ‘double triplet’ 3-galactoside, 3-glucoside and 3-arabinoside of cyanidin and peonidin in cranberries. In grape extracts, the pattern depends on species and fruit cultivar. European grapes Vitis vinifera show primarily the 3-glucosides of 5 common aglycones (lacking pelargonidin and dominated by malvidin) whereas American grapes, e.g. Concord Vitis labruscana show a much more complex pattern, including 3,5 diglucosides, and in which no single anthocyanin is dominant. Both species also show varying degrees of acylation with e.g. p-coumaric acid on the glucose moiety depending on cultivar. Since all these patterns are unique to the plant material in question, they have a powerful value in checking the authenticity of anthocyanins which claim to come from a specific botanical source.

Quantification of anthocyanins by HPLC is vexing since so few standards are available, and those that are may be of doubtful purity and considerable cost. One solution to this problem is to use a surrogate standard such as cyanidin 3-glucoside (a widespread anthocyanin which is also useful as a relative retention time marker) and to express all values as Cy-3-G equivalents. If there is any doubt about the purity of the standard itself, it may be checked by UV/VIS spectrophotometry at pH 1 and compared to the best published extinction coefficients – the purity value may then be corrected as necessary (Francis, 1982; Lee et al., 2005).

In cases where polymeric forms of anthocyanins are also to be accounted for by HPLC it is possible to achieve this to some degree by estimating the area of the hump which lies under the monomeric peaks, and quantifying this as if it were monomeric. Such a procedure is often useful in degradation
time-course studies, e.g. of blackcurrant fruit drinks on shelf-life testing, since both the loss of monomers and the appearance of polymers may be quantified (although the mass-balance by this technique may not add up to 100%!).

An alternative procedure which avoids the use of HPLC is the use of direct spectrophotometry in diluted solution at pH 1 and pH 4.5, and conversion to total anthocyanin content by a set of agreed extinction coefficients. This technique can also provide an estimate of polymeric forms (the so-called degradation index). It relies on the fact that the monomeric forms are essentially uncoloured at pH 4.5 whereas the polymeric forms retain significant colour at higher pH (Francis, 1982). Although the technique is of many years standing and has been in use since the 1960s, it still has value and a newly peer-validated trial has recently been published (Lee et al., 2005). Obviously this technique cannot verify the presence of individual anthocyanins and is therefore of no use in authentication studies, for example, but can still be appropriate in cases where this is not an issue and where no other coloured components such as artificial food dyes are present.

10.3.7 Procyanidins

The oligomeric procyanidins are related to the catechins (see Section 10.3.4), being direct C–C linked combinations of catechin building blocks. In some cases the degree of polymerisation may exceed 30 but generally it is oligomers of \( n = 2–10 \) which are of most interest. Although they are formed by defined biosynthetic means in the plant, degradation during processing also yields ill-defined oxidised and polymeric materials which may quantitatively exceed the native forms. The primary extracted sources of procyanidins for dietary supplements are grape seeds and pine bark (both the waste products of other industrial processes) although many foodstuffs such as red wines, apples, cocoa and blueberries also contain them. (It should be noted that blueberries and cranberries contain A-type double-linked oligomers which are markedly different in structure and properties from the more common single-linked B-type).

Although there are some HPLC methods for estimating individual oligomeric procyanidins \( (n = 2–10) \) using normal phase HPLC with fluorescence or mass-spectroscopic detection, most of them require an intimate knowledge of polyphenolics chemistry to make them effective and are limited by a complete lack of commercial standards. Surrogate standards are not appropriate since the fluorescence response factors are not linear with respect to molecular size. In our experience, the use of the originally-proposed silica columns is not robust, and variable and unpredictable analyte losses can occur on-column. A recent variant method using a diol-bonded stationary phase seems to offer much greater robustness and may be the path for the future if a commercial supply of standards can be overcome (Kelm et al., 2006). For the moment, such methods are limited to the research laboratory and at the time of writing,
only one commercial laboratory, with private access to a source of standards, appears to offer a service using this method. Nor do these methods estimate the large amount of polymeric procyanidins (n>10) and part-degraded forms which may, in practice, outweigh the oligomeric forms several-fold.

For the estimation of total procyanidins, the original hydrolysis method of Bate-Smith as refined by Porter may be considered (Porter et al., 1986). This method (which gave rise to the term ‘procyanidin’ itself) relies on the oxidative cleavage in strong acid of the C–C bond between the flavonoid units to form the red cyanidin cation which is estimated spectrophotometrically at 520 nm. The method is made more robust and reliable by the addition of an iron redox catalyst in the form of ferric ammonium sulphate. As it stands, the Porter method gives only an arbitrary measurement of cyanidin intensity for a nominal 1% procyanidin solution. However, this provides usable relative values and can easily be calibrated against a ‘standard’ procyanidin freed of all low molecular weight material. Typically, Porter values of 25–30 are the maximum obtained for grape seed extracts and could be regarded as close to 100% pure procyanidin.

The great advantage of the Porter method is that it is selective for true procyanidins and is not influenced by interfering non-procyanidin phenolics of low molecular weight. Unfortunately some unscrupulous suppliers of so-called procyanidins, especially from winery waste, assay their products simply as total polyphenols by the Folin–Ciocalteu method (see Section 10.2). This figure includes low molecular weight catechins and gallic acid as well as the true procyanidins, and may serve to grossly inflate the true values, especially in samples which are prepared from undifferentiated grape pomace and not from selectively extracted seeds. To obtain a truer value for procyanidins, it is necessary to carry out RP-HPLC analysis on the same samples to obtain an estimate of the monomeric catechins and their gallate esters and free gallic acid. These figures are then subtracted from the total polyphenols as estimated by Folin–Ciocalteu, to provide a realistic measurement of true procyanidins by difference (Grape Seed Method Evaluation Committee, 2007).

The methods given apply to the estimation of procyanidins in extracts or dietary supplements, not in actual foodstuffs or fortified foods where too many interferences are likely. In those cases, some sort of selective extraction would be required. For instance, the use of the Sephadex gel LH-20 has been widespread amongst polyphenol chemists for this purpose for many years (Lea et al., 1979) and is often used as a pre-treatment/concentration step for procyanidins prior to HPLC. Extraction of the foodstuff by 70% methanol is followed by removal of the alcohol by rotary evaporation. The aqueous extract is then applied to a small column of the gel swollen in 20% methanol. The gel is then washed with 20% methanol until the UV absorbance falls to zero, and the adsorbed procyanidins are then desorbed from the gel with 100% methanol, 70% acetone or similar solvent. This extract, after taking to dryness, may then be assayed by any of the methods given.
10.3.8 Propolis

Propolis is a waxy plant secretion collected by honey bees for various housekeeping purposes around the hive. It has anti-microbial and antiseptic properties and is widely sold as a dietary supplement. In temperate zones of the world, notably Europe, Asia and North America, bees collect propolis almost exclusively from the buds of poplar trees. This is true even in the southern hemisphere, e.g. New Zealand, where both the bees and the poplars have been introduced. In tropical zones such as Brazil and Zambia, bees collect propolis from a wide variety of trees but not from poplars, which are not found in those areas (Tomás-Barberán et al., 1993).

In addition to the wax and benzoic acid which it contains, temperate-zone propolis contains a range of characteristic poplar-derived flavonoids which can be used for characterisation and as a specification against adulteration. Earlier methods for this involved non-specific assays such as colorimetric reactions with aluminium chloride using, e.g. quercetin as a standard. This was unsatisfactory since quercetin scarcely occurs in propolis, but did not guard against its incorporation as an adulterant. Based on work by Markham et al. (1996), the UK Health Foods Manufacturers Association (HFMA) in conjunction with Reading Scientific Services Ltd (RSSL) developed, during the 1990s, a propolis analysis method using RP-HPLC to quantify three major propolis flavanones – chrysin, galangin and pinocembrin. In raw propolis the minimum criteria were set as 1% of each flavanone and in purified propolis after alcohol extraction as 2%. The virtual absence of quercetin was also a criterion for acceptance. This method is now accepted by the UK Food Standards Agency (FSA) and Local Authorities Coordinators of Regulatory Services (LACORS). However, it does not apply to propolis of tropical origin.

The analytical method was validated and is published on the HFMA website (UK Health Foods Manufacturers Association, 2007). In outline, propolis resin is dissolved in an excess of ethanol overnight after ultrasonic treatment and the extract is then analysed by reverse-phase HPLC in a water–methanol gradient (35–80%) acidified with formic acid (acetonitrile gives poor peak shape for this application). UV diode-array detection is used at 268 nm (chrysin and galangin), 280 nm (pinocembrin) and 350 nm (quercetin). Purchased standards are used and, although expensive, have been shown to be stable indefinitely in ethanol solution when stored dark and cold and tightly closed.

10.3.9 Oleuropein

There are a host of relatively minor phenolics, including those specific to one particular plant species or genus, of which oleuropein is a typical example. This, and similar related components, have a complex coumarin-like ‘secoiridoid’ structure which is glycosidically linked (Soler-Rivas et al., 2000). Oleuropein occurs in the fruits, but in much larger amounts in the
leaves, of the olive tree *Olea europea*, and has recently found a niche market as a dietary antioxidant and possible anti-hypertensive. For analysis, dried leaves are extracted by ultrasonication in aqueous methanol and then analysed by RP-HPLC in acidified acetonitrile/water at 233 nm. In a study by Savournin *et al.* (2001), levels of oleuropein in dried powdered leaf of named olive cultivars were found to range from 9–14%. In unpublished work from the author’s laboratory, oleuropein was found to be present in commercial olive leaf extracts from Australia and also in air-dried leaves from a roadside Corsican olive tree, but none in freeze-dried leaves where it was apparently replaced by the isomeric oleuroside which differs in the position of a terminal double-bond. It appears possible that oleuroside is the form biosynthesised in the plant (and conserved by freeze-drying), but that oleuropein is the more thermodynamically stable form formed after processing. This example further demonstrates (as in the case of *Echinacea* described previously) that the complexity of natural product composition may be dependent upon the source and its processing history, and cannot be taken for granted.

10.4 Antioxidant capacity measurements

The free radical concept of degenerative disease implies that cellular components are constantly exposed to damaging levels of oxidatively generated free radicals by normal metabolic processes such as respiration. An example would be the oxidation of low density lipoprotein (LDL) as a precursor to cardiovascular disease. By this hypothesis, the scavenging of free radicals in plasma by plant-derived antioxidants such as Vitamin C and Vitamin E acts to mitigate such effects (Serafini, 2006). The hypothesis has been extended to embrace the action of plant polyphenols, despite the fact that the circulating plasma levels of such materials after dietary administration (even in conjugated form such as glucuronides) are very low. It is also very clear that the relationship between the ingestion of ‘antioxidant-rich’ foods and any subsequent changes in plasma antioxidant status is far from being straightforward (Lotito and Frei, 2006; Prior *et al.*, 2007). Although many workers now believe that the polyphenols more probably act as cell signalling gene regulators rather than as genuine antioxidants (Halliwell, 2006), the measurement of free radical scavenging activity of plant extracts has become widespread and is now commonly used as a marketing tool. These methods are loosely termed ‘antioxidant activity’ or ‘antioxidant capacity’ measurements.

Many such assay systems exist and their chemistries are complex; a detailed discussion is beyond the scope of this chapter but the subject has been well reviewed (Huang *et al.*, 2005; Prior *et al.*, 2005; MacDonald Wicks *et al.*, 2006). Some assays were originally developed in clinical laboratories to measure antioxidant capacity in plasma samples but have been retrospectively applied to foods, and especially to those high in polyphenols. Nearly all of them generate coloured or fluorescent reaction endpoints so they can be
quantified spectrophotometrically. They can be roughly divided into hydrogen atom transfer reactions (HAT) and electron transfer reactions (ET). The former is most related to free-radical chain-breaking antiproliferation ability and the latter to the reducing ability of the substrate. In the first group fall assays such as ORAC (oxygen radical absorbance capacity) and TRAP (total radical trapping antioxidant parameter). In the second group fall assays such as the Folin–Ciocalteu (see Section 10.2), the TEAC assay (Trolox equivalent antioxidant capacity) and the FRAP assay (ferric ion reducing antioxidant power).

Many assays require the generation and quenching of a specific radical and there is much debate on the biological significance of those that are chosen. For instance, the ORAC assay uses a peroxyl radical which is claimed to be more biologically relevant than the synthetic ABTS radical used in the TEAC assay; on the other hand, the reproducible generation of the peroxyl radical itself may not be a robust procedure in practice. Most assays express the results compared to the behaviour of a standard antioxidant, e.g. Trolox, a synthetic water-soluble tocopherol analogue. Some methods (e.g. ORAC) rely on measuring the fluorescence area under the curve to an end point for radical quenching (which may itself introduce some measurement uncertainties), whereas in others (e.g. TEAC), visible absorbance measurements are made at specific time points. Hence the ORAC method requires relatively sophisticated analytical equipment, while the TEAC does not. Most antioxidant measurements are made in aqueous solution, which is appropriate for many foods but not all. The ORAC method has been modified using cyclodextrins to increase the solubility of lipophilic substrates while the TEAC assay can be used directly in lipid solvents (Wu et al., 2004).

It is evident that there is no consensus methodology in this field, which makes comparison between results obtained in different laboratories almost impossible. It is also fair to point out that each assay has been developed largely by individual groups of workers who tend to be rather partisan in promoting their own methods. Thus, the high profile of the ORAC technique is in some part due to its development in the USDA laboratories and its adoption as a quasi-official method in the USA. At the time of writing, though, ORAC has not been subject to critical ring-triailed validation across different laboratories, and there is only one laboratory offering it on a commercial basis. Arguably ORAC and TEAC are the ‘front runners’ although both work using different chemistries. Prior et al. (2005), reporting from a specific meeting on antioxidant measurements, have suggested that these two, together with the Folin–Ciocalteu method, are urgently in need of standardisation and validating. At the end of the day, in any case, the relationship between any antioxidant measurement in food and its capacity for disease prevention or mitigation is a long way from being proven.
10.5 Acknowledgement

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11

Assessing the bioavailability of nutraceuticals

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11.1 Introduction

A targeted delivery system, natural or engineered, for the efficient release of an active compound from a food, nutraceutical or pharmaceutical matrix, can provide a means of optimising both extent and rate of absorption of the compound into the body. Delivery systems may also have considerable benefit by protecting essential nutrients and other ‘bioactive’ food components during food processing and digestion. For example, they might prevent the loss of the component, or adverse interactions with inhibitors of absorption. However, optimum absorption should not be confused with maximum absorption, as so often happens in the bioavailability field. The concept of ‘if a little does you good, more will be better’ has pervaded the nutraceutical market for many years. Even with the essential nutrients, getting ‘more’ into the body at a ‘faster’ rate does not necessarily equate to ‘improved’ function and ‘better’ health; in some instances quite the reverse is the case. Different rates of delivery of the same load may have a profound effect on subsequent health outcome. For example, there are proven health benefits from ‘slow release’ carbohydrate foods; they do not over stimulate the secretion of insulin, cause unnecessary large excursions in blood glucose, or undesirable glycosylation of proteins. By analogy, the slower delivery of other food components may maximise health benefits by not over-loading transport systems, or causing undesirable excursions in plasma and tissue concentration. Furthermore, the fact that some portion of nutrients escape absorption in the small intestine and are ‘lost’ to the colon should not automatically be interpreted negatively, since they may contribute positively to colon health through the maintenance of an appropriate microflora and the production of beneficial products of large bowel fermentation.
Achieving optimum (amount, rate and site) rather than maximum absorption via controlled or targeted delivery is particularly pertinent to the focus of this book and highlights the importance of understanding the bioavailability concept and measuring its components intelligently. The following sections examine the bioavailability concept and approaches to measuring its component parts.

11.1.1 The bioavailability concept

Each year there are approximately 200+ research publications with ‘bioavailability’ in their titles. It is obvious from these publications that ‘bioavailability’ means different things to different people. Its most common usage by far is to describe absorption, or ‘being in a form’ that can be absorbed. For drugs and the correction of nutritional deficiencies in plants and animals, the endpoints are closely monitored and evaluated. Where there is a putative benefit to human health, the endpoints of greatest interest and utility, namely the metabolic consequences and impact on wellbeing, are unfortunately seldom monitored and evaluated adequately. Although changes in plasma concentration, nutrient retention/excretion etc., induced by interventions, may be simply measured, the failure to evaluate holistic health benefit in ‘healthy volunteers’ seriously undermines any subsequent dietary recommendations.

The term ‘bioavailability’ has arisen (i) from the nutritional concept that some nutrients in food are only partially ‘available’, e.g. lysine, niacin, iron, and (ii) in the field of pharmacology, where it is a useful working concept of the ‘rate and extent to which a drug reaches its site of action’.

Clearly, bioavailability has a number of components, embracing absorption, distribution, metabolism, excretion (ADME), with subsequent biochemical and physiological effects. It is therefore a concept and as such has no numerical value or units; it cannot be increased or diminished, only changed. Nevertheless, as a concept, it has value in providing a framework for studying links between the administration of a compound, the metabolic and physiological consequences, and impact on health.

The pharmacological concept is concerned with site-specific drug delivery in order to treat a disorder, where effective and timely treatment requires the drug to reach a site at a concentration for sufficient duration to elicit the desired effect. Within this concept consideration is given not only to absorption but to concentration and persistence in the body, metabolism, and whether the compound (or its effective metabolites) can actually reach the target organ or site and elicit a clearly defined and characterised response. This involves considerations of, for example, toxicity, hydrophobicity, solubility, molecular size and shape, affinity for target, membrane permeability, carriers and transport mechanisms. Some of these properties can be manipulated by the construction of analogues, or derivatives, to achieve the desired outcome while minimising toxicity and loss of active component. Thus it is possible
to make an injected drug more ‘bioavailable’ by improving the affinity of the
target, or constructing a derivative drug with greater affinity for the target.
This amounts to manipulating the way the drug is partitioned into the various
body pools. The selection of drug candidates and their modification for
delivery and efficacy is a very sophisticated process; in contrast, the foods
we eat contain many components about which we know almost nothing and
which do not lend themselves to modification as a way of manipulating
‘bioavailability’.

As with drugs, the concept of nutrient bioavailability should embrace all
these key issues. Bioavailability should not be limited (as so often happens)
to measurement of nutrient release from the food (or ingredient) matrix and
subsequent absorption. However, a drug is generally a known synthetic
construct for a specific purpose. Prior to bioavailability trials, the drug will
not be present (or be present at a very low concentration) in the tissues of
human volunteers, giving a clear baseline. When the drug is administered, it
and its metabolites can be seen clearly to appear and disappear from the
plasma pool, or other assayed tissue. This makes absorption and metabolic
studies much simpler to perform and interpret. Additionally, a drug is normally
consumed in tiny quantities and is less likely to affect satiety, consumption
patterns and lifestyle, whereas foods need to be supplementary to (or replace
items of) habitual diet. A drug is usually incorporated into a simple matrix
with predictable ‘release’ characteristics. Unlike food components, a drug is
not a compound to which the body is normally exposed from the cradle to
the grave. It is, however, possible to treat food components like drugs and to
feed the extracted component, and/or package it in simple or complex
characterised delivery systems. This does not really simplify measurement
in a tissue already containing the same component and its metabolites that
have been derived from the diet. Furthermore, the extrapolation from
populations who derive health benefits from eating a particular diet or food
to inferring those benefits derive from a single component should be critically
examined. Studying and predicting the absorption, metabolic fate and functional
effects of food components is hugely more challenging.

In summary, understanding the concept of bioavailability, how its component
parts are best measured, limitations to interpreting the results of
experimentation, the pros and cons of modifying the extent and rate of
compound release at specific sites in the intestine, and definition of ‘optimum’
is crucial to ethical product development and good nutrition.

11.2 Measuring absorption, metabolism and tissue
targeting

Measuring the quantity of nutrients, or non-nutrient components, consumed
in foods and nutraceutical products may have little predictive value with
respect to their interaction with the human organism, because:
the ‘native’ compound present in the consumed product may not be the chemical form to which human tissues are exposed after digestion and post-absorptive metabolism;

- diet matrix may significantly affect the extent and rate at which compounds are liberated and become available for absorption;

- a proportion of some compounds released into the lumen of the intestine may need a carrier to be potentially absorbable;

- only a proportion of a released compound may be absorbed;

- the absorbed compound may not appear systemically because it is metabolised by the enterocyte or re-excreted back into the gut lumen;

- non-absorbed compounds may still exert physical, physiological and/or biochemical effects on the intestine itself, with subsequent consequences for whole body metabolism;

- the genetic heterogeneity in human populations may result in individual differences in the site, extent and/or rate of absorption, metabolism and tissue targeting, which in turn will influence functional response.

Exploration of the mechanisms of action of food (and nutraceutical) components on human health thus requires an understanding of the factors that constrain their release from the matrix in which they are contained, the rate and extent of conversion or transfer to absorbable species, the extent, rate and site of absorption, and their health impact in genetically diverse individuals.

Measuring how much of a compound (drug, nutrient, toxin or carcinogen) reaches its site of action, irrespective of its route of administration, requires sampling the tissue/cell site and making the measurements. A more pragmatic and less interventionist approach is to administer a range of doses to groups of similar cases, or volunteers, to see which dose is ‘effective’ and to relate this to some more easily performed measurement, for example, concentration over time in whole blood, or a blood fraction such as plasma, erythrocytes or a specified population of white blood cells. This surrogate approach is defined by Schumann et al. (1997) as ‘that fraction of an oral dose (parent compound or active metabolite) that reaches the systemic circulation’. From the drug or nutraceutical perspective the assessment of ‘effective’ is based on some clearly defined and usually short-term objective, for example, suppressing an infection, relieving pain or altering blood chemistry. In the case of foods the time-scale may be short, for example, correcting a vitamin deficiency, but is more often than not long-term. Will the intervention reduce the risk of cancer or cardiovascular disease in 10 or 50 years time, and does the intervention have to be continued over this period? Clearly there is a hierarchy of effectiveness from a 100% short-term ‘cure’ to a lifelong reduction of risk, and this needs to be considered during the design of any study.

Schumann’s definition (1997), however, precludes injection as a route of administration. The injection route is precluded because it bypasses complications of intestinal metabolism and absorption and any effects of time. An injected compound is considered to be 100% ‘bioavailable’, despite the fact that this is erroneous in terms of the drug definition because it does
not take into consideration how much gets to the target. Injections or infusions are therefore more correctly 100% absorbed, although these routes exclude orally administered compounds that are absorbed into, and are metabolised by, the enterocyte, or which enter the enterocyte and are subsequently re-excreted into the gut lumen. In both cases, the authors consider that such compounds are ‘bioavailable’.

This distinction between routes is crucial because many ‘bioavailability’ studies use tracer:tracee methods, where the absorption kinetics of an oral dose are quantified in the plasma pool by using a simultaneous injected (real or virtual) labelled tracer, i.e. absorption is quantified by reference to the 100% ‘absorbed’ injected dose. This approach, however, must be used only for compounds that are not affected by the delivery route; for example, the approach would not be suitable for hydrophobic compounds packaged in chylomicrons or for folates. The plasma Area Under the Curve (AUC) of an injected hydrophilic compound is likely to be much larger than an equivalent oral dose, since the oral dose is absorbed via the portal vein and may be more or less metabolised (cleared) on first pass of the liver. However, clearance kinetics of the compound of interest, once equilibrated throughout the body will be the same in both cases provided metabolism, or clearance mechanisms, are not overloaded.

Where the orally administered compound is strongly hydrophobic, and follows lipid absorption, it will be packaged into lipoproteins and enter via the thoracic duct. This is very different from an injected or infused dose because the injected compound may not be packaged in the same way as that which has passed through the enterocyte and will therefore not be picked up by the lipoprotein receptors. The injected material may therefore have different kinetics in the plasma pool, in which case the percentage ‘comparative’ absorption measurement for the oral dose is of unknown value.

Measuring the absorption and metabolism of isolated food components \textit{in vivo} may be valid if used to address mechanisms and for the construction of models but the results may bear little resemblance to the case of foods where:

(i) most of the compound in the food is not the same as that absorbed and/or appearing in the plasma;
(ii) an absorbed compound may be metabolised by the enterocyte, or re-excreted back into the gut lumen and never seen in the plasma pool;
(iii) rate and limit of production of bioaccessible forms of substances (a term used by the authors to define the amount of a compound released from the matrix during digestion and/or fermentation and thus available for absorption) will be constrained by buccal, gastric and small intestinal processes, diet composition and food matrix interactions;
(iv) non-bioaccessible components may become bioaccessible (in one form or another) through the action of the large bowel biota.

Any attempt to take into account or model the effects of all the variables that
may affect the absorption of a potential product of digestion are bound to fail because there are too many unknown factors. As an example, West and Castenmiller’s (1998) ‘SLAMENGHI’ mnemonic for carotenoids (S = species, L = molecular linkage, A = amount consumed in a meal, M = matrix containing the carotenoid, E = effectors of absorption and bioconversion, N = nutrient status of the host, G = genetic factors, H = host related factors and I = the mathematical interactions) may be a useful way of remembering some of the variables (as applied in this case to carotenoids) but affords no insight into how they can be effectively combined to construct a universal predictive model of ‘bioavailability’ and ‘bioefficacy’ of β-carotene.

Where absorption of nutrients, or other compounds, from foods is concerned, the pragmatic approach is to treat the gastro-intestinal tract as a ‘black box’ in the sense that it is not necessary to know how systems work, only their effect. With input measures (food, food form, composition, etc.) and output measures (absorbed components or surrogates, health measurements) it is possible to train neural networks to accurately predict outcomes (a model of reality) from a selected range of inputs, without knowledge of the algorithms by which the prediction is made. If, however, informed rather than intuitive manipulation is required, for example, ‘What should I do to this food to increase the health benefit?’, Bayesian statistical analytical methods, using our knowledge and beliefs, can be applied to identify the main effectors. The main effectors can then become new, or revised, inputs for the neural network to refine the predictive power of the model (model closer to reality). However, once the nutrient has become systemic, understanding its distribution, metabolism and excretion requires compartmental modelling (Jacquez, 1999) to understand how the component affects, for example, gene expression, mRNA, protein synthesis; and ultimately, some integral of functionality that describes the health outcome.

Compartmental models are used to describe the flux of nutrients and other compounds in vivo into and out of body compartments (Fig. 11.1). The use of compartmental models requires either:

- perturbation of the steady state within physiological limits
- or use of tracers (stable/radio isotope)
- and serial sampling and analysis of as many accessible body pools as possible.

Compartmental models can be constructed from a linguistic description of the process. For example, the following statement can be easily converted to a linear compartmental mathematical model: ‘Most of the nutrient is absorbed from the gut into the blood. It exchanges with liver stores and is eventually cleared into the urine’.

The above description indicates ‘pools’, process, sequence and time, and the equations are derived from a consideration of mass balance and assumption that the nutrient in each pool is well mixed. In this case, that fraction of the component that is excreted in the faeces is unlikely to be ‘bioavailable’, but
that which is excreted in the urine must have been absorbed and passed through the body and hence would be considered ‘bioavailable’. The simplest ‘compartment’ is the whole body, but any number of compartments (at any scale) can be used: for example, plasma, muscle, adipose tissue, bone, liver, faeces, urine. As a general rule, the number of compartments should be kept to the minimum that can be biologically justified. Data can easily be over-interpreted by including more compartments (variables) and although this improves the data fit, it also increases the error in the parameters of interest, i.e. the rate constants. A good data fit obtained by adding additional compartments must be justified by sound physiological or biochemical argument (Jacquez, 1999).

Here, the substance enters into a dynamic pool, or pools, the concentration of which varies as fluxes (mass per unit time, indicated by arrows) in and out change with metabolic demand. The size of a ‘pool’ in such models is the distribution volume or mass. Pool volume or mass can be measured, found in tables of human body data, or predicted from anthropometric data.

If steady state is assumed (the volunteer is not growing, sick or perturbed by intervention), any compound should not be described as ‘used’, ‘utilised’ ‘stored’ or ‘accumulated’ but simply moving into the body from the diet, distributed dynamically between various compartments and excreted unchanged, or as downstream metabolites. After dosing, the ratios of the amount of material in pools does not remain constant because there are always delays in pool mixing during this dynamic period: they should, however, return to normal after an acute dose. If pool ratios are found to have changed after a chronic regimen, then bioavailability has changed. However, there are cases where the rate controlling mechanism is not the creation of absorbable

![Fig. 11.1 Arrows represent the transfer of nutrient from one compartment (i) to another (j) and are associated with the rate constant Kji. Circles represent well mixed pools.](WPNL1103)
species or absorption but is an inherent feature of post-absorptive metabolism. In this case the body will freely and rapidly absorb as much as it is given, pools will become saturated, and the body will ‘dump’ ‘unused’ material into excretory pathways. This can be considered in the same way as exceeding the physiological dose leading to non-linearity. In both these cases excess dose ‘spills over’ into the disposal system (provided it too is not overloaded). In the view of the authors, overloads and non-metabolisable organic compounds should still be considered bioavailable if they require the expenditure of energy for their excretion.

For organic compounds that can be metabolised, the amount metabolised in a pool is simply the difference between influx and efflux over time; hence the importance of accurately establishing the rate constants. It is not normally possible to identify a downstream metabolite as being specific to a particular pool other than perhaps the liver, which has the specific capacity to detoxify otherwise intractable compounds. Thus it will not be possible to measure the carbon dioxide from a particular pool (other than whole body) but it is possible to identify specific downstream metabolites produced by the liver and excreted in the urine.

Where the food or nutraceutical contains isomers of the same compound, the isomer profile should be checked at all points to ensure that statements

**Fig. 11.2** A more generalised compartmental model.
like ‘isomer [A] is selectively absorbed’ are in fact true. Isomer [A] for example, may persist in the plasma longest at a measurable concentration, while the other isomers disappear quickly. In addition, isomer [A] may be formed in the lumen or enterocyte and therefore is the predominant form transferred to the serosal side. Finally, compounds do not ‘accumulate’ in any particular pool, even if there are receptors. The pool is simply a thermodynamically favourable environment – the system is dynamic.

Unfortunately, compartmental modelling is often misunderstood or abused. The most common errors are the use of incomplete area under the curve (AUC), the use of injectable standards or calibrants where this route is inappropriate, and the assumption that changes in plasma concentration are due only to the compound being absorbed from the test food/diet and not a normal physiological response to the meal.

From Fig. 11.3 it can be seen that the IV dose starts to clear from the time of injection, but the oral dose is delayed by the stomach and constrained by the rate of gastric emptying and absorption. The two curves are therefore temporarily displaced and analysis of AUC before return to baseline (for example, point A on the curve) will remove different proportions of the curves. The area under the incomplete curves cannot therefore be compared. Even when two different vehicles are given orally to deliver the same dose, incomplete curves cannot be compared because the delivery vehicles may have different effects on gastric processing, emptying and subsequent absorption kinetics.

The third point, ensuring that the plasma response is derived only from the oral dose, is essential since it has now been demonstrated that oral folate-containing meals stimulate the release of endogenous folate into the plasma.
(Wright et al., 2003, 2005). It is also recognised that the sight, smell or anticipation of foods can trigger responses, so where placebos or blanks are used they should be chosen with care.

Finally, a question that is rarely asked is ‘Are the changes induced by the test meal/compound significantly different from day-to-day variation within an individual?’ In most cases it is not possible to answer this question because only a single baseline measurement has been made. The authors recommend that researchers do not rely on a single baseline measurement but take sufficient measurements to establish a threshold where the intervention becomes significant.

An essential feature of the Compartmental Modelling concept is that the body attempts to distribute nutrients throughout the body (via the plasma pool) to various organs and tissues, and the equilibrium between pools is linear and constant in the steady state. This condition is termed the ‘homeostatic set point’ by the authors. This state is an integration of genetic make up and exposure to environmental factors, and includes body mass, body composition, and physiological and biochemical conditions that an individual attains at steady state. The homeostatic set point does not imply that this is the ‘healthiest’ condition the body can attain given the conditions.

Increasing the amount of a compound entering a pool does not necessarily change ‘bioavailability’, because the compound will partition proportionally to all the pools according to distribution volumes/masses and pool affinities, and will be subjected to the same processes. The equilibrium between pools changes only if the homeostatic set-point moves; for example, growing, gaining or losing weight, changes in habitual energy expenditure, episodes of illness, ageing, environment and lifestyle. A logical conclusion from this equilibrium model is that ‘dose response’ in terms of changes in pool concentration is predictable unless the dose saturates, or overwhelms, normal physiological processes and the model becomes non-linear.

More commonly, the term ‘dose response’ is used to describe how efficacious a dose is at eliciting the desired physiological/health/pharmacological outcome rather than simply a change in a pool concentration. An organism responds holistically (not just as a result of achieving a particular pool concentration) and this response is usually hormetic (exhibits hormesis), that is, response ranges from the no observable effect level (NOEL), through the most effective dose to overt toxicity. Thus, although pool concentrations may be linearly related to dose injected or absorbed, the response of the organism is seldom that simple. ‘Dose response’ can be either a relationship between dose and amount in a pool, or between dose and organism response, and these are not the same. Dose response should, therefore, always be carefully defined and accessible to precise measurement.

As a cautionary note, before embarking on food, diet, or nutraceutical interventions to increase the intake or absorption of ‘beneficial’ compounds, it is as well to know where the subject(s) is/are on the hormetic dose response curve. In the curve illustrated in Fig. 11.4, only those subjects with sub-
optimal exposure will ‘gain’ from an increase in exposure, the rest will ‘lose’. Where dissimilar individual outcomes are observed, the individuals should be stratified and the reasons for differences identified. In this way, the target groups who will, or will not, benefit can be identified.

Within physiological limits, any absorbed substance is 100% available to participate in biochemical processes but its distribution to the different ‘pools’ and ultimate fate(s) is an inherent feature of the individuals’ metabolism. This in turn is a requirement of maintaining the homeostatic set-point of that individual. Absorption is easily defined but ‘bioavailability’ also includes consideration of how efficacious the absorbed dose is at eliciting the desired effect(s), just as in the drug model.

The following section provides some recommendations for best practice in the design and interpretation of nutrient absorption and metabolism studies, using specific nutrients to illustrate approaches for hydrophobic and hydrophilic compounds.

### 11.3 Study design and interpretation

#### 11.3.1 Ethics

All studies involving human volunteers must have received a ‘favourable ethical opinion’ from a recognised Ethics Committee that has jurisdiction in the country or state where that work will be carried out. Ethics Committees
are helpful in providing advice to applicants, as well as passing judgement on the value of the science and its impact on the volunteers. Furthermore, well thought out Ethics Application forms ask a variety of relevant questions which may not have been considered by the researcher but which are vital if the work is to be carried out safely to a publishable and acceptable International standard.

11.3.2 What sort of study?
At the outset there are a number of decisions to be made. These decisions are based on a critical analysis of what is intended and why. Can the intended studies be carried out in human volunteers, time-scale, intervention effects, compliance, chronic or acute study, statistical power, and will the results provide a clear answer?

11.3.3 Chronic studies
Chronic studies are often described as measuring ‘bioavailability’ but, in fact, can be used only to give a comparative indication of absorption. In such studies it is normal to use two or more large, matched groups of ‘free-living’ volunteers, each of which is given a different long-term intervention with the object of determining which intervention is most efficacious in achieving the desired outcome. The regimen usually takes the form of supplementation to the normal diet over a period of weeks with isolated compounds (tablets/capsules), or foods rich in the compound of interest. Concentration changes in the blood plasma or serum are tracked over time until a new plateau is reached and confirmed. The relative absorption from the ‘test’ food is then calculated against a ‘standard food’ or isolated compound. With this design, a new plasma concentration plateau must be reached, the same compound from different sources can be compared, and relative absorption can be determined. With chronic dosing it is essential that the data are not compromised by seasonal fluctuations in diet or differences in the timing of supplement/food ingestion, or taking of blood samples.

It is not usual to use a crossover design with a washout period because of the long term nature of the intervention, although this can be done so that each volunteer is their own control. If a crossover design is used, it is essential that the pool concentration of the compound of interest returns to the ‘habitual’ concentration before embarking on the second arm. The end point may be the mean (group) change in plasma concentration, or this may be coupled with other assessments, for example, changes in body weight, lifestyle, cognition, ability to carry out tasks, incidence of infectious or non-infectious diseases, progression or reversal of disease states and severity of symptoms.

Chronic studies are useful in a more holistic sense, since the time-scale allows the body to adapt and respond. This then permits nutrigenomic, transcriptomic and proteomic studies to be carried out to provide a fundamental
understanding of the effect of the intervention on the whole body and test hypotheses of the functionality of foods and nutraceuticals. Care should be taken to ensure that the intervention has not changed the rate of compound clearance or pool ratios. If there is evidence for either of these effects, then it can be concluded that the ‘homeostatic set point’ has moved and ‘bioavailability’ has changed.

11.3.4 Acute studies
With acute dosing, the objective is to measure either relative or absolute absorption by following the absorption and disposal of a single dose through monitoring appearance and disappearance of the compound in plasma. The experimental design can be a crossover, where each volunteer acts as their own control, or can be based on a single dose if mathematical modelling is to be applied to the data. Fractional absorption of an oral dose can also be assessed by comparison to an IV dose, using isotopic labelling to differentiate the two sources.

\[
\text{Fractional absorption} = \frac{\ln 2}{T_{1/2}} \times \frac{\text{AUC oral} \times \text{Plasma volume}}{\text{Oral dose}}
\]

Absolute absorption can be measured from the peak plasma excursion, or by measuring the area under the complete curve using the clearance rate \((t_{1/2})\) from the IV dose, and it is assumed that unit absorption provokes unit excursion. Absolute absorption may also be measured against the AUC induced by an IV dose, assuming there are no confounding factors. A seldom used alternative is to calculate what the virtual dose would be to simulate the plasma excursions found, and express this as a percentage of the dose given (O’Neill and Thurnham, 1998). This requires that the clearance kinetics and pool size are known.

11.3.5 Mass balance studies
As the name implies, mass balance studies measure intake and excretion over a period of time. They are most commonly acute studies, this being a more practical alternative to chronic studies which can be confounded by the intake of the compound of interest from dietary sources. They are non-invasive, compliance is good and intervention effects are minimal.

Mass balance studies offer a relatively simple way of obtaining information on absorption at the whole body level. They are particularly useful for inorganic ions, and organic compounds that are excreted unchanged, or which have a specific metabolite that can be easily detected and, if necessary, isolated (for isotope measurements) in urine/faeces, or breath. These simple whole body studies can be coupled with the sampling and analysis of body tissues. The model assumes that the compound recovered from the faeces has not been absorbed and that the compound, or downstream metabolites, recovered...
from the urine, represents material that has been absorbed. Point A (Fig. 11.5) represents a variant of the mass balance approach using ileostomy volunteers, where the unabsorbed material is intercepted at the end of the terminal ileum and therefore has not been subject to fermentation by the large bowel microflora. The ileostomy approach is useful for hydrophobic organic compounds that do not have identifiable urinary metabolites. Point B (Fig. 11.5) indicates body pool samples (plasma, biopsy) that may be necessary or provide additional information.

The disadvantage of these studies is that in many cases it is necessary to use isotopically labelled material and mass spectroscopy to enable discrimination between material arising from the test dose and that which arises from the diet or body. The mass balance between food and faecal excretion of unabsorbed material may have to be continued for several days to ensure that all stools containing unabsorbed material from the test diet are recovered, and often timing is erratic depending on bowel habit. With the ileostomy model, gastrointestinal transit is normally complete in about 14 hours and complete effluent collections can be made at regular intervals and rapidly preserved. Absorbed material or downstream metabolites recovered from the urine can give a direct estimate of the amount of material absorbed. Where the half-life of the whole body pool into which the dose has been incorporated is weeks, months or even years, urine and/or faeces recovery will need to continue until a satisfactory decay curve is obtained and the equation established, so that the line can be extrapolated on to baseline for a whole AUC. This is often difficult to obtain because the dose is commonly only a small fraction of the body pool and even with mass spectroscopy of stable isotopes (mass ratio measurements) it may be difficult to obtain accurate data at the high dilutions found days after the test dose.

Because both faecal and urinary excretion can be measured at the same time, absorption measurement can be either (intake – faecal excretion/intake) or (urinary excretion/intake), so it is possible to cross-check the data and to test some of the assumptions; for example, loss during gastrointestinal transit is only due to absorption.

![Fig. 11.5 Mass balance schematic.](image-url)
Mass balance studies provide a value for absorption, they do not measure ‘bioavailability’ and give no information on, for example, plasma concentrations or clearance rates (other than from whole body), which are essential in making the link between intake and health outcome.

11.3.6 Absorption, distribution, metabolism and excretion studies
For all studies, a decision needs to be taken as to whether a controlled clinical study or a study of ‘free-living’ individuals is needed. A controlled study in a clinical environment means that the researcher can exercise control over every aspect of the study and this can be applied to all volunteers and repeated on subsequent occasions. The value of controlling the study closely is repeatability, avoiding confounding factors, ensuring compliance and medical assistance if required. A study of ‘free-living’ individuals will be more variable, so may require a larger number of volunteers to achieve statistical significance. Volunteer compliance may be a problem, timing of samples may be erratic, and complete sample sets may not be available.

The next issue to address is whether the design is a supplementation study, where test material is given in addition to normal diet, or is a replacement study, where one (or more) foods are replaced by the test material. Supplementation studies are generally easier to conduct because, in theory, they are additional to habitual intakes, because the baseline remains constant. Any measured changes are therefore due to the intervention. Nevertheless, some steps should be taken to ensure that the additional intake does not displace foods, or amounts of foods, habitually eaten. This is most easily detected by using intake diaries, before, during, and if necessary, after the study. Diaries will also pick up seasonal dietary changes in long-term studies. Replacement studies automatically preclude those volunteers who do not habitually eat the food. Replacing a food, or a range of foods, with a ‘superfood’ or non-food vehicle will have an ill-defined effect due to the loss of intake of compounds from the habitually consumed foods which may confound the study.

Studies can have different levels of complexity depending on requirements:
- Absorption, distribution, metabolism and excretion (ADME) study
- Definitive absorption study
- Comparative absorption study

11.3.7 The absorption, distribution, metabolism and excretion (ADME) study
The ADME study is the most complex and the most invasive, and may well involve the use of isotopically labelled foods, orally administered isolates (isotopically labelled), intravenous dosing (isotopically labelled), or continuous infusions (isotopically labelled). It will also involve collecting serial blood and perhaps other tissue samples (biopsies), urine, faeces and breath (CO₂)
over extended time periods, during which the volunteer will need to eat and drink. These studies create a large number of different samples with diverse analytical requirements; for example, analysis of the compound of interest and downstream metabolites in different sample matrices, including breath CO₂. Because of the intensive nature of such studies, they are best carried out on one or two highly motivated individuals in a closely controlled clinical environment. Such studies are extremely valuable in resolving the detail and allowing the construction of informed general absorption and metabolic models.

11.3.8 Definitive absorption studies
Definitive absorption studies are needed to measure exactly how much of a compound is absorbed from a food, nutraceutical or drug delivery system. Although it is possible to vary intake and dose to achieve a specific plasma pool concentration or outcome, it is desirable to know exactly how much active component has been absorbed and over what time scale. Without this information, mis-dosing is likely to occur if the vehicle, or route of delivery, is changed. Additionally, where the compound is a precursor to an active metabolite it is not possible to make an estimate of conversion efficiency. In order to establish definitive absorption, it is necessary to know either the clearance rate from the sample pool or to use a comparison to a 100% absorbed standard. To make such a comparison, care should be taken to avoid confounding factors which may be introduced with the 100% absorbed comparator; for example, metabolism or isomerisation in the enterocyte, the in vivo carriers (if any), understand the implications of entry route (portal vein, thoracic duct, IV, IP) and any time effects.

Where it is possible to establish clearance kinetics from the body (or a major body pool and the size of the pool), the absolute amount absorbed can be calculated. This is not always possible for compounds that are very rapidly cleared, or where clearance is disproportionally changed in response to the presence of the compound in the pool, unless the concentration-dependent change can be established (for example, as can be done for glucose). To establish the clearance rate for compounds administered via the oral delivery route, sampling the pool over at least the time taken for the food to pass through the stomach and ileum is required to ensure that at least 5 data points are acquired during the clearance phase, uncomplicated by continuing absorption from the gut. The concentration-dependent clearance rate can then be applied to the whole absorption/clearance curve to establish the total amount absorbed. For such studies, sampling times and frequency should be set to capture an accurate concentration/time curve. Curves can be extrapolated to the point where the pool concentration returns to baseline: the more data available on the clearance-only part of the curve, the better the mathematical fit and more accurate the predicted absorption.

An alternative way of establishing the clearance rate is to load the pool of interest at a single time point, for example by IV injection. After a short
period of mixing throughout the body, ca. 10 min, the change in concentration in the pool sampled is solely due to clearance. Such studies can be designed so that a single volunteer receives a food or nutraceutical preparation on one occasion and an IV on another occasion, or both can be accomplished at one session if one of the doses is isotopically labelled and can therefore be discriminated from the other and from the endogenous background. Care should be taken to ensure that the delivery routes do not have different clearance rates. Although absolute absorption studies are relatively simple, they are best carried out on small, well-defined groups of volunteers, in a carefully controlled environment.

11.3.9 Comparative absorption studies

Comparative absorption studies are perhaps the easiest to perform but they do not provide any information on the absolute amounts absorbed. The only information they provide is that one delivery system or food, when compared to another containing the same (or normalised) load of component, elicits a greater or smaller concentration excursion in the sampled pool. This type of study is often carried out to compare absorption from two or more foods in different physical states, for example, raw vs. cooked, or cooked vs. some processed product, or a standard product with more or less added compound of interest. It is implicit in such studies that the complete concentration vs. time AUC is a measure of absorption because it will take account of any differences in time taken to ingest the food, gastric processing time, gastric emptying rate and differences in delivery profile. Occasionally, it is possible to use the maximum plasma excursion concentration instead of the AUC, provided that delivery profile is a constant.

Studies should be conducted within an individual so that they act as their own control, allowing a paired t-test which will increase the power of the statistical treatment. Both AUC and peak excursion can be normalised to take account of different loads, provided it is known that clearance rate is not unpredictably load sensitive.

11.4 Other considerations

Using an intravenous dose (assumed 100% ‘absorbed’) as a standard for comparison is not always valid for hydrophobic compounds. Such compounds, fed orally, may passively follow lipid absorption and are therefore transferred to the serosal side incorporated into chylomicrons synthesised in the enterocytes. This situation cannot usually be replicated and applied to human studies; thus an intravenous preparation would not behave in the same way as the chylomicron packaged material. Because of the rapid clearance of the chylomicrons (half-life 3–5 min) that carry the newly absorbed compound, it is not easy to provoke significant plasma response curves with a physiological
dose. This has led to the belief that some hydrophobic compounds are poorly absorbed by some individuals. An added complication to using plasma is that hydrophobic compounds carried by chylomicrons are sequestered by the liver and re-exported/transported in other lipoproteins. Thus, distribution among lipoprotein carriers may change rapidly over the period of study.

There are two potential resolutions to this problem: (i) isolate chylomicrons (or triglyceride-rich lipoprotein (TRL) fraction) or (ii) continue the study over several days until the compound has cleared from the long-lived lipoprotein particles. The latter approach is impractical since it would mean maintaining volunteers on a diet free of the compound of interest for several days, and during this time baseline concentration would start to fall. Analysis of the chylomicron (TRL) fraction is therefore preferable since appearance and clearance of the compound in the fraction is over within 12–16 h. This is a tolerable time for the volunteer to fast, or they can be given a meal free of the compound of interest at 5–6 h, when the original test meal has completely cleared the stomach. This procedure has the advantage that, whereas a concentration change may not be seen against the endogenous background in plasma, it is more easily seen in the chylomicrons (TRL), which carry only a small fraction of the total amount in plasma.

As with hydrophilic compounds, a classical approach to predicting the relative absorption of hydrophobic nutrients, or other food derived compounds, is to compare the plasma (or other blood fraction) concentration AUC response of a test dose of an isolated, well-absorbed compound (the ‘reference’ dose) to a test meal containing the compound of interest. Before using this method it is essential to establish that change in post-prandial plasma concentration is directly, or predictably, a response to the oral test dose. For example, recent research has demonstrated that this is not the case for folate and vitamin C, both of which are water soluble.

It is accepted that both synthetic folic acid and natural (or synthetic nature-identical) folates given orally would be bio-transformed in the intestinal enterocytes and passed to the hepatic portal vein only as 5-methyltetrahydrofolic acid (5-MTHF). The liver then removes a fraction of the 5-MTHF and the remainder enters the systemic blood system to create a systemic post-prandial 5-MTHF response. Since the proportion removed by the liver is unknown, the most common approach is to relate the plasma 5-MTHF AUC response to that of an equal reference dose of folic acid, and then calculate relative absorption. Recent publications, using isotopically labelled folate demonstrate, however, that a substantial portion of the total plasma 5-MTHF response to an oral dose is of endogenous origin, i.e. does not originate from the test dose. This endogenous response is large and unpredictable, and is not related to fasting baseline plasma or erythrocyte folate concentration. Thus it was concluded that relative absorption calculated from all previous studies that used unlabelled test doses (or foods) and an unlabelled reference dose, are invalid (Wright et al., 2005).
The same phenomenon has been demonstrated for vitamin C, where at least 50% of the plasma total post-prandial vitamin C response came from an endogenous source, also in an as yet an unpredictable manner (Bluck et al., 2005; Bates et al., 2004). Once more, this invalidates previous studies conducted without the aid of ‘labelled’ material. Whether other food compounds/nutrients behave in a similar manner must be determined prior to the conduct of studies using ‘unlabelled’ compounds.

The intestine is a major organ, works in concert with the body via feedback mechanisms, and has the capacity not only to digest and absorb compounds but also to metabolise, temporarily store and regulate serosal delivery post absorption. There are therefore many potential mechanisms by which a plasma response cannot be reconciled to the absorbed dose. While the gut lumen is empty it may be logical to assume that the gut sequesters and temporarily stores nutrients from the plasma to maintain its high metabolic requirements over extended time periods, only to dump them back into circulation once nutrients start to be absorbed from a meal.

11.5 Health response

There are many epidemiological studies linking the prevalence of non-communicable diseases (NCD) to diet and lifestyle. There is strong evidence that stopping smoking reduces the risk of lung cancer and even some evidence that adopting a diet similar to a population with a low incidence of NCD reduces the risk of developing some of these diseases. Where the difficulty arises is the identification of a single compound or group of compounds found in ‘good’ diets and attaching the putative benefits to these compounds. In other words, it is the holistic response of the individual to all the external factors that determines the health outcome. Optimum health can therefore only be achieved if both internal and external factors are sufficiently flexible and adaptable to attain congruence throughout life.

Most ‘bioavailability’ (absorption, mass balance, retention, tracer:tracee) studies are performed with the volunteer at steady state, although ‘snapshots’ can be taken during periods where the homeostatic set point is moving, for example, during growth, provided the time window for the study is relatively short with respect to the rate of change. A growing child and an adult may both absorb the same amount of a nutrient, such as calcium, but the child is likely to retain more calcium for growth, while the adult uses it for recycling established stores. Thus, calcium distribution, metabolism, requirement and functional response will be different in each case. Absorption is not the same as bioavailability, and bioavailability does not equate with health.

With the ever increasing interest in diet–health relationships creating opportunity for new food products and spawning innumerable programmes for ‘healthy eating’, the questions that must be answered are:
Food fortification and supplementation

(i) Does changing the diet simply change the concentration of the body pools of compounds and thus alter health outcome?
(ii) Does changing the diet move the homeostatic set-points of the body, and hence bioavailability, and thus alter health outcome?

The latter is more likely because a persistent change or larger or more frequent oscillations in the concentration of body pools of compounds will in itself move the set-point (a change in body pool ratios). As a simple example, reducing energy intake in an individual at steady state will alter both the overall size of the pool (body mass) and pool ratios (lean: fat). Thus, eating a diet rich in fruits and vegetables may not confer health benefits simply through increased intake of substances found in these foods *per se*, but by stimulating changes in homeostasis which cumulatively confer health benefits.

It is still a matter of speculation how large or persistent changes in intake and absorption have to be to provoke significant changes in gene expression or the proteome, and how long these changes persist if the diet is returned to pre-intervention conditions. This raises the question, not only of amounts and delivery profile, but also of frequency and duration.

For nutritional studies, the term ‘bioavailability’ should therefore not be used to describe studies from which it is hoped to measure a value; nor should the term be used for comparative studies where absolute absorption is not known. Furthermore, it cannot be claimed that for two foods containing a similar quantity of the same compound that one food induces a greater or smaller perturbation in the measured pool, thus the compound of interest from that food is more or less bioavailable. It is more or less absorbed, which is a function of bioaccessibility. In addition, ‘bioavailability’ cannot be used to compare two different compounds, or even two different isomers of the same compound.

11.6 Implications of controlled absorption for product development

The upper digestive tract has a complexity of control mechanisms, which govern the entire process of ingestion and digestion. The main switches for these processes are oral stimuli, gastric stretch receptors, gut hormone responses to food in the GI tract and the systemic presence of absorbed nutrients.

It is commonly accepted that the half-life of solid foods in the stomach is about 60–90 minutes and that most digestion and absorption occurs in the upper 30% of the small intestine. The potential time window for digestion and absorption, mouth to terminal ileum, is around 6 hours. Foods, nutrients and structures that are resistant to digestion in this 6 hour time frame, are predominantly lost to the large bowel. The examination of terminal ileal effluent from ileostomy volunteers routinely shows recognisable fruit and vegetable structures (leaf, skin, seeds and tissue fragments), nut fragments,
cereal structures and mushrooms. These materials, and much of their potential nutrient content, survive because the cell walls are not digestible. Although most nutrients can be absorbed throughout the small intestine the ability to absorb them diminishes distally, thus glucose infused into the distal ileum is poorly absorbed and may trigger osmotic diarrhoea. It can therefore be envisioned that the presence of absorbable compounds at different points along the small intestine will have different effects on the control and feedback mechanisms that regulate digestion.

With the notable exception of carbohydrate foods, where the ‘Glycaemic Index’ is seen to be important, putative health benefits have been related to overall amount, and not a combination of load and rate/site/limit of digestion/absorption. However, optimal rate of nutrient delivery, as well as optimal amounts, is also potentially important for maintenance of good health, and has long been an important concept in enteral and parenteral feeding in a clinical setting. Experimental studies in patient volunteers have demonstrated significant differences in physiological response associated with altered rates of delivery. Under ‘normal’ feeding conditions, it has been shown that even modest variations in the initial rate of duodenal glucose entry may have profound effects on subsequent glycaemic, insulin, and incretin responses (O’Donovan et al., 2004). Physiological response to different rates and sites of delivery of micro-constituents of food has been poorly researched but such investigations are crucial for the ethical design of controlled or site-specific release products. Modifying how and where nutrients (and other compounds) are released and absorbed can have both beneficial and adverse consequences.

Epidemiological studies provide a regular supply of data indicating associations between dietary habits, specific foods, or specific constituents of foods, and increased/decreased risk of a range of chronic diseases. Well-known examples include dietary fibre and colon cancer, and lycopene and prostate cancer. Following disclosure of these epidemiological associations, dietary supplements, or foods containing more readily absorbed, or higher amounts of these putative beneficial compounds are promoted in the market place. However, the bridge between observation and exploitation may not be supported by rigorous examination of the hypothesis linking a specific compound to altered disease risk and, crucially, there is often inadequate understanding of the effects of dose and rate of delivery of the putative beneficial compound. A recent example of where too little information has resulted in adverse response in potentially ‘at risk’ individuals relates to beta-carotene (Oomen, 1996). The purported health-giving properties of plant polyphenols also represent a case in which enthusiastic marketing claims may far exceed the current scientific evidence. Even when good experimental evidence exists, results need to be interpreted with caution in relation to human health benefits at different time scales.

The analysis of nutrient risk–benefit curves, to define both the optimal intakes and safe limits of consumption of bioactive food components, is a
focus of investigation using the ‘omic’ technologies (transcriptomics, proteomics, metabolomics), which address this issue from a nutritional systems biology perspective. By adopting a multi-parameter approach, it is envisaged that this will allow the quantification of subtle, physiologically relevant changes and establish methodologies for the assessment of effects of bioactive food components in the context of the diet, at relevant intake, and as they occur over long periods of time in the whole human body.

Currently, the approach is seeking to integrate the effects of different components of specific elementary cellular and physiological processes; for example, how different food-derived components improve, or hamper, mitochondrial function. This ‘holistic’ approach distinguishes risk–benefit analysis from disease prevention-directed nutritional sciences, and from toxicology. Nutrigenomic approaches are crucial to this goal, since the technologies allow simultaneous analysis of the large numbers of parameters that need to be assessed. Clearly also, inter-individual variation, gender, age and lifestyle, must be considered, both in relation to personalised nutrition and to identify requirements of specific subgroups. Analysis of gene and subsequent metabolic response in humans requires quantitative measurement of site-specific nutrient delivery and emphasises the paramount importance of definitive measures of nutrient absorption and distribution. This new approach to nutrition is now being explored and developed in many research laboratories around the world. One excellent source of information is the European Nutrigenomics Organisation (see Section 11.8).

### 11.7 Future trends

We are all concerned with the health of society, and consequently the individual, and if nothing else the cost of health care will be a major incentive to optimise our personal health. There is a debate to be had as to what constitutes personal health and the social and economic objectives of society. It is undoubtedly the case that there will be developments in ‘personal nutrition’ but these, at least initially, may be targeted to, or adopted by, only those whose genetic make up predisposes them to a specific disease state. The disease state must have a clear functional relationship between intake/absorption and long-term health outcome, and could be the result of a missing or damaged gene, a suppressed or silenced gene, auto-immune deletion of specific cells or proteins, or production of a dysfunctional protein. The overcoming of a single ‘error’ in an individual whose health will deteriorate if there is no intervention is just the tip of a very big iceberg. At first sight, for a ‘healthy’ person, analysis of the health impact of a specific food or diet that takes into account all of the genes in the human genome, their interactions and all of the interactions possible with diverse lifestyles, age and sex, seems impossible. Such complex interactive systems are notoriously difficult to perturb, simply because there is a high degree of compensation arising from multiple copies
of genes or other systems that can up or down regulate. However, a close analysis of this complexity may reveal that there are only a few key drivers and the rest of the systems are basically slaves. Should this turn out to be the case, it greatly diminishes the number of probable variables that need to be manipulated to attain optimum health.

It is well documented that poor nutrition in utero (Barker, 1998) may have a profound effect on health, with respect to both communicable and non-communicable disease. The reader is directed to the output of a large, ongoing EC supported project concerned with early nutrition programming (EARNEST, FOOD-CT-2005-007036). What is less well documented is that the nutritional status of parents and grandparents may also have heritable epigenetic effects (Pembrey, 2002; Whitelaw, 2006; Richards, 2006).

It is clear that further work needs to be undertaken to evaluate nutrient–gene interactions and the subsequent health effects induced by wide fluctuations of intake within individuals (e.g. seasonal reliance on specific foods, periods of starvation) which lead to cyclical changes in body mass and metabolic profile and which may have transient or heritable epigenetic effects.

The time has come to stop trying to pick ‘magic bullets’ out of foods, discard quick fix diets and adopt flexible (age, sex, lifestyle) dietary regimens that are congruent with our ‘optimum metabolic set-point’ and thus minimise the risk of developing non-transmissible diseases.

11.8 Sources of further information and advice


11.9 References

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12

Codex Alimentarius standards affecting fortified foods and supplements

J. Maskeliunas and K. Miyagishima, Joint FAO/WHO Food Standards Programme, Italy

12.1 Introduction

The Codex Alimentarius Commission (CAC) was established in 1961/1963 by the Food and Agricultural Organization of the United Nations (FAO) and the World Health Organization (WHO) as an intergovernmental body to implement the Joint FAO/WHO Food Standards Programme. The objectives of the Codex Alimentarius Commission include the protection of the health of consumers and the assurance of fair practices in food, as well as promoting coordination of all food standards work undertaken by international governmental and non-governmental organisations.

Codex Alimentarius interprets, literally, as ‘food code’ or ‘food law’. The Codex Alimentarius is a collection of internationally adopted food standards, and other texts presented in a uniform manner. These international food standards and related texts have been systematically developed under the auspices of, and adopted by, the Codex Alimentarius Commission, whose membership represent more than 99% of the world population. Codex standards are developed based on the best scientific and technical advice available. Usually this scientific advice is provided through FAO/WHO expert committees and ad hoc expert consultations. Codex is the only international forum able to bring together scientists, technical experts, government regulators, and international consumer and industry organisations.

Codex standards and related texts have become the benchmarks against which national food measures and regulations are evaluated within the legal parameters of the World Trade Organization (WTO), including its trade dispute settlement mechanisms. More specifically, the WTO Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement) explicitly
recognises Codex standards and related texts as the international standards for food safety. For matters other than food safety, the WTO Agreement on Technical Barriers to Trade (TBT Agreement) makes reference to international standard-setting bodies in general, and WTO Members are obliged to ensure that their technical regulations are not more trade-restrictive than necessary to fulfil a legitimate objective, taking into account the risks that non-fulfilment would create. Members are encouraged to base their technical regulations on international standards where they exist.

Basically Codex standards and related texts are elaborated via a procedure consisting of eight steps; the elaboration process usually takes 3 to 5 years, but the time necessary depends on how member governments go about the negotiation among themselves. The principal developmental work of the Commission is undertaken by its various committees and other subsidiary bodies (Fig. 12.1). Once adopted, Codex standards and other texts are used as a basis for national food regulations in many countries.

The Codex Alimentarius Commission recognised the importance of the work on nutrition since its early sessions.\(^1,2\) The European Codex Committee on Dietetic Foods was established by the 3rd Session of the Commission in 1965, with the terms of reference focused on dietetic foods.\(^3\) The Committee was given the status of a worldwide commodity committee by the 4th Session of the Commission in 1966 and was renamed as the Committee on Foods for Special Dietary Uses.\(^4\)

The matter of how Codex should deal with issues related to nutrition was extensively discussed by the Commission at its 13th and 14th Sessions.\(^5,6\) The 15th Session of the Commission agreed to extend the terms of reference of the Codex Committee on Foods for Special Dietary Uses to coordinate work on nutritional aspects within Codex.\(^7\) The name of the Committee was amended in 1987 to refer to nutrition.\(^8\) Since then, the Codex Committee on Nutrition and Foods for Special Dietary Uses (CCNFSDU) came to be recognised as a general subject committee and not merely as a commodity committee. One could however note that the CCNFSDU has preserved until today its two characters: the one as a ‘commodity committee’ in so far as it elaborates standards for foods for special dietary uses, and the one as a ‘general subject committee’ when it deals with general questions on nutrition.

Currently, the CCNFSDU is the unique subsidiary whose terms of reference explicitly state its role to deal with nutrition, and is responsible for the elaboration of standards and other texts in the area of nutrition and foods for special dietary uses. Among a number of standards and related texts developed and adopted by the Codex Alimentarius Commission, two texts elaborated by this CCNFSDU bring to light the major work so far accomplished by the Commission in the area of nutrient addition/fortification of foods and food supplements.
### Codex Alimentarius Commission

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| Latin America and the Caribbean (Mexico) |
| North America and the Southwest Pacific (Tonga) |
| Near East (Tunisia) |

**Fig. 12.1** Organigramme of the Codex Alimentarius Commission.
12.2 Codex General Principles for the addition of essential nutrients to foods

At the 11th Session of the CCNFSDU, in 1978, it was proposed that the Committee should elaborate the General Principles concerning fortification of foods. However, the Committee started considering this matter substantively only at its 14th Session in 1985. The Codex Alimentarius Commission adopted the text at its 17th Session in 1987; later, amendments to these principles were adopted by the 18th and 19th Sessions of the Commission in 1989 and 1991 respectively.

12.2.1 Introduction and scope

The Codex General Principles for the Addition of Essential Nutrients to Foods are intended:

(i) to provide guidance to those responsible for developing guidelines and legal texts pertaining to the addition of essential nutrients to foods;
(ii) to establish a uniform set of principles for the rational addition of essential nutrients to foods;
(iii) to maintain or improve the overall nutritional quality of foods;
(iv) to prevent the indiscriminate addition of essential nutrients to foods thereby decreasing the risk of health hazard due to essential nutrient excesses, deficits or imbalances (this will also help to prevent practices which may mislead or deceive the consumer); and
(v) to facilitate acceptance in international trade of foods which contain added essential nutrients.

For definitions of terms used in Codex texts, see Table 12.1.

12.2.2 Basic principles

The General Principles state that essential nutrients may be added to foods for the purpose of restoration, nutritional equivalence of substitute foods, fortification and ensuring the appropriate nutrient composition of a special purpose food. The essential nutrient should be present at a level which will not result in either an excessive or an insignificant intake of the added essential nutrient considering amounts from other sources in the diet. The addition of an essential nutrient to a food should not result in an adverse effect on the metabolism of any other nutrient and should be sufficiently stable in the food under customary conditions of packaging, storage, distribution and use. The essential nutrient should be biologically available from the food; should not impart undesirable characteristics to the food (e.g. colour, taste, flavour, texture, cooking properties); and should not unduly shorten shelf-life. Technology and processing facilities should be available to permit the addition of the essential nutrient in a satisfactory manner. Addition of
Table 12.1 Definitions of terms in Codex texts

**Nutrient** means any substance normally consumed as a constituent of food:

(a) which provides energy; or  
(b) which is needed for growth and development and maintenance of healthy life; or  
(c) a deficit of which will cause characteristic bio-chemical or physiological changes to occur.

**Essential nutrient** means any substance normally consumed as a constituent of food which is needed for growth and development and the maintenance of healthy life and which cannot be synthesized in adequate amounts by the body.

**Nutritional equivalence** means being of similar nutritive value in terms of quantity and quality of protein and in terms of kinds, quantity and bioavailability of essential nutrients. For this purpose, nutritional equivalence means that essential nutrients provided by the food being substituted, that are present in a serving or portion or 100 kcal of the food at a level of 5% or more of the recommended intake of the nutrient(s) are present in the substitute or partially substituted food (extender) in comparable amounts.

**Substitute food** is a food which is designed to resemble a common food in appearance, texture, flavour and odour, and is intended to be used as a complete or partial replacement for the food it resembles.

**Fortification or enrichment** means the addition of one or more essential nutrients to a food whether or not it is normally contained in the food for the purpose of preventing or correcting a demonstrated deficiency of one or more nutrients in the population or specific population groups.

**Restoration** means the addition to a food of essential nutrient(s) which are lost during the course of good manufacturing practice, or during normal storage and handling procedures, in amounts which will result in the presence in the food of the levels of the nutrient(s) present in the edible portion of the food before processing, storage or handling.

**Special purpose foods** are foods that have been designed to perform a specific function, such as to replace a meal which necessitates a content of essential nutrients which cannot be achieved except by addition of one or more of these nutrients. These foods include but are not limited to foods for special dietary use.

**Nutrient density** means the amount of nutrients (in metric units) per stated unit of energy (MJ or kcal).

**Standardisation** means the addition of nutrients to a food in order to compensate for natural variations in nutrient level.

**Vitamin and mineral food supplements** derive their nutritional relevance primarily from the minerals and/or vitamins they contain. Vitamin and mineral food supplements are sources in concentrated forms of those nutrients alone or in combinations, marketed in forms such as capsules, tablets, powders, solutions etc., that are designed to be taken in measured small-unit quantities. This refers to the physical forms of the vitamin and mineral food supplements not to the potency of the supplements, but are not in a conventional food form and whose purpose is to supplement the intake of vitamins and/or minerals from the normal diet.

Essential nutrients to foods should not be used to mislead or deceive the consumer as to the nutritional merit of the food. The General Principles also require that the additional cost should be reasonable for the intended consumer and that methods of measuring, controlling and/or enforcing the levels of
added essential nutrients in foods should be available. When provision is made in food standards, regulations or guidelines for the addition of essential nutrients to foods, specific provisions should be included identifying the essential nutrients to be considered or to be required and the levels at which they should be present in the food to achieve their intended purpose.

12.2.3 Nutrient addition for purpose of restoration
The General Principles recommend restoration of the essential nutrients of concern lost during processing, storage or handling, where the food has been identified as a significant source of energy and/or essential nutrients in the food supply, and particularly where there is demonstrated evidence of public health need. A food should be considered a significant source of an essential nutrient if the edible portion of the food prior to processing, storage or handling contains the essential nutrient in amounts equal to or greater than 10% of the recommended nutrient intake in a reasonable daily intake (or in the case of an essential nutrient for which there is no recommended intake, 10% of the average daily intake).

12.2.4 Nutrient addition for purpose of nutritional equivalence
The General Principles also recommend nutritional equivalence in terms of the essential nutrients of concern, where a substitute food is intended to replace a food which has been identified as a significant source of energy and/or essential nutrients in the food supply, and particularly where there is demonstrated evidence of public health need. A food being substituted or partially substituted should be considered a significant source of an essential nutrient if a serving or portion or 100 kcal of the food contains the essential nutrient in amounts equal to or greater than 5% of the recommended nutrient intake. Where there is a clear public health reason to moderate the intake of a specific nutrient, the level of this nutrient need not be equivalent.

12.2.5 Nutrient addition for purpose of fortification
The General Principles stress that fortification should be the responsibility of national authorities since the kinds and amounts of essential nutrients to be added and foods to be fortified will depend upon the particular nutritional problems to be corrected, the characteristics of the target populations, and the food consumption patterns of the area.

The following conditions should be fulfilled for any fortification programme:

- There should be a demonstrated need for increasing the intake of an essential nutrient in one or more population groups. This may be in the form of actual clinical or sub-clinical evidence of deficiency, estimates indicating low levels of intake of nutrients, or possible deficiencies likely to develop because of changes taking place in food habits.
• The food selected as a vehicle for the essential nutrient(s) should be consumed by the population at risk.
• The intake of the food selected as a vehicle should be stable and uniform, and the lower and upper levels of intake should be known.
• The amount of the essential nutrient added to the food should be sufficient to correct or prevent the deficiency when the food is consumed in normal amounts by the population at risk.
• The amount of the essential nutrient added should not result in excessive intakes by individuals with a high intake of a fortified food.

12.2.6 Nutrient addition to special purpose foods
Nutrients may be added to special purpose foods, including foods for special dietary uses, to ensure an appropriate and adequate nutrient content. Where appropriate, such addition should be made with due regard to the nutrient density of such foods.

12.2.7 Application of the General Principles – a few examples
The General Principles were taken into account while developing provisions for iodisation in the Codex Standard for Food Grade Salt. The Standard states that in iodine-deficient areas, food-grade salt shall be iodised to prevent iodine-deficiency disorders (IDD) for public health reasons. In this Standard, the actual levels of iodine compounds are not specified, as it is recognised that the levels required vary considerably from one country to another. It is left to national health authorities to determine these, in view of the local iodine deficiency situation. Similarly, provisions for fortification are included in the Codex General Guidelines for the Utilization of Vegetable Protein Products (VPP) in Foods and the Codex General Standard for Fruit Juices and Nectars.

12.3 Guidelines for vitamin and mineral food supplements
The work on the elaboration of the Guidelines for Vitamin and Mineral Food Supplements, from the very beginning, was quite controversial on all aspects including the scope of the Guidelines as well as the provisions on the composition and contents of vitamins and minerals. Some countries were in favour of their development while others were opposed to it, arguing that worldwide guidelines were not appropriate to address this very complex issue thereby suggesting to leave this matter of regulation to national authorities.

The initial discussion in the CCNFDU started in 1988, when several delegations proposed work in the area of food supplements as part of an overall review of the work of Codex in the area of nutrition. The 18th Session of the Codex Alimentarius Commission in 1989 asked for more
consultation between Member countries before agreeing to undertake the work. It identified the problem of defining supplements as ‘food’ (i.e. falling within the Codex mandate) or as pharmaceuticals. In 1991, in the light of government comments received, the CCNFSDU agreed to undertake new work on guidelines for vitamin and mineral supplements. An initial working draft was prepared by Germany and was circulated for comments in 1992. Eventually it took more than ten years of deliberations at the CCNFSDU before the Guidelines were adopted by the 28th Session of the Commission in 2005.

12.3.1 Preamble and scope
The Guidelines recognise that most people who have access to a balanced diet can usually obtain all the nutrients they require from their normal diet. Because foods contain many substances that promote health, people should therefore be encouraged to select a balanced diet from food before considering any vitamin and mineral supplement. In cases where the intake from the diet is insufficient or where consumers consider their diet requires supplementation, vitamin and mineral food supplements serve to supplement the daily diet. These guidelines apply to vitamin and mineral food supplements intended for use in supplementing the daily diet with vitamins and/or minerals. Food supplements containing vitamins and/or minerals as well as other ingredients should also be in conformity with the specific rules on vitamins and minerals laid down in these Guidelines. These Guidelines state that they apply only in those jurisdictions where products defined in Section 12.2.1 are regulated as foods, and that they do not apply to foods for special dietary uses as defined in the General Standard for the Labelling of and Claims for Pre-packaged Foods for Special Dietary Uses.19

12.3.2 Composition
Selection of vitamins and minerals
The Guidelines state that vitamin and mineral food supplements should contain vitamins/provitamins and minerals whose nutritional value for human beings has been proven by scientific data and whose status as vitamins and minerals is recognised by FAO and WHO. The sources of vitamins and minerals may be either natural or synthetic, and their selection should be based on considerations such as safety and bioavailability. In addition, purity criteria should take into account FAO/WHO standards, or if FAO/WHO standards are not available, international Pharmacopoeias or recognised international standards. In the absence of criteria from these sources, national legislation may be used.

Vitamin and mineral food supplements may contain all vitamins and minerals that comply with the criteria described above, a single vitamin and/or mineral, or an appropriate combination of vitamins and/or minerals.
**Contents of vitamins and minerals**

The Guidelines indicate that the minimum level of each vitamin and/or mineral contained in a vitamin and mineral food supplement per daily portion of consumption as suggested by the manufacturer should be 15% of the recommended daily intake as determined by FAO/WHO.

Maximum amounts of vitamins and minerals in vitamin and mineral food supplements per daily portion of consumption as recommended by the manufacturer shall be set, taking the following criteria into account:

(i) upper safe levels of vitamins and minerals established by scientific risk assessment based on generally accepted scientific data, taking into consideration, as appropriate, the varying degrees of sensitivity of different consumer groups;

(ii) daily intake of vitamins and minerals from other dietary sources.

When the maximum levels are set, due account may be taken of the reference intake values of vitamins and minerals for the population. This provision should not lead to setting of maximum levels that are solely based on recommended nutrient intakes (e.g. Population Reference Intake or Recommended Daily Allowance values).

**12.3.3 Packaging**

The Guidelines require that the product be packed in containers which will safeguard the hygienic and other qualities of the food. The containers, including packaging material, shall be made only of substances which are safe and suitable for their intended use. Where the Codex Alimentarius Commission has established a standard for any such substance used as packaging material, that standard shall apply.

**12.3.4 Labelling**

The section on labelling of the Guidelines sets out detailed provisions on how vitamin and mineral food supplements should be labelled, presented, stored and used to consumers. The label should not state or imply that supplements can be used for the replacement of meals or a varied diet.

**12.4 Ongoing work**

At the 28th Session of the CCNFSDU held in Chiang Mai, Thailand (2006), one delegation indicated that the General Principles were adopted in 1987 and since then there had been changed approaches or philosophies related to controlling the addition of vitamin and minerals for foods, changes in technologies employed for achieving addition or enhancement of vitamin and mineral levels in foods and an increased interest in the addition to foods
food fortification and supplementation

of non-nutrient bioactive substances. The delegation pointed out that all these suggest that a review of the General Principles may be timely to ensure that these principles continue to be relevant and useful, and proposed new work that would address three separate issues within the Principles: non-traditional addition of vitamins and minerals to foods; discretionary addition of vitamins and minerals to food to provide consumers with a greater variety of foods with added vitamin and mineral nutrients; and addition of bioactive substances that are essential constituents to foods. The Committee agreed to consider the proposal for new work to amend the Codex General Principles for the Addition of Essential Nutrients to Foods at the 2007 session on the basis of a discussion paper.

12.5 Conclusions

Fortification programmes of foods have been and will be used in many countries of the world to eliminate or reduce existing micronutrient deficiencies and to promote better health of their populations. The recommendations of the Codex Alimentarius Commission on food fortification will continue to provide useful guidance for governments, food industry and other partners for implementing food fortification in a meaningful and safe manner. Furthermore, in view of the increasing emphasis placed on combating non-communicable, diet-associated diseases in both developing and industrialised countries, nutrition will most probably remain one of the areas where the Codex Alimentarius Commission will continue to be active in coming years by setting standards and related texts as a basis for international harmonization. In order for Codex to continue to develop standards, guidelines and other recommendations based on sound science, it is indispensable that FAO and WHO continue to provide to Codex relevant, independent expert advice on risk assessment/safety assessment in the area of nutrition, in a timely manner. In this regard, the interaction between the CCNFSDU and FAO/WHO should be maintained and be strengthened where necessary.

The views expressed in this chapter are those of the authors and do not necessarily represent the official positions of FAO, WHO, the Codex Alimentarius Commission or their member governments.

12.6 References

13

European legislation on fortified foods and supplements

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13.1 Principles and evolution of EU food legislation

The European food supplements directive (2002/46) (FSD), adopted in 2002, represents a first step in the development of a European-wide harmonised legal framework governing the production, labelling, quality and marketing of food supplements. It regulates food supplements as a specific category under food law. This means that all requirements that apply to foodstuffs also apply to food supplements. Since July 2007, the addition of vitamins and minerals to foodstuffs is also harmonised by a specific regulation (1925/2006). The main question addressed in this chapter is why this has taken so much time and why the harmonisation exercise is far from complete.

Traditionally, nutrients and other substances, including botanical ingredients, have been used in the European Union both in foods (including food supplements) and medicinal products. The 27 member states have regulated such products in a variety of different ways, either by covering them under food law, medicinal law or creating new product categories (e.g. natural remedies, etc.). Such approaches include different ways of authorisation and monitoring of such products on their territory. In some member states, no procedure or constraints exist whereas in others, procedures such as notification and pre-marketing authorisation are required. In some member states, positive lists exist, containing substances that are permitted to be used as well as the conditions for their use, including maximum and minimum levels, warning statements, etc. In other member states, no such lists exist. More fundamentally, there are two different approaches underlying the various legal initiatives of the member states. Some take a safety approach, leading to a system where consumers have the choice between numerous products containing levels that may greatly exceed the recommended daily allowances.
(RDA). Maximum levels, if any, are based on a scientific risk assessment and no restrictions are imposed if the products or levels of nutrients and other substances are safe. Other member states take the view that consumers need to be protected and put nutritional factors to the forefront. This leads to a marketplace where the number and level of nutrients and other substances is restricted, because it is the view of the authorities that the national diet is sufficient for meeting the dietary needs of their population. They consider that levels higher than the RDA are not necessary. This leads to a centrally directed system where consumers do not have the possibility of choosing products in accordance with their wishes.

It is obvious that with such national differences, there was only limited free movement of such products between the member states. The development of a European harmonised system seemed extremely problematic. Nevertheless, in 2002 such an EU system was established, creating a legal framework for food supplements. It was a remarkable accomplishment, primarily because of three major achievements:

(i) It established for all member states that Food Supplements are to be regulated under food law as a specific category of foodstuffs, in conformity with the general food law regulation (178/2000) (GFLR).
(ii) It established the underlying principles for setting at a later stage maximum and minimum levels of vitamins and minerals to be used in food supplements.
(iii) It laid down in positive lists the vitamins and minerals and their chemical sources that are permitted for use in food supplements.

Although these are important milestones in the development of a European Market, full harmonisation will be achieved only when the maximum and minimum levels are established, and harmonising rules are developed for other substances, including botanicals. In relation to the establishment of maximum and minimum levels, the European Commission (EC) launched a consultation in June 2006. For this purpose the EC issued a discussion paper, inviting comments on a number of questions by 30 September 2006. In relation to the further harmonisation of other substances (i.e. non-vitamins and minerals), the FSD requires the EC to submit to the European Parliament and the Council a report on the advisability of establishing specific further rules.

This chapter will describe the current practice in relation to the application of the FSD and elaborate on further steps in the harmonisation process of food supplements and fortified foods in the European Union.

13.2 Barriers to trade in the EU relating to fortified food products and food supplements

In the absence of harmonisation, as is currently the case for maximum and minimum levels of vitamins and minerals in food supplements and fortified
foods, the principle of mutual recognition applies. This principle is enshrined in Article 28 of the European Treaty, relating to the prohibition of quantitative restrictions between member states. It means literally that member states are not allowed to prohibit import of products from another member state if such a product is lawfully manufactured or marketed in the exporting member state. They cannot, in principle, prohibit the sale of such products on their territory, not even when those products are produced to technical or qualitative specifications that differ from those required in the importing member state. There are only few exemptions. Article 30 of the European Treaty specifies that only the following aspects are sufficient basis for import restrictions: public morality, public policy or public security; the protection of health and life of humans, animals or plants; the protection of national treasures possessing artistic, historic or archaeological value; and the protection of industrial and commercial property. Furthermore, the European Court of Justice (ECJ) has established criteria for the application of restrictive measures. Any measures taken must be compatible with the principles of necessity and proportionality. The burden of proof to demonstrate the valid ground for one of the conditions spelled out in Article 30 (including safety concerns) is on the member state.

The ECJ first laid down the grounds for this principle (although it was never actually called ‘principle of mutual recognition’ by the ECJ) in the famous ‘Cassis de Dijon’ case in 1979. It has since been confirmed by many subsequent cases. Nevertheless, the European Union has been struggling with the application of this principle ever since the EC published their communication on the consequences of the ‘Cassis de Dijon’ judgment in 1980. In its ‘Action plan for the single market’, adopted on 4 June 1997, the EC identified the application of the principle of mutual recognition as one of the measures to be taken to improve the performance of the internal market. In 1999, the EC adopted a communication on the application of the mutual recognition principle. It was based on a detailed analysis of the cases of incorrect application of mutual recognition handled by the EC in recent years. This analysis was set out in the ‘First biennial report on the application of the principle of mutual recognition’, which accompanied the communication. A ‘Second biennial report on the application of the principle of mutual recognition in the single market’, adopted in July 2002, assessed the progress made in the application of mutual recognition in the single market since 1999 and highlighted the fields in which mutual recognition continues to pose problems. On the basis of this communication, the Council adopted a resolution on mutual recognition, which was incorporated into the ‘Agreement on the European Economic Area’. The communication has also inspired the Economic and Social Committee to draw up an opinion on mutual recognition.

In 2003 another extensive interpretative communication on the practical application of mutual recognition was published. This communication is a practical guide to enable member states and economic operators to benefit from the free movement of products in the many sectors where there are no
European legislation on fortified foods and supplements

‘harmonised’ rules, thereby ensuring the free movement of goods within the EU. The document makes clear that member states must allow the placing on their markets of any product lawfully manufactured and/or marketed in another member state, unless a member state has technical or scientific proof that the product constitutes a risk for human health, safety or the environment. The communication also clarifies the burden of proof and summarises when and how the free movement of goods can be restricted.

In spite of all the efforts by the EC to have mutual recognition correctly applied by the member states, such application has been highly problematic in the field of fortified foods and food supplements. This is because of the different national practices and the use of alleged grounds for health protection. Although a procedure exists within the EC to deal with complaints relating to the non-application of mutual recognition, companies are often reluctant to use it.

13.3 EU harmonisation to resolve barriers to trade in the field of food supplements and fortified foods

The divergent national approaches and resulting barriers to trade have triggered the European Union to launch a harmonisation exercise. This was cautiously prepared with a number of extensive discussion papers in 1991 and 1997. After much discussion the directive was finally adopted and published in 2002. It established a definition of food supplements: ‘foodstuffs, the purpose of which is to supplement the normal diet and which are concentrated sources of nutrients or other substances with a nutritional or physiological effect, alone or in combination, marketed in dose form, namely forms such as capsules, pastilles, tablets, pills and other similar forms, sachets of powder, ampoules of liquids, drop dispensing bottles, and other similar forms of liquids and powders designed to be taken in measured small unit quantities.’

This definition contains a number of important elements, especially in relation to the similarity that may exist with medicinal products in the presentation of such products:

(i) Food supplements are foodstuffs. This means that the whole food regulatory framework applies to food supplements. This includes the application of the GFLR covering responsibilities of business operators, notification duty, etc. and aspects covered by vertical food legislation such as additives, flavouring substances, contaminants, residues, GMOs and irradiation.

(ii) Food supplements are destined to supplement the normal diet and are concentrated sources of nutrients or other substances with a nutritional or physiological effect, indicating that their effect on the body should be nutritional or physiological. It clearly extends the interpretation of
food from the more traditional concept, still favoured by some member
states, that foodstuffs would only cover edible constituents that are
taken for nourishment or pleasure. Such interpretation is clearly outdated
since the principle that foodstuffs also include edible material with an
effect on human physiology was taken as the basis for the Nutrition
and Health Claims Regulation (NHCR) (1924/2006).\(^{18}\)

(iii) Food supplements are marketed in dose form and designed to be taken
in measured small unit quantities. This part of the definition serves to
distinguish food supplements from regular foodstuffs fortified with the
same active constituents. This latter category of products is covered by
the regulation relating to the addition of vitamins and minerals and
of certain other substances to foods (AVMOSR).\(^{2}\) This part of the
definition also signifies that the form in which food supplements
are marketed may be indistinguishable from the form of medicinal
products.

Although the directive states in its preamble and in the definition that a vast
range of vitamins, minerals and other substances with a nutritional or
physiological effect, including but not limited to amino acids, essential fatty
acids, fibre and various plants and herbal extracts may be present in food
supplements, at this stage the directive contains detailed rules on vitamins
and minerals only. On other substances, the directive imposes upon the EC
the duty to submit to the European Parliament and the Council a report on
the advisability of establishing specific rules, including, where appropriate,
positive lists, on categories of nutrients or of substances with a nutritional or
physiological effect other than those vitamins and minerals, accompanied by
any proposals for amendments to the directive which the EC deems necessary.
And even with regard to the use of vitamins and minerals, the directive is not
yet finalised, with two aspects still pending: the completion of the list of
nutritional substances and the setting of maximum levels.

13.3.1 The list of nutritional substances
The FSD contains two annexes. Annex I lists the vitamins and minerals
which may be used in the manufacture of food supplements. It may be
observed that a number of minerals are not included (e.g. boron, nickel,
vanadium, silicon). Annex II lists the vitamin and mineral substances (i.e.
chemical form), which may be used in the manufacture of food supplements.
This list was compiled from substances that had already been evaluated by
the Scientific Committee on Food (SCF) and the European Food Safety
Authority (EFSA) in the framework of their use in foodstuffs for particular
nutritional uses (dietetic foods). At the time the directive was adopted, many
more substances were used by manufacturers in a number of member states,
especially in the UK. A general ‘grandfathering’ of all these substances was
not acceptable from a public health protection perspective. Member states
were, however, given the possibility to continue to allow in their territory the
use of the vitamins and minerals not listed in Annex I, or in forms not listed in Annex II. This was provided that the substance in question was used in one or more food supplements marketed in the Community on the date of entry into force of the Directive and the EFSA had not given an unfavourable opinion in respect of the use of that substance, or its use in that form, in the manufacture of food supplements. A dossier supporting the use of the substance in question had to be submitted to the EC by the member state not later than 12 July 2005. At that date the EC was confronted with some 700 dossiers of which about 400 have been submitted to EFSA for assessment.

Some public parties (representing mainly UK based manufacturers and distributors) deemed that the compilation of these dossiers proved a costly and time-consuming exercise, which was unnecessary and disproportionate in relation to the ultimate goal of the directive and took a challenge to the European Court of Justice (ECJ). The ECJ rejected their grounds and confirmed the validity of the directive, including the use of positive lists.19 Companies now have to rely on the results of the EFSA assessments and EC approval for the missing substances, which will become gradually available in the course of the coming years.

13.3.2 The setting of maximum levels
The FSD creates the framework that allows EU-wide maximum levels to be established for the use of vitamins and minerals. The determination of the criteria for the setting of such maximum levels was a difficult exercise in view of the diverging approaches between the member states. In some member states, such as the UK and the Netherlands, few regulatory maximum levels are established. Safety is therefore the primary basis for companies to develop food supplements. A number of national and international organisations have established safe upper levels for vitamins and minerals (UL) or have published guidance on how to establish them. These include the SCF and EFSA20, IADSA21 and WHO.22 These maximum levels consider total intake from the diet. However, intake of vitamins and minerals (and many other dietary compounds) can stem from three sources. Those that are naturally present in foodstuffs, those that are added to foodstuffs and those that are ingested through food supplements to supplement the diet. Levels of nutrients present naturally in the diet are more or less stable and can be quantified. Intake through fortified foodstuffs and food supplements is very variable and is dependent on the product’s content and the amount ingested by the individual consumer. The scientific reports establishing safe ULs cover all sources from the diet, so when developing food supplements, intake from other sources should be taken into consideration. The possibilities and factors to be taken into consideration in the setting of maximum levels are further discussed in Section 13.4.
13.4 Factors to be considered in the setting of maximum levels in food supplements and fortified foodstuffs

The FSD does not give a deadline for establishing minimum and maximum levels for vitamins and minerals in food supplements. The AVMOSR, however, imposes a deadline to the EC of two years following the entry into force of that regulation, so it is 19 January 2009. In order to start the work on this, the EC launched a public consultation in June 2006 by publishing a discussion paper on the establishment of maximum and minimum amounts of vitamins and minerals in foodstuffs. The factors influencing the setting of maximum levels are of a legal, scientific and political nature.

13.5 The legal basis as set out in the Food Supplements Directive and the Addition of Nutrients to Foods Regulation

The basis for the establishment of maximum levels of vitamins and minerals in food supplements and foodstuffs are laid down in the FSD and the AVMOSR. These principles are quite similar. For food supplements, Article 5 of the directive specifies that maximum amounts of vitamins and minerals shall be set, taking the following into account:

(i) ‘upper safe levels of vitamins and minerals established by scientific risk assessment based on generally accepted scientific data, taking into account, as appropriate, the varying degrees of sensitivity of different consumer groups’;
(ii) ‘intake of vitamins and minerals from other dietary sources.’

It also specifies that when the maximum levels are set, due account should also be taken of reference intakes of vitamins and minerals for the population. For fortified foods, Article 6 of the regulation specifies that maximum amounts of vitamins and minerals shall be set, taking into account:

(i) ‘upper safe levels of vitamins and minerals established by scientific risk assessment based on generally acceptable scientific data, taking into account, as appropriate, the varying degrees of sensitivity of different groups of consumers’;
(ii) ‘intakes of vitamins and minerals from other dietary sources.’

It also specifies that when the maximum amounts are set, due account shall also be taken of reference intakes of vitamins and minerals for the population. Furthermore, it specifies that when the maximum amounts are set for vitamins and minerals whose reference intakes for the population are close to the upper safe levels, the following shall also be taken into account, as necessary:

(i) ‘the contribution of individual products to the overall diet of the population in general or of subgroups of the population’;
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(ii) ‘the nutrient profile of the product established as provided for by the
nutrition and health claims regulation.’

In both cases, the European legislator has considered safety as the basis for establishing maximum levels. To that end, the EC has asked the EFSA to undertake a scientific risk assessment of all minerals and vitamins that can be used in food supplements and foods. These safe ULs are established by scientific risk assessment and the EFSA completed their work in 2006. The report containing the opinions on all vitamins and nutrients is available from the EFSA website.20

The European legislator, however, also included the requirement that due account should be taken of reference intakes of vitamins and minerals for the population. This is a clear compromise, made to consider also the views of those member states that have a nutritional based approach on their territory and do not want to expose their citizens to higher levels of vitamins and minerals, even if such products would not entail health or safety risks.

Because these two essentially conflicting requirements are put into the legislation, the maximum levels that will ultimately be established may require some sort of compromise based on the safe but high levels, currently found in the UK and The Netherlands, but also taking into account the current situation in more restrictive countries such as France and Denmark. Care should be taken, however, that such a decision is based on sound scientific arguments and that the maximum levels are not established on an arbitrary basis.

13.6 Scientific considerations in relation to the setting of maximum levels of vitamins and minerals in foods

The EC discussion paper included as annex, summaries of five models that have been developed by a number of member states and other organisations.5 These models describe possible risk management approaches for the establishment of maximum levels. Interestingly, only two of the five models fully consider food supplements as part of their methodology. Also noteworthy is that the models are in very close agreement on the classification of nutrients into safety classes. No risk of exceeding the UL by intake from all sources is considered for vitamin B₃, vitamin B₂, biotin, vitamin B₁₂, pantothenic acid, vitamin K and chromium. Low risk of exceeding the UL is associated with vitamin B₆, vitamin C, folic acid, vitamin D, vitamin E, nicotinamide, molybdenum, phosphorus, selenium and magnesium. Finally vitamin A, beta-carotene (for smokers), calcium, copper, fluoride, iodine, iron, manganese and zinc have a high risk for exceeding the UL.

The five models, however, show different approaches as regards the methodology to establish maximum amount of products.
13.6.1 French AFSSA model
The French Food Safety Agency (AFSSA) published their model in 2003.\textsuperscript{23} It covers specifications for the selection of a ‘nutrient-vector food pair’ and therefore only considers fortification. This model is a good example of a purely nutrition based system. It starts from the presumption that food fortification should serve only a nutritional purpose and lists a number of considerations that would need to be assessed to decide what nutrients can be added to what foods and in what amounts to serve public health. Such considerations include:

- exclusion of heavily consumed nutrients (based on 97.5th percentile);
- establishment of a nutritional benefit for the consumer (the objective of fortification should be nutritional benefit in relation to clinical deficiency or inadequate intake);
- identification of groups at risk of inadequate intake;
- and nutritional characteristics of the vector food (overall nutritional composition of the food and benefit/safety ratio of fortification).

Elements guiding the selection of the vector food are also included (ability to reach target population; priority for foods naturally containing the nutrient), as are elements to be validated in the case of fortification of foods not naturally containing the nutrient. Verification of the safety of the fortification, simulations based on consumption data, verification of the utility of the fortification, simulations based on consumption data and monitoring changes in the consumption of the fortified products are further elements to be taken into consideration. It is clear that such an approach, while valid for addressing mandatory fortification in relation to a structural deficiency in the population (e.g. iodine deficiency), would not be in line with the principles as laid down in the AVMOSR.

13.6.2 ILSI model
The International Life Sciences Institute (ILSI) model (2003)\textsuperscript{24} also deals only with food fortification, which considerably limits its applicability in relation to food supplements, but this model starts from a safety-based approach. The nutrients are classified in risk categories that are in conformity with the classification given in Section 4.2: Safe addition of more than one times the RDA for vitamin B\textsubscript{12}, vitamin C, vitamin E, riboflavin, pantothenic acid, niacin and thiamine; safe addition between 50 and 100\% of RDA for vitamin B\textsubscript{6}, vitamin D, folic acid, biotin, copper, iodine and selenium; safe addition between 10 and 40\% of RDA for iron, zinc, calcium, phosphorus and magnesium. Vitamin A is considered a specific case as high intake levels are close to the UL. The model then assumes that the intake of food by the human individual is self-limited by nature if energy intake is considered as a measure. It takes a worst-case scenario of 3600 kcal per day (95th percentile of energy intake in Europe), consisting of 36 portions of 100 kcal. Given that
50% of the total energy content of the diet is delivered by foodstuffs that are not eligible for fortification, and that it should be possible to fortify all fortifiable foodstuffs to the max level, the following equation is used for calculation of the acceptable level of fortification per 100 kcal (FAn):

\[ FAn = \frac{UL - CI95}{(0.5 \times 36 \times PFFn)} \]

where the UL is the tolerable upper safe level (in adults) as established by SCF or EFSA; CI95 is the 95th percentile of daily intake from non-fortified foods; and PFFn is the percentage of products on the market that will be fortified in relation to the total number of fortifiable foods, to be defined on a case-by-case basis. While the model estimates that of the 50% of foodstuffs that actually can be fortified, 50% might in practice be fortified, the model allows for the use of other assumptions. However, since intake from food supplements is considered to be negligible, the model is difficult to apply in reality. The model also does not consider age-specific issues but it indicates that because of the conservative estimations, it would be suitable also for children. Finally, calculation on 100 kcal basis is not applicable for low-calorie products. It is argued that since such products do not contribute to energy-intake, their consumption is not self-limiting and therefore only a low level of fortification would be appropriate, to be defined per portion.

13.6.3 Danish Institute for Food and Veterinary Research model
The model presented by the Danish Institute for Food and Veterinary Research (2005) is based largely on the ILSI model.\(^{25}\) It includes a similar risk characterisation of the nutrients, based on ULs set by SCF and EFSA. Where no UL has been set, specific guidance levels and temporary guidance levels are developed. The model bases itself on Danish intake data and the most sensitive population. The formula used for calculating the acceptable level of fortification per 100 kcal is:

\[ ALA = \frac{MA}{EI95 \times PFFn} \]

where \( MA = UL - (CI95 + SI) \) and UL stands for tolerable upper safe level (in adults) as established by SCF or EFSA; CI95 is the 95th percentile of daily intake from non-fortified foods; SI is the daily intake from food supplements; MA is the highest allowed intake from fortified foods. EI95 is the 95th percentile of daily energy intake from the daily diet (expressed in portions of 100 kcal); and PFFn is the percentage of products on the market that will be fortified in relation to the total number of fortifiable foods, set at 25%. The model deals with fortified foods only, but assumes a high intake of food supplements. The resulting maximum levels therefore are low.

13.6.4 German BfR model
The model proposed by the German Federal Institute for Risk Assessment (BfR) considers both food supplements and food fortification.\(^{26,27}\) The model
starts with a risk assessment, classifying nutrients according to their risk, quite similar to the other models. It then, however, does not apply this to the majority of nutrients, but takes a conservative worst-case multi-exposure approach. The amount the model proposes as available for safe addition to fortified foods and food supplements (R) is calculated using the following formula:

\[ R = UL - DINF \]

and \( R = \text{‘Gesamt. NEM’} + \text{‘Gesamt. ang. LM’} \), where UL stands for the tolerable upper safe level (in adults) as established by SCF or EFSA; DINF is the daily intake from normal non-fortified foods (95th or 97.5th percentile of intake); Gesamtzufuhrmenge NEM is the amount of intake from food supplements; and Gesamtzufuhrmenge ang. LM is the amount of intake from fortified foods.

The model assumes that that the sum of intake from food supplements and fortified foods should not exceed 100% of the amount available for safe addition to fortified foods and food supplements, i.e. a 50% split is proposed. Furthermore, it takes as its basis that an individual may consume two food supplements at maximum levels per day, so the maximum amount in food supplements is therefore to be divided by 2:

\[ TLNEM = \text{‘Gesamtzufuhrmenge NEM’}/2, \]

where TLNEM is the acceptable maximum level in a food supplement.

The same is used for fortified foods, i.e. consumption of two fortified foods at maximum level, divided by 2:

\[ TLang.LM/portion = \text{‘Gesamtzufuhrmenge ang. LM’}/2. \]

Because of its assumption of a theoretical worst-case scenario of an assumed intake of two maximum level supplements per day, a number of questions could be asked as to the applicability of the model for setting maximum levels for vitamins and minerals in food supplements and fortified foods. Such assumptions are not based on intake data, nor on consumption surveys. Furthermore, for most nutrients (except for vitamin B₆ and potassium) the model is not applied and a conservative RDA approach is selected, based on nutritional need. The reasons used for not applying the model are given and include those cases where:

- EFSA did not set a UL. For those nutrients, no qualitative risk assessment was performed and neither were UL established.
- There was a lack of intake data. No data available from other sources were used.
- The UL was judged to be higher than the assumed therapeutic dose for vitamin K.
- The UL, established by EFSA, was criticised by the BfR, as for vitamin E and iodine, for which BfR proposes their proper UL (but in the case of iodine the BfR UL is not even used in the model).
13.6.5 ERNA/EHPM model

The model proposed by the European Responsible Nutrition Alliance (ERNA) and the European Federation of Associations of Health Product Manufacturers (EHPM) is the last model that is included in the EU discussion paper.\(^28\) It covers both food supplements and food fortification. As with most of the other models, this model also performs a categorisation of the nutrients: a category where no evidence of risk for human health is demonstrable within ranges currently consumed (vitamin B\(_1\), vitamin B\(_2\), biotin, vitamin B\(_{12}\), pantothenic acid, vitamin K and chromium) and where consequently no reasons exist to establish maximum levels in supplements; a second category where a low risk exists of exceeding the UL (vitamin B\(_6\), vitamin C, vitamin D, vitamin E, nicotinamide, molybdenum, phosphorus and selenium). For these nutrients maximum levels should be established, taking into account changing dietary patterns in the population (it should be noted that for magnesium and folic acid, EFSA already established a maximum level for supplementation in their risk assessment exercise); and a third category where the potential risk of exceeding the UL exists (Vitamin A, beta-carotene (in the case of smokers), calcium, copper, fluoride, iodine, iron, manganese and zinc). In these cases, maximum levels would also need to take into account the reference intake value or RDA for that nutrient to address also the potential risk of deficiency in the population. This categorisation is performed using the UL as established by SCF and EFSA, and by the calculation of a population safety index (PSI). This PSI is a measure for assessing the space that exists between RDAs and the UL:

\[
\text{PSI} = \left( \frac{\text{UL} - (\text{MHI} + \text{IW})}{\text{RLV}} \right)
\]

where the UL is the UL as established by SCF and EFSA; MHI is the mean highest intake, i.e. the mean of the 97.5th percentile of intake of the nutrient from foodstuffs, including fortified foods (but excluding food supplements) from those member states where such data are available (Ireland, Italy, The Netherlands, United Kingdom); IW stands for the intake from water (relevant for minerals); and RLV are the reference labelling values as established by the SCF, which can be used as a measure for judging the level of adequacy of intake in the population (quite similar to the population reference intakes (PRI) or RDA).\(^29,30\) The values derived by the model for the nutrients are presented in Table 13.1. The best measure for estimating the relative contribution of fortified foods to the intake of nutrients from the diet is to look at the 97.5th percentile of intake in a country with no restrictions as to the kind, number and level of nutrients that can be added to foods. Intake figures from the UK show that the relative part of fortified foodstuffs in the daily diet is far below 50% of the RDA for those nutrients where data are available. Therefore a cut-off value of PSI = 1.5 was chosen by the authors of the model to decide if a nutrient has a low or a potential risk of exceeding the UL. For those nutrients where no UL was established by SCF and EFSA, a qualitative risk assessment was performed, looking at the reasons why no
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UL was developed and including an assessment of descriptive sources for the absence of adverse effects. For all but manganese and beta-carotene in smokers, no indications of adverse effects were observed at current levels of intake.

The model then proposed a calculation model for establishing maximum supplement levels (MSL) for the low risk category of nutrients that take into consideration potential future trends of intake. For vitamins:

\[
MSL = UL - (MHI \times 150\%) 
\]

and for minerals:

\[
MSL = UL - [(MHI \times 110\%) + IW] 
\]

The factors 150% and 110% are ‘safety’ factors, derived from the trend in intake that is observed between the consecutive dietary surveys of 1986/87 and 2000/01 (intake for adult men) in UK. From these data, an increase of intake of more than 20% was apparent only for vitamin B₆ and vitamin C. The model proposes a value of 50% to take this into consideration. Likewise, for minerals, where fortification is self-limiting for technological and taste reasons, a factor of 10% was used.

Although the model in this way proposes values only for maximum amounts of vitamins and minerals in food supplements, it considers the current practice

Table 13.1 Maximum Supplement Levels derived using the ERNA risk management model

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Proposed MSL</th>
<th>Nutrient</th>
<th>Proposed MSL</th>
<th>Nutrient</th>
<th>Proposed MSL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin B₁</td>
<td>–</td>
<td>Vitamin B₆</td>
<td>18/93 mg</td>
<td>Vitamin A</td>
<td>800–1000 µg</td>
</tr>
<tr>
<td>Vitamin B₂</td>
<td>–</td>
<td>Vitamin C</td>
<td>1750 mg</td>
<td>Beta-carotene</td>
<td>4.8–7 mg</td>
</tr>
<tr>
<td>Biotin</td>
<td>–</td>
<td>Vitamin D</td>
<td>35 µg</td>
<td>Calcium</td>
<td>1000–1500 mg</td>
</tr>
<tr>
<td>Vitamin B₁₂</td>
<td>–</td>
<td>Vitamin E</td>
<td>270/970 mg</td>
<td>Copper</td>
<td>1–2 mg</td>
</tr>
<tr>
<td>Pantothenic acid</td>
<td>–</td>
<td>Nicotinamide</td>
<td>820 mg</td>
<td>Fluoride</td>
<td>3.5 mg</td>
</tr>
<tr>
<td>Vitamin K</td>
<td>–</td>
<td>Molybdenum</td>
<td>350 µg</td>
<td>Iodine</td>
<td>150–200 µg</td>
</tr>
<tr>
<td>Chromium</td>
<td>–</td>
<td>Phosphorus</td>
<td>1250 mg</td>
<td>Iron</td>
<td>14–20 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Selenium</td>
<td>200 µg</td>
<td>Manganese</td>
<td>2 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Magnesium</td>
<td>250 mg</td>
<td>Zinc</td>
<td>10–15 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Folic acid</td>
<td>600 µg</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Classification based on those sources currently approved in Annex II of Directive 2002/46/EC and 2001/15/EC. Ranges reflect widely divergent ULs from international risk assessments. No UL set by SCF.
of intake of fortified foods in the most liberal member state of the EU where intake data shows that such current practice is safe.

### 13.6.6 UK EVM and Dutch RIVM models

Two other models that were available at the time the discussion document was published have not been included. These are the models proposed by the UK Food Standards Agency Expert Group on Vitamins and Minerals (EVM) and the Dutch National Institute for Public Health and the Environment (RIVM).

The EVM report was published in 2003.31 It contains advice on additional intake (both in relation to fortification and food supplements). For eight vitamins and minerals (vitamin B₆, beta-carotene, vitamin E, boron, copper, selenium, zinc and silicon) ULs were established; for the twenty-two other cases, guidance levels are indicated. Depending on the nature of the available evidence, these guidance levels are expressed as total intake, with an estimated safe margin for intake from both supplements and fortification within current high-level dietary intake or expressed as a supplemental intake with an estimated safe total intake. The model is a good example of a purely safety based system, using actual data from the National Diet and Nutrition Survey conducted with UK adults in 1986/87, which enables the consumer to have access to safe products.

Finally, the RIVM described a model covering both food supplements and fortified foods in 2005.32 It builds essentially upon the ILSI model, which it applies to the situation in The Netherlands. The acceptable level of addition of a nutrient to a food (per 100 kcal) is calculated using the following formula:

\[
\text{ALA} = \frac{(\text{UL} - (\text{CI95} + \text{SIhoog}))}{(\text{EI95}/100)} \times \text{PFFn}
\]

where UL is the tolerable upper safe level (in adults) as established by SCF or EFSA; CI95 is the 95th percentile of age-specific daily intake from non-fortified foods; SIhoog is the daily worst-case intake from food supplements in the absence of 95th percentile intake data; EI95 is the 95th percentile of daily energy intake from the daily diet (expressed in portions of 100 kcal); and PFFn is the percentage of products on the market that will be fortified in relation to the total number of fortifiable food. This value is set at 15% (estimation that only 30% of all foodstuffs are fortifiable and that 50% of these will actually be fortified in a worst-case scenario. Such estimate would even allow for growth of the market in the next 2–3 years).

The intake from food supplements cannot be derived from intake data available in The Netherlands, therefore a worst-case scenario precautionary approach is taken assuming high intake. The model concludes that vitamin A, vitamin D, folic acid, selenium, copper, zinc and iodine are considered the critical nutrients and that maximum levels should be based upon the most vulnerable population groups.
In summary, all the models come more or less to the same safety classification of the nutrients. But the approaches for setting maximum levels range from purely on nutritional need basis to purely safety based.

### 13.7 Legal considerations of setting maximum levels of vitamins and minerals in foods

Because of the existence of the many barriers to trade and the non-application of the principle of mutual recognition in this area, some cases have been taken to the European Court of Justice. The ECJ has treated five notable cases, which give insight into the principles for decision-making, in line with the principles of the European Treaty.

In Case 387/99, the European Commission brought charges against the Federal Republic of Germany for infringement of Article 30 of the treaty by automatically classifying as medicinal products vitamin and mineral preparations which are lawfully produced or marketed as food supplements in the other Member States where they contain three times more vitamins and minerals than the daily amount recommended by the Deutsche Gesellschaft für Ernährung.33

In Case 24/00, the European Commission brought charges against the French Republic for hindering the marketing in France of certain foodstuffs, such as food supplements and dietetic foods containing the substances L-tartrate and L-carnitine, and confectionery and drinks to which certain nutrients have been added, without establishing that the marketing of such foodstuffs entails a real risk for public health.34

In Case 150/00, the European Commission brought charges against the Republic of Austria for infringement of Article 28 of the Treaty by systematically considering vitamin or mineral preparations that are manufactured or marketed lawfully in other member states as medicinal products when they contain more vitamins, other than vitamins A, C, D or K, or minerals, other than chromates, at a level higher than one times the recommended daily allowance of these nutrients or when they contain Vitamins A, D or K, irrespective of their dose.35

In Case 192/01, the European Commission brought charges against the Kingdom of Denmark for infringement of Article 28 of the Treaty by applying an administrative practice which entails that enriched foodstuffs lawfully produced or marketed in other member states can be marketed in Denmark only if it is shown that such enrichment with nutrients meets a need in the Danish population.36

In Case 41/02, the European Commission brought charges against the Kingdom of The Netherlands for maintaining in force its legislation and for applying a derogation scheme under which, where applicable, account is not taken of the substitutability of certain fortified food products. This had the
result that food products lawfully prepared and marketed in another member state, fortified with vitamin A, vitamin D, folic acid, selenium, copper or zinc, which are not substitution products or reconstituted products within the meaning of the legislation may not be marketed in The Netherlands unless that addition does not pose a risk for public health and meets an actual nutritional need.\(^3\)

The ECJ upheld these five complaints. In all cases the ground of the infringement was the fact that the respective national authorities automatically (i.e. without a case-by-case assessment) prohibited import of specific products that were lawfully on the market in other member states unless specific authorisation procedures were followed (e.g. medicinal product authorisation when content of nutrients exceed simple multiples of the RDA). The two arguments to defend such practice – that there may be a risk of exceeding a safe level of intake and that the nutrient may be classified as medicinal by function – were rejected by the ECJ:

- Firstly, in conformity with many previous court cases, the ECJ states that the status of a product as a medicinal product can be ascertained only by the national competent authorities on a case-by-case basis, having regard to all of their characteristics, in particular their composition, their pharmacological properties (to the extent to which they can be established in the present state of scientific knowledge), the manner in which they are used, the extent of their distribution, their familiarity to consumers and the risks which their use may entail. Thus, the risk posed by a product is only one of the properties to be considered. Such risk must be established on a case-by-case basis and the level at which the nutrients would become dangerous would need to be assessed based on scientific data. In the case of vitamins and minerals, this has been done by the SCF and EFSA in the scientific risk assessment that led to the establishment of ULs. Furthermore, if measures taken by national authorities restrict or block the free movement of these goods, they can only do so on the basis of Article 36 of the Treaty. They therefore have to show in each case, in the light of national nutritional habits and in the light of the results of international scientific research, that the measures are necessary to give effective protection and, in particular, that the marketing of the products in question poses a real risk to public health.

- Secondly, the ECJ acknowledged that the absence of a safety risk does not preclude the fact that a product may still have an effect on the functioning of the body and be considered a medicinal product ‘by function’. Nevertheless, in order to classify a product as a medicinal product ‘by function’, national authorities need to ascertain that the product is indeed intended to restore, correct, or modify physiological functions, and thus have consequences for health. A systematic categorisation of nutrients based on multiples of the RDA may result in classifying certain products as medicinal, while such use would not
result in products that pose a health risk or restore, correct or modify physiological functions.

These ECJ court cases therefore laid down important boundaries for the establishment of maximum levels in foods and food supplements. Such levels cannot be set arbitrarily, based on nutritional need, since the level of safety needs to be the guiding principle and be determined on a case-by-case basis.

The ECJ has also investigated the competences of the member states in relation to the protection of health. It ruled that (in non-harmonised matters) it is for the member states to decide on the degree of protection of the health and life of humans they intend to ensure. However, such discretion of the member states is not absolute. It is only valid in so far as there are uncertainties in the present state of scientific research, the requirements of the free movement of goods within the Community is taken into consideration, and the measures imposed are proportionate. The means that they choose must therefore be confined to what is actually necessary to ensure the safeguarding of public health; they must be proportional to the objective thus pursued, which could not have been attained by measures which are less restrictive of intra-Community trade. In the cases detailed, the ECJ ruled that a general abstract approach for all vitamins, applying the strictest criterion, goes beyond what is necessary to achieve the objective of protection of health permissible under Community law and thus is not proportionate.

The FSD is harmonising the rules on food supplements within the EU. It thereby overrules the discretion of the member states to choose a higher degree of protection of health than that laid down within the FSD. This should therefore not lead to adopting the most restrictive approach within the member states, as it is obvious that in the light of the SCF and EFSA opinions and practice in the other member states that such an approach would not be based on scientific risk assessment, does not respect intra-community trade and would most probably be considered disproportionate. Furthermore, Article 12 of the FSD provides member states with the possibility to temporarily suspend or restrict the application of the provisions of the FSD on its territory, when it has detailed grounds for establishing that a product endangers human health even though it complies with the FSD, as a result of new information or of a reassessment of existing information made since FSD or the adoption of one of the implementing Community acts. In this light, a restrictive precautionary approach would clearly not be in line with the ECJ rulings.

13.8 Political considerations in relation to the EC discussion paper

In order to be guided on what policies to follow, the EC issued its discussion paper in mid-2006. In this paper, the EC put to public consultation nine questions and invited the stakeholders to provide comments on the options
given (see Section 13.9). The reason underlying these questions is the fact that the organisations that have addressed safe ULs for the addition of vitamins and minerals to foodstuffs and in food supplements arrive at such divergent positions. And this in turn is caused mainly by a number of scientific uncertainties that are replaced by political assumptions.

13.8.1 Establishment of a safe UL
A first uncertainty is the safe UL itself. The SCF and EFSA have not been able to establish ULs for a number of nutrients mainly because of lack of evidence of adverse effects. This leaves room for interpretation, discussion and arbitrary decisions. One of the most often heard arguments is that where a UL has not been set, maximum levels should be based on nutritional need, as if a value that is established for judging the lower end of population intake will suddenly be relevant also for judging the higher end! Furthermore, ULs are often considered as safety levels, which they clearly are not. They are values indicative for chronic safe daily consumption from all sources, judged to be unlikely to pose a risk of adverse health effects to humans over a lifetime. It is an estimate of the highest level of intake which carries no appreciable risk of adverse health effects. For a consumer or population group to occasionally exceed the UL would not give rise to adverse effects. The consequences would depend on the magnitude and the duration of the excessive intake.

13.8.2 Lack of intake data and uncertainty about the intake of vitamins and minerals from the diet
Lack of intake data and uncertainty about the intake of vitamins and minerals from the diet is a second factor that may lead decision makers to overcautious or arbitrary decisions. It is a fact of life that there are only limited data on the intake of vitamins and minerals from the diet. There are many factors accounting for this. Traditional dietary surveys focus on energy and macronutrients. The methodology used, such as the EPIC methodology, based on two 24-hour recall surveys and food frequency tables, are not sufficiently precise to address accurately the many sources of vitamins and minerals from the diet. Furthermore, calculation is strongly hampered by the lack of data on vitamin and mineral content in food composition tables and the substantial variability of vitamin and mineral content of fruit and vegetables depending on origin, soil composition, varieties, mode of preparation, etc. It is clear that the assessment of intake from fortified foodstuffs and food supplements is virtually impossible if no link can be established with the individual food product composition. Finally, the status of certain vitamins and minerals in the population may be unrelated to dietary intake but can better be estimated by using biological parameters of body fluids or other appropriate assessment methods. But in spite of these factors, data are available in some member
13.8.3 Estimation of multi-exposure

A major factor leading decision makers to over-cautious measures is the estimation of multi-exposure. This is basically the approach of the German BfR model. If safe ULs are applied to individual consumers, it is easy to assume that a limited number of products may suffice to cover the range of safe intake below the UL. In the case of a safe UL of, for instance, four times the RDA, and assuming that the consumer would get one RDA from normal foods, the rest could be covered by two portions of a foodstuff enriched to one times the RDA and two food supplements at one times the RDA. This would lead risk managers automatically to setting low maximum levels. However, such logic is based on a number of theoretical assumptions, e.g. that all consumers would consume every day two portions of such fortified foodstuffs and two food supplements; that all foods are fortified at the maximum levels and that all food supplements contain the maximum level; and that consumers are unable to read the instructions of use of these products. Reality shows that not all fortified foods are fortified up to the maximum level. In fact, most fortified foods are fortified to 15% of RDA, the lower legal limit for claiming that a vitamin or mineral has been added. The criteria for ‘source of’ and ‘high in’ in the NHCR will in most cases determine levels added in future. Furthermore, the models state that only a limited fraction (30–50%) of all foods are fortifiable, and that of these foods certainly not all are actually fortified. There are cost implications and technological restraints (e.g. taste, stability). Also, not all nutrients are used for food fortification and not all fortified foods are consumed daily. This is demonstrated by the fact that the contribution of fortified foods to the highest intake is rather limited (less than 50%). Also, for food supplements, there are many arguments that overrule a theoretical worst-case multi-exposure scenario approach. First of all, not all consumers take food supplements. In fact, the number of food supplement users is rather low (15–20%). Furthermore, not all food supplements are used daily or over long periods. Also, not all food supplements contain all nutrients at their maximum level. An important factor in relation to food supplements is that consumption of food supplements is a conscious act, which means that labelling is a valid risk management option. Food supplements may exist in many different doses, enabling the consumer to choose the product he deems most appropriate for his use, since they clearly indicate the dose and any precautions for their use. Interestingly, in Case 150/00, the ECJ ruled that the argument of the Austrian authorities that food supplement users often take higher doses than indicated on the label is not valid for imposing lower levels, as nearly all products are dangerous when consumed in excess, so that only normal usage should be considered when taking decisions.\(^{35}\)
13.8.4 The assumption that all consumers already consume minimum levels of vitamins and minerals from the diet

Some risk managers assume that all consumers already consume minimum levels of vitamins and minerals from the diet. It is clear from dietary surveys, however, that a substantial part of the population does not reach the RDA of a certain number of vitamins and minerals, especially in specific sub groups such as children, adolescents, pregnant women and elderly people. This is particularly the case with folic acid, vitamin D, calcium, iodine and iron. Furthermore, there are many indications of benefits of levels of vitamins and minerals exceeding the RDA. People wanting to supplement their diet with nutrients because they want to reach a status of optimal nutrition, should be able to do so.

13.8.5 Vulnerable population groups

Specific attention should be paid to vulnerable population groups. There is a case for taking into consideration intake in vulnerable groups, including children, when setting maximum levels for fortification. This is basically because such foods are purchased and used for the whole family, so no differentiation is made by its users. It is not clear how far such consideration should go since the absolute intake in terms of volume or energy levels of children is lower, which proportionately reduces intake of added nutrients also. In the case of food supplements, manufacturers already formulate their products in relation to the intended population. In food supplements destined for use by children, levels of nutrients are already adjusted to this age group. Supplements intended for adults are not consumed by children and vice versa. And if such risk would nevertheless exist, appropriate labelling could be imposed.

13.8.6 Consumer protection

Finally some risk managers argue that consumers must be protected against their own choice because they cannot judge what they need or what is beneficial for them. This stems primarily from the assumption that certain diets are in themselves balanced and sufficient to guarantee the health of the population. The southern Mediterranean diet is often given as an example of a healthy diet. Also the northern European Countries are quite focussed on the adequacy of their national diets. It is argued that fortification of foodstuffs may negatively influence the national dietary patterns and lead to changes in the intake of traditional foods. Such assumptions are seldom supported by intake data and consumption surveys but are nevertheless political factors that come into the discussion.
13.9 Conclusions on the setting of maximum levels in the future harmonised EU framework: The EC discussion paper on setting maximum and minimum amounts of vitamins and minerals in foods

From the situation as described so far and the questions posed in the EC discussion document, one can identify the key issues and options open for the future maximum levels of vitamins and minerals in food supplements (Questions 8 and 9 of the discussion paper refer to the setting of minimum levels and will therefore not be discussed in this chapter).5

**Question 1:** Where there are not yet scientifically established numerical tolerable upper intake levels for several nutrients, what should be the upper safe levels for those nutrients that should be taken into account in setting their maximum levels?

As illustrated previously, there are several potential reasons why EFSA has not established numerical ULs. In order to assess these reasons, a case-by-case analysis of the EFSA opinion can be carried out. If, from such analysis, it becomes clear that for most of the nutrients where no UL has been established this is because at current intakes from foods, fortified foods and food supplements, no evidence of adverse effects has been found, the only logical conclusion should be that there is no scientific basis to set a maximum level for fortification and food supplements.

In those cases where ULs were not established because of limited data and indications exist that adverse effects might be possible in case of excessive intake (e.g. beta-carotene and manganese), evidence from international risk assessments and ULs established by other organisations may be taken into consideration (e.g. Food and Nutrition Board, EVM, IADSA).21,31,38 A case-by-case qualitative risk assessment could be performed and a review mechanism put in place so that any maximum level could be re-evaluated and changed in the light of new evidence.

**Question 2:** For some vitamins and minerals the risk of adverse effects, even at high levels of intakes, appears to be extremely low or non-existent according to available data. Is there any reason to set maximum levels for these vitamins and minerals?

For such nutrients there appear to be neither a scientific basis nor any objective grounds for setting a maximum level for food fortification or for food supplements based on safety consideration. However, if the risk manager would judge that setting of maximum levels in food supplements and fortified foods to avoid unlimited additions would be an appropriate measure, such levels should be set sufficiently high to reflect current safe practice and avoid reformulation of products. One example could be the maximum levels established by the UK EVM group, which are the current standard in the UK.31
Question 3: Where we set maximum levels, do we inevitably also have to set maximum amounts for vitamins and minerals separately for food supplements and fortified foods in order to safeguard both a high level of public health protection and the legitimate expectations of the various food business operators? Are there alternatives?

Some risk managers would seem in favour of a worst-case scenario where each individual consumer ingests on a daily basis a number of food supplements and fortified foods which, when combined, reach the UL. However, it is not clear why another approach other than the population based approach as used in risk assessment for residues and contaminants would need to be applied. Furthermore, the use of food supplements and fortified foods is inherently different. The development of a ‘maximum total intake’ from which arbitrary proportions are split between fortified foods and food supplements assumes a number of theoretical presumptions that are hard to be maintained in reality. It must be considered that the vast majority of foods and food supplements will not contain vitamins and minerals at the maximum allowed levels. For many nutrients, particularly minerals used in food fortification, the levels used are self-limiting for technical and taste reasons. Furthermore, the amounts of nutrients to be added for making a ‘source’ and ‘high’ content claim under the Nutrition and EU’s Health Claims Regulation, namely 15% RDA and 30% RDA per 100 g/100 ml, respectively, represent another bench mark for addition of vitamins and minerals to foods.

In contrast, a scientific approach, based upon a categorisation of the nutrients in risk categories following a case-by-case assessment, would seem to be the best way forward. This will result in maximum levels that are based on actual intake data and reflect current practice in currently unrestricted markets.

Question 4: The EC would appreciate receiving available information on intakes of vitamins and minerals or indications of the best sources providing such data at EU level.

There are intake data available, although not always readily accessible because they are not published. Member states should bring these data into the discussion for application in the different models.

Question 5: If such existing data refer only to the intake in some member states, can they be used for the setting of legitimate and effective maximum levels of vitamins and minerals at European level? On the basis of what adjustments, if any?

The most representative data for applying a model would be the member state with the highest intake from all sources, for example the UK, because they reflect intakes of nutrients in a liberal market place, where both fortified foods and food supplements coexist.

Question 6: Should the intake from different population groups be taken into account in the setting of maximum levels of vitamins and minerals?
The setting of maximum levels will undoubtedly consider the most vulnerable population group. This is more relevant for fortified foods than for food supplements as the former are eaten by all consumers, including children, while the latter are not.

*Question 7: Taking into account all the above-mentioned considerations, how far should PRIs/RDAs be taken into account when setting maximum levels for vitamins and minerals?*

Although the setting of maximum levels should be based on scientific risk assessment, as is specified in the criteria of the legislation, some member states will undoubtedly refer to the use of arbitrary multiples or fractions of RDAs/PRIs or other nutritional considerations. This would not be appropriate both in the light of the criteria laid down in the legislation itself and of the ECJ court case rulings. The RDA, however, needs to be carefully considered when establishing maximum levels for those nutrients where the RDA is close to the UL and both risk of excessive intake and deficiency may exist in parts of the population.

The responses to the discussion paper are available on the EC web-site ([http://ec.europa.eu/food/food/labellingnutrition/supplements/resp_discus_paper_amount_vitamins.htm](http://ec.europa.eu/food/food/labellingnutrition/supplements/resp_discus_paper_amount_vitamins.htm)). On the basis of these responses the European Commission, in July 2007, issued an orientation paper to guide the Member States in further discussions that will lead to a formal proposal in the course of 2008.

### 13.10 References


16 European Communities: *Diet Integrators, A Discussion Paper*; III/3767/91; December 1991


19 European Court of Justice: Joint Cases C-154/04 and C-155/04: Judgment of the Court (Grand Chamber) of 12 July 2005. References for a preliminary ruling under Article 234 EC from the High Court of Justice England and Wales, Queen’s Bench Division (Administrative Court), made by decisions of 17 March 2004, received at the Court on 26 March 2004, in the proceedings The Queen, on the application of: Alliance for Natural Health (C-154/04), Nutri-Link Ltd v. Secretary of State for Health and The Queen, on the application of: National Association of Health Stores (C-155/04), Health Food Manufacturers Ltd v. Secretary of State for Health, National


30 Scientific Committee for Food (SCF): *Opinion of the Scientific Committee on Food on the revision of reference values for nutrition labelling (expressed on 5 March 2003)*; SCF/CS/NUT/GEN/18 Final, 6 March 2003; http://europa.eu.int/comm/food/fs/sc/scf/out171_en.pdf


14

Safety of vitamins and minerals added to foods: An overview of international expert opinions on micronutrient safety

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14.1 Introduction

It is nearly one and a half centuries since the first general pure food law in the English-speaking world was passed in the UK in 1860: An Act for Preventing the Adulteration of Articles of Food or Drink. This act initiated the first legislation and the scientific approach to food problems.

Since the passing of that Act there has been a growing momentum to ease barriers to trade and a continuing progression towards international agreement on pure food legislation leading, in 1955, to the proposal to form a Codex and, in 1958, to the formation of a permanent Council for the proposed Codex. Its purpose was to establish the basic principles required: to protect the health of the consumer, to guarantee the identity of the products, to govern the treatment and processing of goods and to prevent trading in products which are neither genuine nor marketable. It was anticipated by the sponsors that the Codex would be regarded as: a compendium of definitions and standards which, by common consent of the governments and the food industry, are to be recognized as binding on all the parties concerned. This Codex has become the Codex Alimentarius Commission of the Food and Agriculture Organization of the United Nations and the World Health Organization. Amongst its many specialist committees is the Codex Committee on Nutrition and Foods for Special Dietary Uses (CCNFSDU), which has met annually since its inception in May 1966 under the auspices of the host nation, Germany. This Committee includes food supplements prepared and sold for nutritional purposes.

Nutritional supplements containing vitamins, minerals and trace elements may be sold under food law and without medical supervision, provided that
no medical claims are made, and subject to the observance of national laws. Consequently, it has to be assumed that the supplements may be consumed on a daily basis for life, although from market data it could be concluded that this is rarely the case.

Arising from this situation are many scientific issues, including:

- Is consumption of micronutrients in amounts greater than the Recommended Daily Allowance (RDA) potentially harmful?
- Is consumption in amounts greater than the RDA potentially beneficial?

In either case, are there confounding factors; for example, complications arising from genetic susceptibilities and other causes for groups of a population to be at risk, product instability, problems of bioavailability, product interactions and so on.

- Can these questions be answered in part or in whole by scientific risk assessment? (A scientifically assessed risk must be managed by legislators and translated into regulation).
- Can this be done without interaction with the scientific risk assessment?
- Can this be done in a manner which provides confidence to manufacturers and consumers and which, in the process, remains fully consistent with the scientific basis of the risk assessment?

In the review of current and future developments in assessing the safety of micronutrients which follows, these questions are discussed in the context of the recently published scientific reviews on vitamin, mineral and trace element nutrition, with particular reference to the use of scientific risk assessment as a basis for managing and regulating the safe use of nutritional supplements, published by the Food and Nutrition Board of the USA, the Food Standards Agency of the United Kingdom and the former Scientific Committee for Food of the Commission of the European Union, whose responsibilities are now managed by the European Food Safety Authority.

14.2 Risk analysis of essential dietary micronutrients: Background to the issue of safety

Until almost the middle of the 19th century, the prime objective of nutritional standards was to prevent hunger and to sustain the health of the armed forces, the unemployed and manual workers. The evolution of standards developed in the UK from the 17th century through to the ‘Lusk’ energy standard and its development by the British Medical Association, culminating in the international standard of 12 nutrient allowances of the League of Nations in 1938 (energy, protein, fat, Ca, P, Fe, I, Vitamins A, B₁, B₂, C, D). This publication of the United Nations marked the end of the period in which the motivation was that of preventing hunger and opened the way for the
development of a new concept of using allowances to promote health by identifying and specifying key nutrients. The concept of the promotion of health was carried further in the USA when, in 1941, the National Research Council declared, in its first publication on nutrient allowances, that their objective was ‘To achieve buoyant health … and the building in the USA of dietary allowances to a level of health and vigour never before attained or dreamed of.’

With the realisation that micronutrients were not merely good for health, but were essential for the maintenance of life, came the idea that ever increasing consumption might lead towards ever increasing health: the attainment of vigour never before attained or dreamed of. As consequences of this realisation there followed the fortification of foods with micronutrients and the creation of the vitamin and mineral food supplement industry, providing the opportunity to consume large amounts of micronutrients in tablet and capsule form.

Inevitably the safety to the consumer of unlimited access to micronutrients was challenged, especially in Europe, where a tradition arose in some countries of establishing a dividing line between supplements considered to be foods and those regulated as medicines, by applying an arbitrary multiple of the RDA. This approach persisted into the 21st century in some countries, not only in Europe, but also in many other parts of the world. It was notably absent from regulatory practice in the USA and the UK, where this pattern of thinking was challenged on the grounds of the absence of any evidence of correlation between multiples of RDA and the safety of micronutrients.

In Europe, the European Federation of Associations of Health Product Manufacturers (EHPM) commissioned a review of the safety of vitamins and minerals provided in nutritional supplements for free sale by self-selection by customers. The published review established upper safe levels for daily supplementation for thirteen vitamins and β-carotene and for calcium, phosphorus and magnesium and eight trace elements. The basis of the recommendations was that: Any micronutrient may be consumed in an amount that is less than that for which an adverse effect has been confirmed either in peer reviewed scientific literature or in responsibly monitored practice. Where no adverse effect had been recorded, the upper level for free sale was described as a marker of safety.

Two following publications, from the EHPM and the Council for Responsible Nutrition (CRN) in the USA, reached broadly similar conclusions using the NOAEL (no observed adverse effect level) and LOAEL (lowest observed adverse effect level) approach of the US Environmental Protection Agency with the use of uncertainty factors where the published scientific data was judged to be inadequate.

These earlier studies were followed by three major studies on the safety of micronutrients and the establishment of recommended upper levels of consumption for adults on a daily basis for life. In each case the study took several years and was carried out by experts working under the auspices of official bodies. They were, in the USA, the Standing Committee on the
Scientific Evaluation of Dietary Reference Intakes of the Food and Nutrition Board of the Institute of Medicine (FNB)\(^3\); in the UK, the Expert Group on Vitamins and Minerals of the Food Standards Agency (EVM)\(^4\); and, in the EU, the Scientific Committee on Food (SCF)\(^5a,b\) continued by the Scientific Panel on Dietetic Products, Nutrition and Allergies of the European Food Safety Agency (EFSA). These three studies form the body of international expert opinion and it is on these that the following overview is based. Common to all three is the method of scientific risk assessment based on the establishment of NOAELs and LOAELs with the use of uncertainty factors. An outline of the procedure used in these studies follows.

### 14.3 Scientific risk assessment of nutrients

There are four steps in the risk assessment process:

(i) **Hazard Identification** The collection and evaluation of all scientifically published information, priority being given to that published in peer reviewed journals, relating to adverse effects.

(ii) **Dose Response Assessment** The relationship between incremental levels of consumption and thresholds for the onset of adverse effects of differing intensities and most particularly a threshold for an irreversible adverse effect. From this data the Upper Safe Level (UL) is determined.

(iii) **Exposure Assessment** An evaluation of total daily nutrient intakes in the population in which it is desirable to establish both the median intakes for particular nutrients as well as the 2.5 percentile and 97.5 percentile intakes.

(iv) **Risk Characterisation** A judgment formed from the three previous steps and from which some recommendations for caution may be made; for example, in relation to particular sections of a population who may have unusual sensitivities because of genetic, physiological or cultural reasons.

#### 14.3.1 Hierarchy of data

The heart of the assessment lies in the identification and characterisation of hazard. It is customary to organise both the search for data and their evaluation within an established hierarchy of preference. The hierarchy which is usually followed is:

- Human before animal
- Long-term clinical trials before short term-trials
- Trials with large numbers of participants before those with fewer participants
- Clinical trials that are double blind before trials without such control
- Data for both NOAEL and LOAEL before either alone
- Data for NOAEL before LOAEL
If the purpose is to establish ULs for nutritional supplements, supplementary use before total intake.

### 14.3.2 Uncertainty factors

Once the data have been selected, then it is necessary to assess its reliability as a predictor of the response of an entire population to consumption up to the level of the derived provisional UL and to consider whether it is appropriate to modify this by the application of an uncertainty factor (UF). The conventional factors are:

- NOAEL derived from LOAEL \( UF \leq 3 \)
- Animal data to human equivalent \( UF \leq 10 \)
- Clinical trials with few participants or of short duration \( UF \geq 1 \leq 10 \)

In all cases the selection of the primary data and the subsequent decision of whether or not to apply an uncertainty factor and, if so, of what size, is a matter of judgment and a cause of difference between the conclusions of experts and expert groups. In particular, such judgment must take into account the nature of the adverse effect: its nature and severity and whether or not it is reversible.

There are several recent reviews of the application of risk analysis to assess the safety of nutrients and other ingredients added to foods\(^{13,14,15}\) in addition to the accounts given in the three studies (FNB, EVM, SCF).

### 14.4 Overview of three major international studies

These are the studies already referred to and conducted by the FNB in the USA, the EVM of the Food Standards Agency in the UK and the SCF/EFSA of the European Union. Their assessments and recommended upper safe levels (ULs) are broadly in agreement and they are not disparate with those previously published by the EHPM in Europe and the CRN in the USA. Nevertheless there are differences, and five particular instances illustrate the problems that can arise in the scientific assessment of risk relating to nutrients which can be added to foods through fortification or directly as supplements. The five instances relate to the establishment of ULs for the following:

   No adverse effects had been reported in the scientific literature, and as a consequence due to the lack of data neither a LOAEL nor a NOAEL could be established. This has resulted in the reviewing bodies taking different approaches. The FNB and SCF concluded that no safe upper level (UL) could be established and no limit was set. However, the EVM based its conclusion on the highest recorded NOAEL. An uncertainty factor of 10 was applied to this because the trial quoted...
was for a small number of individuals and only for 3 months. The resulting limit was recommended as a level for guidance, but noting that higher daily intakes could be safe.

(ii) Vitamin B₆ – differences in the selection of peer reviewed publications. The FNB derived its UL from human data, applying an uncertainty factor of 2 resulting in a UL of 100 mg. The EVM and the SCF both derived their ULs from LOAELs obtained from animal data but applied different uncertainty factors to the LOAEL of 3000 mg. The EVM used the default values of 3 (LOAEL to NOAEL) × 10 (Animal to human) × 10 (Trial weaknesses), resulting in a UL of 10 mg, whilst the SCF also used default values of 3 and 10 for LOAEL to NOAEL and for animal to human, but chose 4 for human variation, giving a UL of 25 mg.

(iii) Folic acid – differences in the approach to the selection of a NOAEL or LOAEL for the derivation of the UL. All three studies concluded that an appropriate UL was 1000 µg, but this was obtained in each case by the use of different assumptions. The FNB selected a LOAEL and applied an uncertainty factor of 5 to derive a UL direct from the LOAEL. The EVM selected a NOAEL to which it applied an uncertainty factor of 1, whilst the SCF derived a NOAEL from a LOAEL and then applied an uncertainty factor of 1.

(iv) β-Carotene – differences in the interpretation of peer reviewed publications. The FNB concluded that β-carotene should not be used as a nutritional supplement because of published data indicating that severe adverse effects in smokers were associated with the use of nutritional supplements of synthetic β-carotene. Hence, based on this view, the UL was considered to be zero (0 mg) in the context of the addition of β-carotene to supplements and foods. The SCF concluded that the data was inadequate, set no UL and advised caution for any who chose to use β-carotene in a nutritional supplement. The EVM concluded that the human data were strong and that a LOAEL could be derived from them without the application of an uncertainty factor. The NOAEL was obtained from this LOAEL by use of the default value of 3 to give a UL of 7 mg.

(v) Vitamin A – publication of new data. The FNB recommended a UL of 3000 µg, noting that revision may be necessary depending on the nature of the ongoing research indicating a possible relation between vitamin A and increased risk of fracture in the bones of limbs. The SCF also recommended a UL of 3000 µg and advised, on the basis of the most recent research, that caution should be observed until further work had been completed. The EVM recommended a lower UL, of 1500 µg, because the most recent reports, published since the FNB and EVM had reached their conclusions, confirmed the fears that increased incidence of bone fracture was associated with daily intakes of vitamin A in excess of 1500 µg.
Given the nature of the data, these differences, apart from those relating to vitamin B₆, are not significantly different and the causes of the variation illustrate the critical role of scientific judgment in interpreting what is inevitably an incomplete database.

14.5 Current developments in the scientific risk assessment of micronutrients

The current focus is on the management of the scientifically assessed risk following the publication of the three major international studies and in parallel with continuing research into methods of assessing the safety of nutrients. A chosen ideal is for risk assessment and risk management to be carried out by different groups of individuals in isolation from each other. This ideal emphasises the difference between science which, in concept, leads to conclusions based on fact, and management, in which conclusions may also be influenced by judgment. In practice, the difference is rarely so sharp and clear cut. There are many interventions of judgment in the scientific risk assessment of nutrients. The analysis of the causes of difference in conclusion between the three international studies illustrates the complex interaction between recorded facts and a scientist’s judgment of their relevance to the task in hand. For the risk manager, there is a significant risk of reaching an incorrect conclusion if there is a lack of awareness of the judgments and the reasons for them that have already been made in the scientific risk assessment. The resolution of this dichotomy is key to the current major activity: to derive Upper Safe Levels (ULs) for the inclusion of vitamins, minerals and trace elements in food, either as fortifying agents, or directly as nutritional supplements. Furthermore, these two activities are not mutually exclusive but potentially additive to a variety of national diets.

The problems underlying this dichotomy are discussed in the following sections where the issues are common to managers in both industry and government, although most attention is currently focused on risk managers in government.

14.5.1 Adverse effects

A key part of scientific risk assessment is that of adverse effects. These are used to determine both the NOAELs and the LOAELs used to establish safe levels. It is essential that both risk assessors and risk managers understand the nature of adverse effects, their severity and their reversibility. It is also essential to have an understanding of the uncertainties and limitations associated with arriving at a LOAEL and a NOAEL for a micronutrient. In particular, to understand the reduced certainty of either value when it is deduced from animal data and applied to the human condition.
A reality check must be applied at each stage of the assessments and, in particular, an awareness of the current experiences of consumers is important. For example, if the recommended upper safe level is less than the recommended dietary intake, it could expose some of the population to the possibility of a deficiency disease.

14.5.2 The significance of groups at risk
An assessment of the implications for a population group who may be at risk needs to be undertaken in the context of the following questions:

- Are the numbers so large that further caution – the precautionary principle – should be applied?
- Are the numbers so small that consumer protection can be provided from information, including labelling, without further modification of the risk assessment?

14.5.3 Understanding the reasons for differences of conclusion between different scientific risk assessments of the same micronutrient
As already discussed in Section 14.4, risk assessment groups can appear to come to different conclusions in relation to safe upper levels. It is important that the reasons for the differences are fully understood. There is a need to relate the value of the uncertainty factor chosen to what is known about the variation in daily use in the community, and also to recognise whether the methodology is sufficiently sensitive for the reported differences to be real or whether they are within the error of the technique.

14.5.4 Calculating exposure
In calculating exposure, it is necessary to take account of the cumulative effect of supplementation and fortification together with the diet. For example, a diet rich in liver is unlikely to be able to tolerate, without risk, both supplementary vitamin A and foods fortified with vitamin A such as edible fats. The majority of foods currently fortified contain added micronutrients to restore processing losses, but the more recent functional foods may contain relatively large amounts of added micronutrients, added for perceived health benefits.

14.6 Overall guidelines for risk management
The following general principles should be applied to a risk management exercise:
The risk assessment applied must be that which is most relevant to the circumstances of the application.

A conclusion which would significantly reduce consumer choice should not be accepted without strong evidence of consumer risk.

Conclusions must be reviewed as new evidence comes to hand and modified when that evidence significantly extends or changes the perspective of previous data or competently refutes an earlier interpretation of the data then available.

There needs to be an awareness that it is not always possible to eliminate judgment from scientific conclusions and hence these may already be in part, or even in whole, risk management decisions.

14.7 Future trends and developments

Scientific risk assessments and risk management of micronutrients are relatively new procedures and are derived from safety assessments of food additives. Questions which still need to be answered are:

14.7.1 Risk assessment

How do we assess the risk of a nutrient for which no adverse effect has been reported?

An approach to this problem has recently been published by a working group of FAO/WHO\textsuperscript{12} within an analysis of the three international studies (FNB, EVM, SCF) on risk assessment of vitamins and minerals as nutritional supplements and in food fortification. In such cases, the Working Group considered that the biological threshold for an adverse effect, if one existed, could be many times higher than the highest intake studied. The Group’s proposed solution was to determine the Highest Observed Intake (HOI) defined as follows:

\textit{The highest observed intake (HOI) is derived only when no adverse health effects have been identified. It is the highest level of intake observed or administered as reported within (a) study(ies) of acceptable quality.}

How do we establish, by scientific risk assessment, safe upper levels of consumption for other bioactive nutrients and micronutrients?

This issue is being studied by the Scientific Group of The International Alliance of Dietetic and Supplement Associations (IADSA).\textsuperscript{13} Here the risk analysis has to be assessed from a limited database where the data is insufficient to derive either a NOAEL or LOAEL, but is sufficient to give confidence in a level of consumption but without the knowledge of either an adverse effect or, where one is recorded, of the threshold for the effect. The term proposed for risk assessment carried out under these circumstances and by the rigorous criteria for admission of data for evaluation is Observed Safe Level (OSL).
The numbers of bioactive nutrients currently being investigated by the international food industry is growing and continually increasing. Each new category can be expected to present new challenges for the identification of potential causes of adverse effect and the consequent task of appropriate risk assessment.

**How do we assess the consumption of a specific nutrient by a population in such a way that it is applicable between communities nationally and internationally?**

A critical component of the risk assessment procedure is the establishment of exposure – that is, the various origins of the nutrient under consideration and the routes of its transmission to the consumer (food, drinks, fortified foods and drinks, nutritional supplements, water, environment, for example) from particular manufacturing processes. In all cases the exposure is a product of the concentration of the nutrient in the foods, drinks and environment under study and the length of time of the consumer’s exposure. For foods and drink this necessitates an accurate and complete account of total consumption and so far none of the methods in use is without significant limitations, not least being the unknown level of reliability of the records submitted by consumers, with the exception of those residential in laboratories.

There are two dominant requirements: (i) internationally agreed and consistent procedures for the compilation of databases and (ii) the assessments of consumption of each food and hence each nutrient within the base. Within the European Union, the European Food Safety Authority (EFSA) has initiated work in this field to establish a European database as an ideal, but has recognised that the differences in eating habits between north and south Europe may be so large that a single database may not be achievable. The issues and the planned stages to develop an *EU Food Consumption Database* were discussed in an EFSA Colloquium in 2005.\(^{14}\)

**How do we identify metabolic markers of adverse effects together with their derivatives, and the physiological, biochemical and pharmacological characteristics of both markers and derivatives?**

There is little detailed information in this area. Whilst there are some data on some vitamins and trace elements, there are little on the many bioactive nutrients being considered, or already in use, as fortifiers of selected foods or as nutritional supplements. These issues have been discussed at successive workshops and in research publications in recent years, but adequate research funding for progress to continue has not yet been allocated from government, research councils or industry. Consequently this remains a trend for development that is very much in the future.
14.7.2 Risk management

How do we translate the risk assessments of expert groups into workable safe upper levels for regulatory purposes?

There has been significant development in the sources of micronutrients in diets since the conclusions of these groups have been published. The use of fortification to improve the health of populations has been extended, one example being the fortification of flour with folic acid. Another route for introducing micronutrients to the diet is the development of functional foods that are rich in particular micronutrients associated with the improvement of health. For these reasons risk managers have to consider how to manage multiple dietary sources of micronutrients so as to optimise their potential for improving health whilst ensuring that there is virtually no risk. Consumer choice consistent with consumer safety is the challenge and two approaches have been made: Either establish upper safe levels separately for supplementation and fortification combined with an upper limit for each application, or consider the totality of the diet and, within this, all potential sources of micronutrients.

Following the first approach, the International Life Science Institute, Europe (ILSI), published a model in 2003 relating the maximum amount of micronutrient for fortification to the energy value of the food to be fortified. Modifications of this were published later from research groups in Germany and Denmark, and the most recent modification of the Danish research group has been adopted as a basis for regulation of fortification within Denmark.

A research group in the Netherlands has recently proposed a new model for determining upper safe levels for fortification based on the amount of micronutrient per 100 kcal of food (MSFL) defined by the following equation:

\[ MSFL = \frac{[UL - (CI_{95} + SI)]}{EI_{95}/100} \times PFF_n \]

where UL is tolerable upper intake level, CI_{95} is 95th percentile of habitual intake, SI is realistic high intake from dietary supplements, EI_{95} is 95th percentile of habitual energy intake, and PFF_n is proportion of total energy intake that comes from fortified foods. They propose the use of this approach in the Netherlands, where there is a comprehensive population nutritional database.

In 2004, the scientific advisors to the European Responsible Nutrition Alliance (ERNA) and the European Federation of Associations of Health Product Manufacturers (EHPM) published a model for the risk management of micronutrients from all sources: food and water, nutritional supplements, fortified foods and functional food. The model establishes a Population Safe Index (PSI) in which account is taken of the RDA/PRI by use of the Reference Labelling Value (RLV), the Mean Highest Intake from all sources (MHI) and the intake from water (IW) which is significant for many trace elements. The relationship is defined by the equation below in which UL is the Upper Safe Level of daily intake from all sources:
\[ \text{PSI} = \text{UL} - \frac{\text{MHI + IW}}{\text{RLV}} \]

In this model, the MHI was calculated from 97.5th percentile consumption data of the male populations of Ireland, Italy, the Netherlands and the United Kingdom, because this was the group with the generally highest intake and hence it provided a further element of caution in an approach that intended to provide a reasonable picture of the varied patterns of consumption within Europe rather than a complete reflection of dietary patterns which, at this time, is no more than a long term goal.

In 2007, the European Commission published an Orientation Paper\(^{20}\) in which it noted that the ERNA/EHPM model had received ‘more support’ (than others) and extended the consequent categorisation of the vitamins and minerals from the two groups proposed – low risk of excessive intake and risk of excessive intake to a third category, no setting required. The three groups so defined are:

<table>
<thead>
<tr>
<th>No setting of MSL required</th>
<th>MSL taking account of changing dietary patterns</th>
<th>MSL taking account of deficiency/excessive intake</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin B(_1)</td>
<td>Vitamin B(_6)</td>
<td>Vitamin A</td>
</tr>
<tr>
<td>Vitamin B(_2)</td>
<td>Vitamin C</td>
<td>(\beta)-Carotene</td>
</tr>
<tr>
<td>Vitamin B(_{12})</td>
<td>Vitamin D</td>
<td>Calcium</td>
</tr>
<tr>
<td>Biotin</td>
<td>Vitamin E</td>
<td>Copper</td>
</tr>
<tr>
<td>Pantothenic acid</td>
<td>Nicotinamide</td>
<td>Fluoride</td>
</tr>
<tr>
<td>Vitamin K</td>
<td>Molybdenum</td>
<td>Iodine</td>
</tr>
<tr>
<td>Chromium</td>
<td>Phosphorus</td>
<td>Iron</td>
</tr>
<tr>
<td></td>
<td>Selenium</td>
<td>Manganese</td>
</tr>
<tr>
<td></td>
<td>Magnesium</td>
<td>Zinc</td>
</tr>
<tr>
<td></td>
<td>Folic acid</td>
<td></td>
</tr>
</tbody>
</table>

This orientation paper is currently (2007) under discussion within the EU, initially at a meeting of its ‘Expert group on food supplements and on the addition of vitamins and minerals and of certain other substances to foods’.

**How do the regulatory conclusions for a specific country or group of countries relate to regulations of wider applicability between countries within a region of different habits of food consumption, in order to facilitate regional free trade and still more broadly to facilitate word-wide free trade?**

A consultative electronic workshop\(^{21}\) is in progress under the auspices of the Codex Alimentarius\(^{7}\) to develop risk analysis principles and possibly guidelines for application to the work of the Codex committee on nutrition and foods for special dietary uses. These terms of reference for the workshop of 2006 demonstrate the interaction between assessment and management.

For the work of Codex, providing a reference for standards for the World...
Trade Organisation (WTO), the emphasis is on the long term goal of deriving workable standards suitable for international trading without significant risk and with significant benefit.

*How do we interpret risk assessments for specific and short term uses when those assessments have been made on the assumption of lifetime use?*

The suggestion that scientifically assessed risk could be managed differently when the intended use was for a short period compared with the conventional assumed adult lifespan was made in a publication of the EHPM in 1997 and was suggested as a possibility for vitamin B₆ in the EVM Report of 2003, with further discussion during the IADSA conference in Prague in the following year. There had also been a brief publication during this period, outlining a possible approach to modifying a risk analysis for life to one for a specified short-term use. A resolution of this problem is becoming more urgent because of the increased use of supplements for training by sportsmen and women. Their use is for a relatively short part of their life and not always continuously within the years of active training.

### 14.8 An optimistic summary of the future trend

- A comprehensive international network of awareness of the biology of nutritional advances relating to opportunities to improve health.
- Translation of the conclusions of the international network into a scientifically based understanding from which the safety of products could be regulated to ensure global freedom of production, access and consumption, within the framework of the World Trade Organisation (WTO).

### 14.9 References

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