6
Biomarkers of Intake

Gunter G C Kuhnle

University of Reading

Key messages

- Nutritional biomarkers offer an alternative assessment method for dietary intake.
- Nutritional biomarkers can be measured objectively and can be used independently – or in combination with other assessment methods – to estimate intake.
- Recovery biomarkers – that is, urinary nitrogen, sodium and potassium – can be used to determine dietary intake directly, while most other biomarkers only allow ranking of intake.
- Biomarkers require careful planning of specimen collection and analytical method to avoid the introduction of additional bias.

6.1 Introduction: Biochemical markers of intake

Biomarkers – short for biochemical markers – are commonly used as surrogate markers for an event that cannot be observed directly. These events are mainly clinical endpoints, for example disease progression or mortality, or exposure, for example diet.

A nutritional biomarker, or biochemical marker of intake, is an indicator of nutritional status that can be measured in any biological specimen. It is not restricted to a specific compound or groups of compounds, and can be interpreted broadly as a physiological consequence of dietary intake. These markers can be used to assess different aspects of nutrition, for example intake – or status – of micronutrients, specific foods or dietary patterns. In nutritional epidemiology, these biomarkers are commonly used as reference measurements to assess the validity and accuracy of other dietary assessment instruments (Prentice *et al*. 2009; Kuhnle 2012).

An ideal nutritional biomarker should reflect dietary intake – or status – accurately and should be specific, sensitive and applicable to most populations. The biomarker should allow the objective and unbiased assessment of intake, independent of all biases and errors associated with individuals and other dietary assessment methods. However, such an ideal biomarker does not exist, and all biomarkers available have some limitations. Nevertheless, they provide useful information and are commonly used in nutritional epidemiology and other research areas where dietary assessment is important. The three main applications for biomarkers in nutritional sciences are as a measure of nutritional status; as a surrogate marker of dietary intake; and to validate other dietary assessment instruments. These different applications will be discussed in the next section.

6.2 Types of biomarkers and their application

Biomarkers are commonly divided into categories depending on their relationship with intake (Jenab *et al*. 2009). *Recovery biomarkers*, based on the total excretion of the marker over a defined period of time, have a well-known relationship with intake and this relationship is consistent between individuals, with low inter-individual variability. *Predictive biomarkers*, a category introduced 2005 (Tasevska *et al*. 2011), also have a consistent, well-known relationship with intake, but an incomplete and low recovery. In *concentration biomarkers*, this relationship

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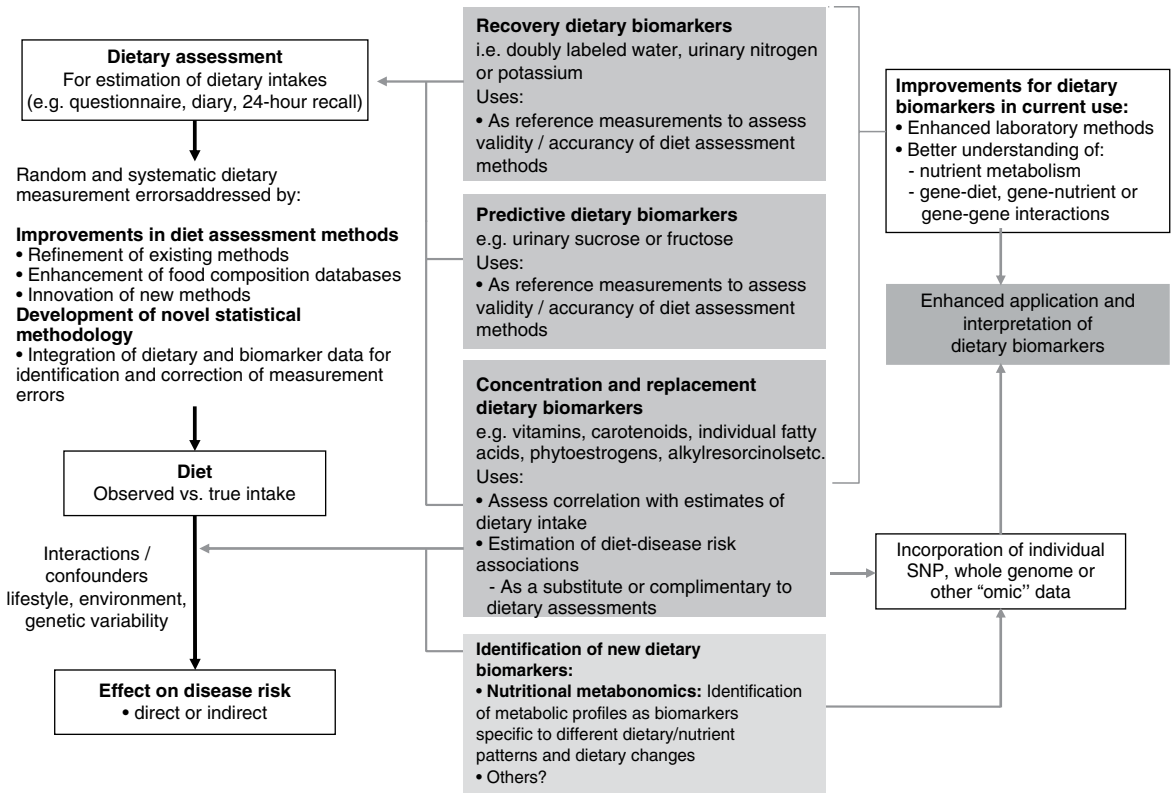

Figure 6.1 Overview of different types of biomarkers of intake, their main properties and application to investigate associations between intake and diseases risk. Jenab *et al.* (2009) Biomarkers in nutritional epidemiology, *Human Genetics,* **125** (5–6), 507. With kind permission from Springer Science and Business Media.

is less well known and more variable, with very high inter-individual variability. A summary of different types of biomarkers is shown in Figure 6.1.

Recovery biomarkers

Recovery biomarkers are the most important category of biomarker available, because they can provide an estimate of absolute intake levels. This type of marker requires a metabolic balance between intake and excretion over a defined period of time and a precise, quantitative knowledge of the physiological relationship. As suggested by the name, recovery markers are based on compounds that can be recovered completely – or almost completely – following consumption, mainly in 24-hour urine samples. For these biomarkers, the inter-individual variability in the excretion of the marker is negligible: for example, the excretion of urinary nitrogen in 24-hour urine is approximately equal to 80% of nitrogen intake in the same time period in any individual in energy and protein balance. A limitation of these biomarkers is that they are only suitable for individuals who are in a steady state; that is, individuals who do not increase or decrease in body mass as do young or old people or pregnant women. These biomarkers are also sensitive to a number of diseases that affect their excretion, in particular kidney diseases. Currently, only a few recovery biomarkers of dietary intake are available, for example urinary nitrogen, potassium and sodium.

Concentration biomarkers

In contrast to recovery markers, which rely on the total excretion of a specific compound over a defined period of time, concentration biomarkers are only based on the biomarker concentration in the respective specimen. While recovery biomarkers can normally only be measured in 24-hour urine samples, concentration biomarkers can be measured in almost all specimens available. These biomarkers do not have a consistent relationship between intake and excretion, and therefore high inter-individual variability; they also do not have a time dimension. For

this reason, concentration biomarkers cannot easily be translated into absolute dietary intake, but are only utilised to compare different levels of intake; additional information is usually required to provide a reference.

Concentration biomarkers are often used to investigate associations between diet and disease risk, as these markers can lead to a better ranking of intake than other assessment instruments that rely on self-reporting. In contrast to dietary data, biomarker concentration determined in blood or urine takes into account bioavailability, metabolism, nutrient–nutrient interaction and excretion, and therefore might provide better information on the bioavailable nutrient than dietary data.

These biomarkers are the most common type of biochemical marker currently available, as they can be measured in a wide range of specimens and do not require the collection of 24-hour urine samples. Many micronutrients can be used as concentration markers of their own intake (see later in this chapter). Other concentration markers include urinary phytoestrogens (isoflavones and lignans) and alkylresorcinols (for wholegrain).

Predictive biomarkers

Predictive biomarkers are the latest category of biomarkers that have been developed. They have an incomplete recovery but a stable and time-dependent correlation with intake. While they allow an estimation of absolute intake, they are not as reliable as recovery markers. Currently, urinary sucrose is the only predictive biomarker available.

Functional markers

Functional markers are an alternative type of biomarker. In contrast to most biomarkers described here, functional markers measure the physiological effect of specific foods as a surrogate marker of intake. A commonly used functional marker is EGRAC (erythrocyte glutathione reductase assay coefficient; Dror, Stern and Komarnitsky 1994) for Vitamin B2 status (see later in this chapter), but other markers are also available.

6.3 Specific biomarkers

Macronutrient and energy intake

There is only one recovery biomarker currently available to assess the intake of the primary three macronutrients, urinary nitrogen for protein intake. While the intake of fat can be determined to some extent by the analysis of fatty acids (see later in this chapter), there is currently no biomarker for the intake of total carbohydrates except for sucrose.

Urinary nitrogen as a biomarker of protein intake

The assessment of protein intake by total urinary nitrogen is based on the assumption that subjects are in nitrogen balance and that there is neither accumulation nor loss due to growth, starvation, diet or injury. The application of urinary nitrogen was described in 1924 by Denis and Borgstrom in a study to investigate the temperature dependence of protein intake in medical students. Since then, this biomarker has been investigated further and it is now commonly used (Bingham 2003).

Several validation studies have been conducted to investigate the association between intake and excretion, and urinary nitrogen is probably one of the best-validated biomarkers available. When taking into account nitrogen losses via faeces and skin, there is an almost complete agreement between long-term intake and urinary nitrogen (as shown by a correlation coefficient of 0.99 for a 28-day diet). The biomarker underestimates intake at higher levels of protein intake and overestimates it at lower levels, but when taking into account the factors described above, urinary nitrogen excretion is on average around 80% of dietary intake, and this ratio can be used for its determination.

Daily individual variations require the collection of urine samples on several days, as an individual is unlikely to be in nitrogen balance on any one day. When using only the sample of a single day, a correlation between intake and biomarker of approximately 0.5 can be expected, with a coefficient of variation of 24%. However, this improves to a correlation coefficient of 0.95 and a coefficient of variation of 5% when using 8 days of urine collection and 18 days of dietary observation.

A key limitation of 24-hour urinary nitrogen is the collection of 24-hour urine samples, which is often not feasible in studies. Urinary nitrogen in partial 24-hour urine collections, and even spot urine samples, has been used, but the results depend on the timing of diet and meal consumption.

The standard method for urinary nitrogen is the Kjeldahl method, developed by the Danish chemist Johan Kjeldahl in 1883. In this method, all nitrogen present in the sample is converted into ammonium sulphate and then analysed; it therefore determines not only protein nitrogen, but all nitrogen present in the sample. The method is very robust and reproducible, and can be automated for the analysis of larger numbers of samples.

An important disadvantage of this method is that it can only provide information on total protein intake, not intake of specific amino acids or the source of proteins (such as plant or animal protein). Additional markers,

such as stable isotope ratios, are required to obtain more information on protein sources, although there is still a paucity of validated markers.

Fatty acids as a biomarker of fat intake

While nitrogen intake can be measured with a single biomarker, urinary nitrogen, there is no such biomarker for total fat intake. Individual fatty acids can be measured in a variety of different specimens, and the fatty acid composition can be used to make inferences regarding dietary fat intake.

Fatty acids are mainly present as triacylglycerol, phospholipids and cholesterol esters, and they are found in membranes, adipose tissue and also plasma (as free fatty acids). Their distribution –among both different molecules and tissues – depends largely on the type of fatty acid, and it is mainly the fatty acid profile that is used to make inferences on intake. As fatty acids undergo extensive metabolism, it is important to take this into account when interpreting different biomarkers. While many fatty acids – in particular saturated fatty acids (SFA) – can be synthesised endogenously, this is rare in people consuming more than 25% of their energy as fat and thus storage in adipose tissue tends to reflect dietary consumption. Essential polyunsaturated fatty acids (PUFA), such as members of the ω – 6 (linoleic acid) and ω – 3 (α-linoleic acid) family, cannot be synthesised *de novo* by humans, and therefore they can also be used as a marker of intake. However, the majority of fatty acids in human tissues are non-essential and can be either endogenously produced or supplied via the diet. The transport of fatty acids into adipose tissue is presumed to be non-selective, and therefore the relative distribution of fatty acids there is often considered to be the strongest biomarker of long-term intake. However, non-selective transport cannot be assumed for all tissues, and this has to be taken into account when interpreting data.

Fatty acids in adipose tissue and blood

Adipose tissue has a half-life of approximately 1–2 years, therefore the fatty acid composition in adipose tissue reflects intake within this period of time (Hodson, Skeaff and Fielding 2008). While this is advantageous when assessing diet, it makes the validation of these biomarkers more complicated, as assessing diet over such a long time frame is difficult. In contrast, the half-life of erythrocytes is approximately 60 days and therefore erythrocyte membrane fatty acids are more suitable to assess medium-term diet. Studies have shown significant correlations between the relative intake of PUFA and PUFA content in adipose tissue, erythrocytes and plasma, in particular n-3 and/or n-6 fatty acids. However, the chain length can affect the association between intake and concentration, for example the plasma concentration of ALNA (alpha-linoleic acid, C18:3 n-3) does not reflect intake, while EPA (C20:5 n-3) and DHA (C22:6 n-3) do. Strong correlations between intake and biomarker in adipose tissue or blood have also been observed for other types of fatty acids, such as *trans* fatty acids, SFA and monounsaturated fatty acids (MUFA), although the observed correlation coefficients vary widely between less than 0.1 and more than 0.7 (Hodson, Skeaff and Fielding 2008). Concentration of pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0) in adipose tissue correlates well with dairy intake, with correlation coefficients of approximately 0.3.

Fatty acid analysis

The analytical methods for lipids depend to some extend on the specimen used. Thin layer chromatography (TLC) or silica cartridges are commonly used to separate different lipid fractions; alternatively, samples are extracted using a chloroform:methanol mixture (1:1) and purified by solid-phase extraction (SPE). Fatty acids are then normally converted into their methyl-ester (FAME, fatty acid methyl ester) by transesterification and analysed using gas chromatography (GC) or high-performance liquid chromatography (HPLC). While mass spectrometric detection facilitates the identification of individual fatty acids, flame-ionisation detectors (FID) are most commonly used and fatty acids are identified by their relative retention time. This is sufficient for most applications; however, it is often difficult to separate *cis*- and *trans*-isomers of fatty acids and to identify compounds with low abundance.

The results of fatty acid analyses are usually given as a fatty acid profile with the relative contribution of each fatty acid, either as mol% (mol fatty acid per mol total fatty acids) or weight%. The former, mol%, are biologically more meaningful, in particular when molecular ratios of fatty acids or long-chain fatty acids are being considered (Hodson, Skeaff and Fielding 2008). Alternatively, the absolute concentration of individual fatty acids can be given, and this data can also be used as a fatty acid profile. However, the absolute concentration cannot be used as a marker of total dietary fat intake.

Recent developments in mass spectrometry have resulted in novel methods for the analysis of fatty acids that do not require laborious sample preparation. In *lipidomics*, samples are usually separated by HPLC and identified by their fragmentation spectrum using tandem mass spectrometry (MS). While this method can provide detailed information about lipids present in the sample, there are several disadvantages, for example differential loss of lipids and lipid–lipid interactions that can affect ionisation efficiency. An alternative method

is shotgun lipidomics, where samples are analysed without prior separation and lipids are identified using tandem MS. This approach has several advantages, for example no differential loss of compounds during liquid chromatography (LC) separation and – when using nano-electrospray – virtually unlimited analysis time, even with small amounts of sample. However, lipidomics relies extensively on bioinformatics for the identification of lipids and interpretation of results, as the generated data can be very complex (Griffiths and Wang 2009; Han, Yang and Gross 2012).

Urinary sugars

There is currently no biomarker for total carbohydrate intake, partially due to the complex nature of these nutrients and their extensive metabolism. However, a *predictive* biomarker exists for total sugar intake – the sum of urinary sucrose and fructose. This biomarker has been validated in several dietary intervention studies as well as the Observing Protein and Energy Nutrition (OPEN) study. The correlation between mean sugar intake and mean sugar excretion is approximately 0.84, even though the recovery is low (~0.05% of total intake). However, this biomarker is more sensitive to extrinsic sugars than to intrinsic sugars. In 24-hour urine samples, the association between total sugar excretion and dietary intake has been estimated to be:

$$\log M_i = 1.67 + 0.02 \times S + 1.00 \log T_i - 0.071 \times A_i + u_{Mi} + \varepsilon_{Mi}$$

where M: biomarker; S = 0 for men; S = 1 for women; A: age; T: true intake; u: person-specific bias; ε: within-person random error

This association can be used to estimate dietary intake.

While urinary sugars are a *predictive* biomarker when determined in 24-hour urine, they are *concentration* biomarkers when determined in spot urine samples. While urinary creatinine can be used to adjust for difference in urine volume in most applications, this is not possible when investigating associations with body mass or body mass index because of the strong correlation between body mass and creatinine excretion. It has therefore been suggested that the ratio of urinary sucrose and fructose should be used as a biomarker of sugar intake.

Urinary sugars are traditionally determined using enzymatic assays, as these are readily available in most clinical laboratories. Alternatively, urinary sugars can be analysed by chromatographic methods such as GC or HPLC. Chromatographic methods have the advantage that they can be used to determine sugars for which no enzymatic methods have been established. In the absence of high-throughput clinical robots, chromatographic methods can also provide a faster sample analysis (Tasevska *et al.* 2005, 2011; Bingham *et al.* 2007).

Fibre and wholegrain

Fibre is an important constituent of diet, but there is still a paucity of easily accessible biomarkers, in particular because of the varied nature of fibre. Two classes of compounds found in fibre-rich foods have been proposed as potential biomarkers of intake: lignans (Lampe 2003) and alkylresorcinols (Marklund *et al.* 2013). As lignans are also found in a number of other foods such as tea and coffee, alkylresorcinols are currently the main candidate biomarker. Alkylresorcinols are phenolic lipids found in particular in cereals, and they are therefore mainly biomarkers of cereal or wholegrain. Validation studies with different assessment instruments, as well as human intervention studies, have shown a significant correlation between wholegrain and the alkylresorcinol metabolite 3-(3,5-dihydroxyphenyl)-1-propanoic acid (DHPPA) and 3,5-dihydroxybenzoic acid (DHBA). In 24-hour urine, alkylresorcinol metabolites correlate well with the intake of both wholegrain (r = 0.3–0.5) and total cereal fibre (r = 0.5–0.6). Alternative biomarkers of fibre intake are stool weight, which correlates well with fibre intake (r = 0.8), and faecal hemicellulose, which also shows a good correlation (r = 0.5). However, these biomarkers require the collection of stool samples and this is not always possible in nutrition studies.

Micronutrient intake

Many micronutrients can be used as their own biomarker of intake, and micronutrient status is often assessed in a number of different specimens. However, for many micronutrients no proper validation studies have been conducted, and there is insufficient information regarding the association between intake and biomarker concentration. Most micronutrients can only act as *concentration* biomarkers – with the exception of potassium and to some extent sodium – as their concentration is affected by a number of different factors.

Vitamin A

Retinol is the bioactive form of vitamin A and can be measured in serum and plasma. Its main dietary sources are retinyl esters, provitamin A carotenoids and vitamin A, the latter mainly from animal sources. Retinol can be measured in blood (serum and plasma), but the concentration has only limited value as it is tightly controlled by the liver. Retinol concentrations are therefore only useful markers of vitamin A status when the liver stores are either saturated or depleted; intervention studies with high intake of retinyl esters in healthy individuals showed only modest changes in plasma concentrations. However, retinol concentrations can be used to detect

vitamin A deficiencies and hyporetinolaemia (retinol concentration < 0.7 μmol/L).

Vitamin A – and other carotenoids – are usually analysed using HPLC with ultraviolet (UV), fluorescence or mass spectrometric detection. Carotenoids are sensitive to light and heat, so it is important to store the samples at low temperatures (below –70 °C) and in the dark.

B vitamins

Thiamine (vitamin B1) can be measured in 24-hour urine samples and correlates well with long term (r = 0.7) and short term (r = 0.6) intake. However, because of high between-subject variability, urinary thiamine cannot be used as a recovery biomarker. Similar correlations between short-term intake and urinary excretion (r = 0.4 – 0.7) were also found for other B vitamins except for vitamin B12. The excretion of vitamin B12 appears to be dependent on total urine volume.

The status of vitamin B2 (riboflavin), an important precursor of FAD (flavin adenine dinucleotide), is often determined using a functional biomarker, the erythrocyte glutathione reductase assay coefficient (EGRAC). In this assay, the activity of erythrocyte glutathione reductase is determined with and without the addition of FAD. In subjects with adequate riboflavin intake, only a slight increase occurs, as sufficient FAD is available. However, the ratio increases with lower intake. Alternative measures for vitamin B2 status are plasma or urinary riboflavin, but while EGRAC reflects long-term intake, these measures are more suitable for short-term intake assessment.

Studies using blood samples showed weaker and non-significant associations for many B vitamins. However, there are strong correlations between intake of folic acid and folate in red blood cells (r = 0.5), serum (r = 0.6) and plasma (r = 0.6). Erythrocyte folate is generally preferred, as plasma folate varies greatly depending on metabolism and intake, while erythrocyte folate is a measure of long-term intake. Although vitamin B12 status (measured in plasma) has been used as a surrogate marker of intake, there is a paucity of information on the association with intake. Low total serum vitamin B12 (the sum of B12 bound to transcobalamin II and haptocorrin) can be used as an indicator of deficiency.

The standard clinical screening test for the diagnosis of vitamin B12 deficiency, measurement of plasma or serum vitamin B12, has low diagnostic accuracy, while plasma levels of total homocysteine (tHcy) and methylmalonic acid (MMA) are considered more sensitive markers of vitamin B12 status. Holotranscobalamin (holoTC), the portion of vitamin B12 bound to the transport protein transcobalamin (TC) and the related TC saturation (the fraction of total TC present as holoTC), represent the biologically active fraction of total vitamin B12 and have been proposed as potentially useful indicators of vitamin B12 status.

Vitamin C

Vitamin C concentration in blood and urine can be used as a biomarker of overall vitamin C intake, although there are some limitations of urinary vitamin C concentration and this biomarker is mainly assessed in plasma. Even though plasma vitamin C is a commonly used biomarker and is often considered to be well established, there is only a modest correlation between intake and biomarker (r = ~0.4) with a large variation between populations, although this might also be due to other factors such as genotype and lifestyle factors. As vitamin C is one of the most labile vitamins, sampling, storage and analytical techniques are of great importance. The annual loss of vitamin C in plasma during long storage periods has been estimated to be between 0.3 and 2.4 μmol/L depending on the baseline concentration. The stability of vitamin C can often be improved by the addition of protein-precipitating agents such as metaphosphoric acid, but this might not be always possible.

The pharmacokinetic properties of vitamin C are well known, and there are a number of sources of inter-individual variability: vitamin C is absorbed both by diffusion and active transport, the sodium-ascorbate-Co-transporters (SVCT 1 and 2, transporting the reduced form) and hexose transporters (GLUT1 and 3, transporting dehydroascorbic acid), and genetic polymorphisms are a large source of variation. Other factors such as age and smoking status can also affect vitamin C status, as well as the consumption of certain foods and drugs. Furthermore, the relationship between intake and absorption is linear only for intakes below approximately 100 mg/day and reaches a plateau at intakes above 120 mg/day; at lower plasma vitamin C concentrations, renal excretion is minimised, further affecting biomarker concentration (Jenab *et al*. 2009).

Vitamin D

Vitamin D can either be absorbed from the diet or synthesised in the skin using UV radiation; the precursor of endogenously formed vitamin D, 7-dehrydrocholesterol, is thereby converted into cholecalciferol (vitamin D3). The main sources of dietary vitamin D are fortified foods, in particular margarines, animal products (vitamin D3) and plant-based foods (vitamin D2, from the irradiation of ergosterol). The activated form of vitamin D, 25-hydroxyvitamin D (25(OH)D), is commonly used as a measure of vitamin D status. Due to the combination of endogenous and exogenous sources of vitamin D, the plasma level of 25(OH)D can only provide information on total status but not dietary intake (Jones 2012; see also Figure 6.2 for details).

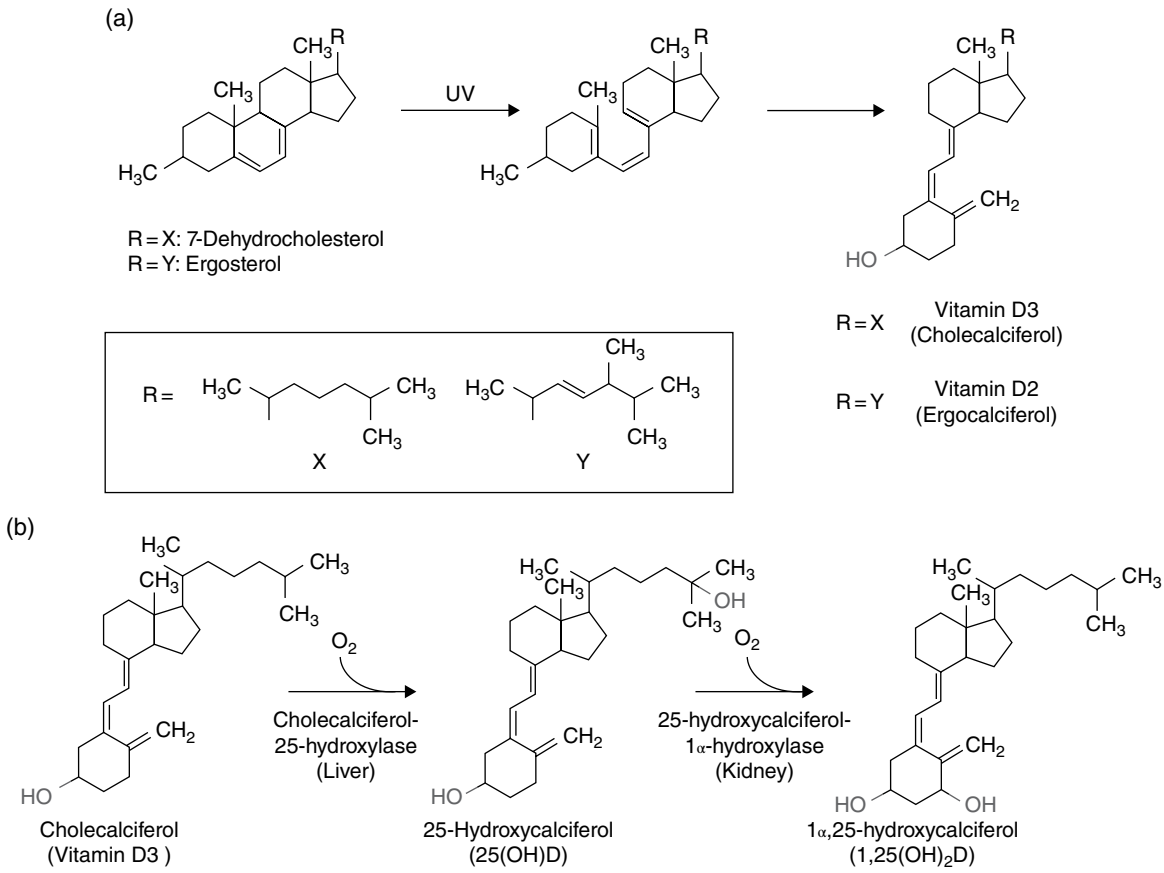

Figure 6.2 Formation and activation of vitamin D. (a) The precursor of vitamin D (7-dehydrocholesterol) is formed non-enzymatically in the skin through photolysis to vitamin D3. The plant sterol ergosterol is activated is also formed by UV irradiation in the plant to form vitamin D2. (b) Vitamins D2 and D3 are active and need to be activated in the liver and kidney to 1α,25-hydroxycalciferol ($1,25(OH)_2D$). 25-hydroxycalciferol (25(OH)D) is the main circulating metabolite and is therefore often used as a biomarker of vitamin D status.

Vitamin E

The main dietary forms of vitamin E are α- and γ-tocopherol. Both can be determined in blood, but there is a strong correlation between them and blood lipids, in particular total cholesterol; it is therefore important to adjust measurements for blood lipids. Using repeated measures, the correlation between lipid-adjusted α-tocopherol and intake adjusted for blood lipids is approximately 0.5, lower than without adjustment (0.6). As a lipid-soluble vitamin, vitamin E can also be measured in erythrocytes (highly correlated with plasma vitamin E) and adipose tissue; however, in adipose tissue γ-tocopherol shows a higher correlation intake (0.4) than α-tocopherol (0.2).

Vitamin K

Vitamin K is a group of lipid-soluble vitamins that are involved in the clotting cascade. Dietary sources (meat, dairy products and green leafy vegetables) and the intestinal flora are the main sources of vitamin K. Significant correlations between the intake of phylloquinone – vitamin K from dietary sources – and plasma concentration have been reported, although the strength of the correlation was very variable (0.1–0.5).

Calcium

Blood calcium is under tight homoeostatic control and is therefore not suitable as a biomarker of intake. While urinary calcium depends to some extent on dietary intake, the association is very weak. In observational studies with 7-day diet diaries and 24-hour urine collection, correlation coefficients of less than 0.2 between dietary and urinary calcium were observed.

Chromium

Plasma chromium levels are very low and changes are therefore difficult to detect, even without deficiency.

Urinary chromium appears to be associated with intake, but the relationship is not sufficient to allow urinary chromium to be used as a biomarker of intake. Chromium concentrations in hair are associated with exogenous and endogenous (e.g. hip replacement) exposure, and therefore might be suitable as a biomarker of intake.

Copper

Copper status is usually assessed using plasma copper or ceruloplasmin. These concentrations can indicate deficiency states, but they are not suitable to determine intake at normal levels of intake. Urinary excretion of copper is also very variable and therefore not suitable as a marker of intake. Copper can also be detected in hair and nail samples, but there are currently insufficient data to show an association with intake.

Iodine

The standard method to determine iodine status is the measurement of urinary iodine, as more than 90% of dietary iodine is excreted via the kidneys. Urinary iodine reflects dietary intake within the past days, and concentrations unadjusted for creatinine are often considered to be sufficient for population screening.

Iron

The metabolism of iron is tightly controlled, and intestinal absorption depends on the iron available in the body's stores. Iron status can be determined in several different ways: serum ferritin is the principal iron storage protein and can therefore be used as a marker of stored iron. Increased serum ferritin can be a marker of iron overload, but otherwise there are no significant associations between the intake of iron and its concentration; however, serum ferritin is associated with the intake of haem iron ($r = 0.1$) and red meat ($r = 0.2$). In contrast to the storage protein, serum iron is highly variable, even within a short time (20% within 10 minutes in one study) and therefore is not a suitable marker of intake. Other markers of iron status are erythrocyte protoporphyrin, mean corpuscular volume and haematocrit. Apart from dietary intake, the major determinant of iron status is blood loss – for example from blood donations or menstruation – and this has to be taken into account when interpreting the relationship between iron status and intake.

Magnesium

Magnesium is homoeostatically controlled, but there is a weak correlation with intake ($r = 0.2$). As urinary excretion is one of the methods of homoeostatic control, urinary magnesium is a better marker of intake, and stronger correlations ($r = 0.3$) with intake have been found in 24-hour urine collections.

Manganese

The plasma concentration of manganese reflects dietary intake. However, as it is primarily excreted via bile, urinary manganese is unlikely to be a suitable biomarker of intake. Toenail and hair manganese have been used to measure environmental exposure, and it is possible that – depending on intake and exposure – they can be used as markers of intake as well.

Molybdenum

No direct biomarkers of molybdenum deficiency are currently known, but there are decreased levels of urinary sulphate and uric acid and concomitantly increased levels of sulphite, hypoxanthine and xanthine.

Potassium

Potassium in blood is tightly regulated and therefore is not suitable as a marker of intake; however, urinary potassium excretion – approximately 77% of intake – can be used as a *recovery* marker of potassium intake. As with other biochemical markers, several measurements are required to obtain a reliable estimate of intake.

Selenium

Selenium has an important role in many biochemical pathways and can be found in many selenoproteins. Blood selenium levels are correlated with intake, and erythrocyte selenium is a suitable measure for long-term intake. Other markers of long-term selenium intake are hair and nail samples, which also show a good correlation with intake.

A functional marker of selenium intake is the activity of selenium-dependent glutathione peroxidase, which increases with increasing selenium status. However, this marker is only useful for individuals with low selenium intake, as the activity plateaus with medium and high selenium intake. Furthermore, the activity of the glutathione peroxidase can decrease during storage, while selenium, as an inorganic element, is not prone to degradation.

Sodium

Sodium is a tightly regulated electrolyte, and therefore blood sodium levels provide only very limited information on intake. In contrast, urinary sodium levels are strongly associated with intake and therefore are a good measure of short-term intake, considered to be one of the few *recovery* biomarkers available, with a urinary excretion of 86%. Average urinary output is a better indicator of intake, however, as urinary excretion is directly dependent on recent intake and therefore has very high day-to-day variation; furthermore, sodium excretion follows a long-term (monthly and longer) cycle. Multiple measurements are therefore necessary to obtain reliable estimates

of intake (Stamler 1997). As multiple 24-hour urine samples are often difficult to collect, overnight urine samples are sometimes used as a surrogate, and calibration studies have shown a very strong correlation between these samples (r = 0.8). Where only casual or spot urines are available, sodium excretion can be standardised using urinary creatinine (see later in this chapter).

Zinc

Dietary zinc can affect plasma levels, but the association is not suitable for making inferences on dietary intake, in particular since zinc levels do not always decrease following deprivation. The concentration of zinc in blood does not only depend on intake, but also on non-dietary factors, and it is subject to tight homoeostatic control. However, despite its poor sensitivity and specificity, plasma zinc is still the most widely used biomarker of zinc status. Zinc in hair is associated with intake, and this might be an alternative biomarker, in particular for long-term exposure.

Other compounds

Many other food components have – or are likely to have – an effect on health and accurate assessment of intake is therefore important. In contrast to many micronutrients, these compounds are rarely under homoeostatic control; concentrations in plasma are very variable and therefore might be a good indicator of intake. However, many of these compounds undergo extensive metabolism, both by the gastrointestinal microbiome and on absorption, and consequently extensive research might be required to identify suitable candidate biomarkers (Figure 6.3). Furthermore, the absorption of many of these compounds is affected by other factors, in particular food composition, and therefore high inter- and intra-individual variability can be observed. For these reasons, all of these biomarkers are *concentration* markers and can only be used accordingly.

Many of these compounds are not only used as a marker for their own consumption, but also as a marker for specific foods, food groups and dietary patterns, and they are therefore discussed in the following section.

Foods, food groups and dietary patterns

Fruit and vegetables

The evidence from observational studies showing beneficial effects of fruit and vegetable intake is often weak, and the World Cancer Research Fund (WCRF) downgraded the likely protective effect of fruit and vegetables in its 2007 report due to accumulating evidence tending towards null, in particular in observational studies. While this effect – or lack thereof – might be real, it contradicts the opinion of many experts and might be due to the attenuation of risk associations because of measurement errors associated with dietary assessment instruments. For this reason, alternative assessment instruments of fruit and vegetable intake are important for future research. The most commonly used biomarkers, carotenoids, vitamin C and polyphenols, are discussed in this section. The limitations of each of these biomarkers – in particular the bias introduced by differences in food composition – make it important to assess results carefully and combine data from several biomarkers.

Carotenoids

Carotenoids are lipid-soluble pigments that are synthesised exclusively by photosynthetic organisms. Structurally, all carotenoids are tetraterpenoids and because of their conjugated double bonds are usually yellow, brown, red or violet. Only about 10% of all known carotenoids can be converted into vitamin A and therefore carotenoid status is distinctly different from vitamin A status; major carotenoids with vitamin A activity are the carotenes and cryptoxanthin. Despite the large number of known carotenoids, food composition tables usually only contain data for a few compounds, primarily β-carotene and lycopene.

The bioavailability of these compounds is largely determined by their lipophilicity, and therefore the fat content in the food matrix can affect absorption; other factors such as colonic metabolism and in particular hormonal factors affect absorption as well. Indeed, intervention studies have found absorption rates of less than 10% to more than 50% of intake, with a modest correlation between intake and blood concentrations (0.2 to over 0.5). This correlation is stronger in normal-weight than in obese individuals, possibly because of differences in the distribution volume. Despite this large variability, blood carotenoid levels are very sensitive to intake as they are not under homoeostatic control. As some carotenoids can be metabolised to retinol, blood carotenoid levels can be influenced by vitamin A status, in particular in individuals with lower status. There is also competition between different carotenoids and β-carotene – given as supplements – which can result in lower blood levels of other carotenoids.

The application of carotenoids as biomarkers of overall fruit and vegetable consumption is impeded by differences in food composition. While carrots and (red) peppers contain large amounts of carotenoids (4–12 mg/100 g), only small amounts are found in foods such as onions or beetroot. Validation studies to investigate the association between carotenoids and fruit and vegetable intake are often conducted using self-reported dietary assessment instruments and might therefore not provide sufficient information. However, these studies show that plasma

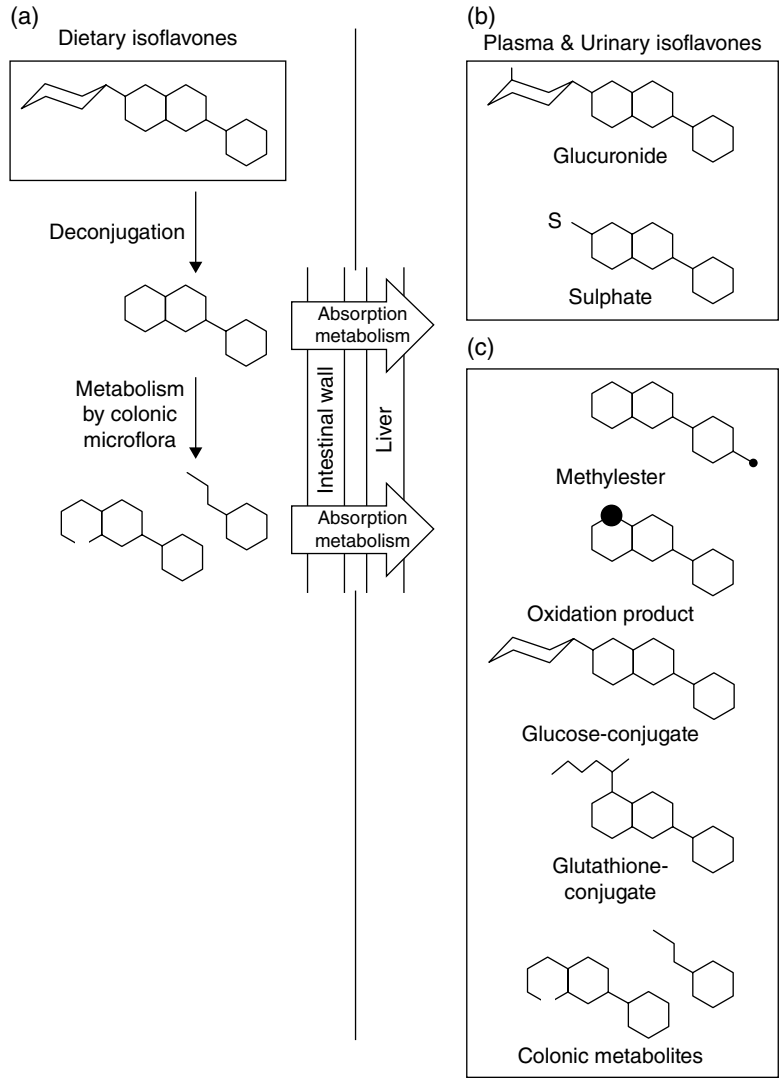

Figure 6.3 The effect of absorption and metabolism on biomarker analysis. Dietary isoflavones (a) are deconjugated and metabolised in the intestinal tract and on absorption. Therefore a variety of different metabolites are circulating. However, most analytical methods only analyse a small number of metabolites (b) and all other metabolites remain undetected (c), resulting in increased variability depending on metabolism and an underestimation. Reprinted from Ward, H.A. and Kuhnle, G.G. (2010) Phytoestrogen consumption and association with breast, prostate and colorectal cancer in EPIC Norfolk, *Archives of Biochemistry and Biophysics*, **501** (1), 170–175, with permission from Elsevier.

concentration is associated with dietary intake; although the correlation coefficients found were modest (0.1–0.4) they were significant, especially for fruit intake.

Flavonoids

Flavonoids are secondary plant metabolites that are ubiquitous in most plant-based foods. They are a diverse group of compounds, comprising monomeric (for example anthocynanidins, isoflavones, flavan-3-ol, flavonols or flavanones) and polymeric (for example proanthocyanidins, theaflavins) compounds. While these compounds are normally present as glycosides in food, they are deconjugated in the intestinal tract and undergo extensive metabolism by the intestinal microbiome and on absorption. Even though flavonoids can be found in most plant-based foods, the actual composition of individual foods is very variable and can be very specific: for example, anthocynanidins are mainly found in berries and isoflavones in legumes. For this reason, flavonoids are not only used as summary markers for total fruit and vegetable intake, but also to identify the intake of specific food groups or even individual foods.

Total excretion of phenolic compounds, measured with a modified Folin–Ciocaltaeu assay, showed a significant relationship with total fruit intake in an observational study, but there is still a paucity of data to validate this biomarker properly. More research has been undertaken to investigate the relationship between diet and biomarker for individual compounds; however, the correlation between intake and biomarker is rather modest. For soy, the main source of dietary isoflavones in the European diet, the correlation coefficient in many studies is below 0.3, and this is similar for other food and biomarker combinations: the correlation between self-reported apple intake and urinary phloretin concentration is between 0.2 and 0.3, and for self-reported citrus fruit intake and the citrus flavonoid hesperetin it is 0.4. A possible explanation for these modest correlations is not only the large variability in food composition and extensive metabolism, but also the very short half-life of these compounds.

Vitamin C

Plasma vitamin C is currently the most commonly used biomarker of fruit and vegetable intake. This is based on the assumption that the main sources of vitamin C in the diet are fruit and vegetables, and its intake is therefore associated with fruit and vegetable intake. As with other biomarkers of food groups, differences in food composition are a major source of variation and are likely to lead to misclassification; in the case of vitamin C, this is exacerbated by the frequent use of vitamin C–containing food supplements. Despite the limitations of vitamin C as a biomarker, it is still the most commonly used biomarker of fruit and vegetable intake.

Meat and fish

Meat intake and fish intake have both been associated with opposing health effects. While fruit and vegetables contain very specific compounds that can be used as candidate biomarkers, this is more difficult with foods of animal origin. Modified amino acids, in particular 1- and 3-methyl-histidine, have been proposed as biomarkers of meat, and they show a significant relationship in dietary intervention studies. As 3-methyl-histidine is also a marker of muscle breakdown, 1-methyl-histidine is more suitable as a biomarker of meat intake.

The ratio of stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotopes is commonly used in archaeology to assess meat and fish intake, and these biomarkers are becoming more commonly used in the nutritional sciences (Kuhnle *et al.* 2012). The urinary isotope ratio changes quickly with dietary changes and can therefore be used to assess short-term dietary intake. Conversely, the longer half-life of albumin and in particular red blood cells makes the whole-blood isotope ratio a better marker of medium-term intake. Isotope ratio analyses can also be conducted on hair and nail samples and they thereby provide long-term information on dietary intake. As this method assesses mainly the protein source, it is not easily possible to distinguish between meat and dairy intake.

6.4 Methodological considerations

Biochemical markers of intake can be analysed in a wide range of specimens. A key advantage of biomarkers is that they can be analysed retrospectively in samples for which no other – or no relevant – dietary information is available. However, careful consideration of collection, storage and analytical methods is necessary to avoid any additional bias.

Specimens

Biochemical markers can be analysed in many different specimens, and each specimen has specific advantages and disadvantages (Table 6.1). While it is not possible for practical reasons to change the type of specimens collected in studies conducted in the past, the choice of specimen in current or future studies is often dictated by the funding available and the feasibility of storage and collection.

There are a number of factors to consider when choosing a specimen:

- The distribution of biomarkers in different specimens depends on a number of factors, e.g. homoeostatic control, active excretion and physico-chemical properties such as lipophilicity. It is therefore important to choose a specimen where the biomarker can be detected.
- Biomarker concentration in blood is often metabolically controlled and is therefore affected by not only dietary intake but also metabolism. The excretion in 24-hour urine samples is often a better indicator of intake, especially for compounds that are readily excreted.
- While 24-hour urine samples are often preferable, they are difficult to collect and many studies rely on spot urine samples. This can increase the variability, in particular for biomarkers with a very short half-life. Furthermore, spot urine samples require an adjustment for urine volume, which can often be achieved by using creatinine.
- Many biomarkers – in particular those with a low lipophilicity – have only a short half-life in the usual specimens such as urine and blood. Hair and nail samples provide an alternative specimen to measure long-term intake (see Figure 6.4 for a comparison of the time frame covered by different dietary assessment methods).

Table 6.1 Summary of main specimens used for biomarker analysis and their advantages and disadvantages.

Specimen	Advantages	Disadvantages
Adipose tissue	Long-term marker of fat intake	Invasive, requires specialist staff for collection
Whole blood, serum and plasma	Most commonly used specimens, infrastructure for collection is often available; reflect directly bioavailability of biomarker; can also be used for genotyping and to determine clinical markers	Invasive, require specialist staff for collection; need to be stored at very low temperature (often liquid nitrogen) to ensure long-term stability; homoeostatic control can affect many candidate biomarkers; timing of sample collection can affect outcome (especially with biomarkers with short half-life); only limited volume available
Hair	Long-term marker of intake, non-invasive collection	Only limited number of biomarkers available; can be affected by hair treatment or lack of hair
Nails	Long-term marker of intake, non-invasive collection	Only limited number of biomarkers available
24-hour urine	Non-invasive collection, does not require specialist staff; large volume available for multiple analyses; stable at –20 °C; no homoeostatic control of most candidate biomarkers	Large volume often requires aliquotation; collection is often considered tedious by volunteers; difficult to assess completeness
Spot urine	Non-invasive collection, does not require specialist stuff; commonly used specimen; stable at –20 °C	Requires adjustment for total urine volume (e.g. using creatinine); biomarkers with short half-life might not be found in all specimens; timing of sample collection can affect outcome (especially with biomarkers with short half-life)

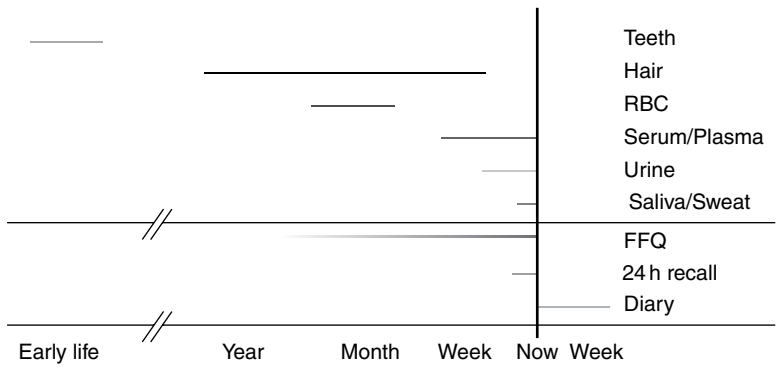

Figure 6.4 Time frame of biomarkers detected in different specimens and different dietary assessment methods (not to scale). Reprinted from Kuhnle, G. (2012) Nutritional biomarkers for objective dietary assessment, *Journal of the Science of Food and Agriculture*, **92** (6), 1145–1149.

Blood

Capillary blood samples can easily be collected in most study settings, as they do not require venepuncture. These samples are ideally suited to rapid analyses such as blood glucose or blood haemoglobin. Capillary blood is usually stored as dried blood spot on filter paper developed for blood spot storage. Dried blood spot can be stored in the dark at room temperature, and many analytes remain stable under these conditions for a long time, for example vitamin D (as 25OHD) or retinol.

Venous blood samples are more commonly collected in nutrition studies, and facilities for their collection and processing can be found in most laboratories. Venous blood samples can then be processed into a number of different specimens, in particular serum, plasma, buffy coat and red blood cells. When collecting blood samples, it is important to specify the type of tube and in particular the type of anticoagulant used, if any. Whether fasting blood samples are required depends on the nature of the analysis and they are often not necessary. Blood samples should be processed quickly and then stored under appropriate conditions, usually at –80 °C or in liquid nitrogen. To avoid frequent freeze–thaw cycles, these samples should be stored in multiple aliquots.

Blood samples are not suitable for all biochemical markers, in particular not for those under tight homoeostatic control, as their concentration is not affected much by intake. For biomarkers that are rapidly eliminated by the kidneys, urine samples are preferable as they can provide information over a long period of time. However, blood samples are ideal for biomarkers with a long half-life, in particular fatty acids or lipophilic compounds,

which can be found in erythrocytes. Recent research has also shown that some hydrophilic biomarkers such as flavonoids can be found in erythrocytes, but more research is required to investigate this. As erythrocytes have a lifetime of 120 days, biomarkers detected in erythrocytes can provide dietary information for approximately 60 days.

Urine

Urine samples are also commonly used for biomarker analysis. While the availability of blood samples is often limited by the volume that can be collected, such a restriction rarely applies to urine samples. In contrast to blood samples, the concentration of biomarkers in urine samples is not controlled homoeostatically and, indeed, compounds underlying homoeostatic control are excreted via the kidneys and their presence in urine is often related to intake. Urine samples are suitable for all hydrophilic – and to some extent also lipophilic – biomarkers that are excreted by the kidneys. Urine samples are mainly useful for markers of short-term intake, as urinary excretion is often more directly related to dietary intake, in particular when the body is in equilibrium.

For the analysis of nutritional biomarkers, 24-hour urine is generally preferable as the total excretion in 24 hours can be measured. There are different protocols for the collection of 24-hour samples, some including the first morning urine, some excluding morning urine samples altogether. While 24-hour urine is less invasive than venous blood samples, volunteers often find the collection of urine samples tedious and prefer venepuncture. To ensure the completeness of 24-hour urine sample collection, *p*-amino-benzoic acid (PABA) tablets are often given. PABA is excreted readily and can be recovered from urine. A quick photometric test can be used to measure recovery, and often a recovery of less than 85% is used to identify incomplete collections (Bingham and Cummings 1983). However, PABA might not be suitable in elderly participants, as the recovery is lower, and it is not suitable in participants with certain allergies. It is also not known whether PABA affects the metabolism of biomarkers or the stability of urine samples.

Spot urine samples can easily be collected and are therefore preferable – from a volunteer's point of view – when compared with 24-hour urine samples. Biomarker concentration in spot urine samples depends on urine volume; to be able to compare concentration between individuals, concentrations are often normalised using urinary creatinine. This normalisation is based on the assumption that creatinine excretion is constant and therefore any difference in concentration is only based on total urinary volume. However, creatinine excretion is not constant and depends on a number of factors, in particular body mass. Normalising urinary concentrations with creatinine can therefore introduce additional bias, in particular when investigating associations with body mass or obesity, or related factors.

Faeces

Faeces are not commonly used as specimens for nutritional biomarker analysis. The main biochemical markers of intake analysed in faeces are markers of fibre intake, although other markers can be detected in faecal samples as well, in particular compounds excreted via the bile. The collection of faecal samples can be facilitated with specially designed collection devices such as the Fecotainer® to minimise the contact with the sample for the study participant.

Tissue

Tissue samples are more often used for the analysis of markers of exposure than for markers of intake. However, some biochemical markers of intake, such as carotenoids or fatty acids, can also be measured in adipose tissue. Tissue samples can often provide information on long-term dietary intake – with an average half-life of 1–2 years, fatty acids in adipose tissue can provide information about average dietary intake within this period.

Hair

Hair samples are more commonly used to detect exposure to drugs than for dietary intake, but with a growth rate of approximately 1 cm/month, hair can provide detailed information on long-term dietary exposure. As the composition of hair does not change after formation, a longitudinal analysis of hair can be used to identify dietary changes, such as those between seasons. Biomarker concentration in hair depends not only on dietary intake but also on other factors, in particular colour and thickness. For hair analysis, careful cleaning is necessary to avoid any contamination from environmental compounds or cosmetic products. Hair samples can be stored at room temperature in the dark, but care must be taken to ensure that information on the direction of growth is maintained.

Nails

Like hair, nail samples can be used to obtain information on long-term exposure. Unlike hair samples, nails have to be collected regularly to obtain this information.

Sample collection and storage

The stage of collection and storage of samples is often the first to introduce bias and increase the measurement error. It is therefore important that sample collection and storage should follow a standardised protocol to

minimise measurement error. The following aspects need to be addressed:

- The sample-collection procedure should be standardised. For urine samples, this should specify what type of urine (morning, mid-stream etc.) should be collected; for blood samples, this should specify whether capillary or venous blood should be collected and what type of anticoagulant – if any – should be used. Furthermore, it is important to specify whether fasting or non-fasting samples are collected; a record of the collection time and the time of the previous meal can help to explain additional variability.
- Preservatives, such as boric acid or iodoacetamide, might be useful, but they can affect analytical techniques. While the techniques intended for use might not be affected by preservatives, it is possible that other techniques are, and it is prudent to keep unmodified samples in case they are required for future research.
- Samples should be processed without any delay and the process (e.g. separation of plasma) should follow standardised procedures.
- The storage condition – both before processing and for long-term storage – should prevent any degradation of the sample, including degradation by light. For example, vitamin C deteriorates rapidly in the absence of preservatives such as metaphosphoric acid. While some samples – such as hair – can be stored at room temperature, other samples require storage in liquid nitrogen. Long-term stability tests should be conducted to establish the best type of storage.
- Samples should always be stored in aliquots to prevent unnecessary freeze–thaw cycles. The size of each aliquot should be sufficient to conduct one or several analyses.
- Samples should be stored in containers that are easily retrievable while requiring as little space as possible. For the European Prospective Investigation into Cancer (EPIC) study, plastic straws were used to store more than 6 million plasma, serum, red cell and buffy coat samples in liquid nitrogen; these straws allow the storage of large numbers of samples in a small place. Recent developments in automated storage systems allow the storage of specimens in vials identified by barcode or RFID tags, as well as the automated retrieval of samples.

Sample stability

Sample stability is an important aspect to consider, in particular when analysing specimens from prospective studies. These samples are often stored for long periods of time and – depending on the number of aliquots available – undergo several freeze–thaw cycles. Furthermore, it is often not feasible to analyse the entire cohort for specific biomarkers and therefore not all samples will have had the same number of freeze–thaw cycles. For this reason, it is important to investigate the stability of candidate biomarkers in the specimens used. While it is often not possible to test the stability directly over a long period of time, alternative methods are available such as accelerated ageing (using higher temperatures and humidity).

Analytical methods

The analytical method used for biomarker analysis depends on a number of different factors, for example the availability of equipment and expertise, and the required sensitivity. However, the choice of analytical method can have a significant effect on the outcome, not only because of differences in the sensitivity of different methods, and thus in the level of quantification, but also because of differences in variability and specificity.

Sensitivity and selectivity

The sensitivity of analytical methods can have a significant influence on the interpretation of biomarker data, in particular when biomarkers are present at low concentrations. A high lower limit of quantification (LLOQ) will reduce the variability and therefore the observed relationship between biomarker and intake. Furthermore, a low sensitivity often increases the noise – in particular at low concentrations – and thereby introduces additional measurement error. Even though sensitive methods normally require more elaborate sample preparation and are often more expensive, more sensitive methods are usually preferable.

While a higher sensitivity is usually desirable, this is not always the case for selectivity. Some compounds used as biomarkers are extensively metabolised, and it might be necessary to determine the sum of all metabolites; with very selective methods, this requires the identification of each analyte and the development of an appropriate method. The Kjeldahl method for total urinary nitrogen is an example of a non-selective (or only element-specific) method that provides more useful information than the analysis of each individual nitrogen-containing compound. For other biomarkers very selective methods are crucial, as isomers or even enantiomers have to be separated. This degree of selectivity often requires either the use of enzymatic methods or of specialised equipment such as chiral or affinity chromatography.

Throughput

The throughput of analytical methods is of particular concern when analysing large numbers of samples from clinical trials or observational studies. High-throughout

methods are usually highly automated and therefore require standardised protocols. While the set-up and maintenance of high-throughput equipment are expensive, results are usually very consistent and of high quality, as there are fewer sources of variation.

Comparability, validation and quality control

Good laboratory practice requires a thorough validation of any analytical method, and a cross-validation when combining data from different laboratories or different methods. Details on method validation can be found elsewhere, for example in the US Food and Drug Administration's *Guidance for Industry*.

Cross-validation is very important for multi-centre studies or when results are also used for diagnostic purposes. It is often sufficient to analyse the same samples in different laboratories or with different methods and compare the results, but for more complex studies this should be done regularly and with blinded samples. An important factor to consider – in particular when comparing different methods – is difference in sensitivity and selectivity. While results might be in agreement across the common range of both methods, the introduction of an artificial cut-off point – that is, the LLOQ of the less-sensitive method – might have an adverse effect on the results.

In addition to the usual quality control samples in analytical batches, it is common practice to place up to 10% blinded QC samples randomly within the normal samples. This will provide additional information about the quality of the analytical method (in particular intra-batch variability) and about the analysis.

6.5 Biomarker development

The development of new nutritional biomarkers is important not only for nutritional research, but also for clinical practice, for instance to monitor compliance with dietary prescriptions. This development can be broadly divided into two parts: biomarker discovery and biomarker validation.

Biomarker discovery is often divided broadly into *hypothesis-driven* and *discovery-driven* approaches (Figure 6.5 shows a work flow for both). In the *hypothesis-driven* approach, candidate biomarkers are selected based on information available about the respective foods. This requires detailed knowledge of the composition of foods or food groups, as well as the *in vivo* metabolism of these compounds, as only the most common metabolites are chosen as candidate biomarkers. Conversely, the *discovery-driven* method does not require any a priori knowledge of potential candidate biomarkers; it uses multivariate techniques to identify candidate biomarkers by comparing metabolites in specimens following intervention diets high and low in respective foods (an example of this is the discovery of proline betaine as a biomarker of citrus fruits; Heinzmann *et al.* 2010). As multivariate techniques are very sensitive, it is important to ensure that the only difference between intervention groups is diet and not other factors. Standardised protocols must therefore be followed.

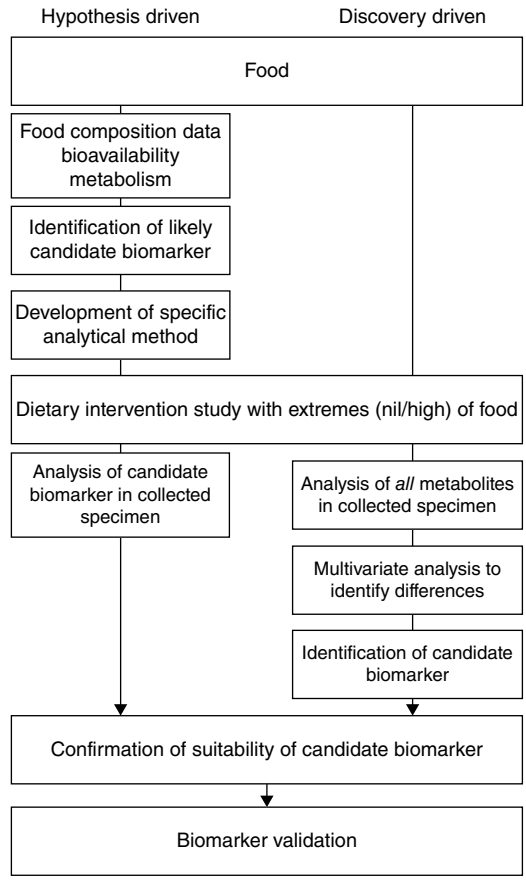

Figure 6.5 Biomarker discovery normally follows either a hypothesis- or a discovery-driven strategy. Both strategies require carefully controlled dietary intervention studies, but use different approaches for the identification of candidate biomarkers. Reprinted from Kuhnle, G. (2012) Nutritional biomarkers for objective dietary assessment, *Journal of the Science of Food and Agriculture*, **92** (6), 1145–1149.

6.6 Biomarker validation

The validation of biomarkers prior to use is important in order to be able to interpret results properly. However, despite this necessity, many biomarkers are used without

or with insufficient validation. Of particular concern is that many biomarkers are only validated against self-reported intake, a method that is known to be biased. The validation of a biomarker should address the issues discussed in this section.

Relationship with intake

A relationship with intake is the most important requirement for a biochemical marker of intake, and any validation process must establish this relationship. It does not necessarily have to be direct and a specific metabolic response to intake might also be suitable as a biomarker. Blood concentrations are often controlled by homoeostatic mechanisms, therefore the sensitivity of a biomarker to intake can only be established in some specimens, not all.

The relationship with intake can be established either experimentally with intervention studies or using prior knowledge of the metabolism. A combination of both is generally preferable, as for many candidate biomarkers only limited knowledge of their metabolism is available and intervention studies are often too small to allow generalisation. The relationship with intake of the *predictive* biomarker of sugar intake, urinary sucrose, has been established using a combination of prior knowledge – that is, the sucrose is absorbed intact from the intestinal tract and excreted unmetabolised – and intervention studies where a dose–response effect was established. These dose–response studies were extended to participants with characteristics important for future research – that is, obese participants – to ensure that this relationship was not dependent on body mass.

In addition to a direct dose–response relationship, it is important to estimate the time frame covered by individual biomarkers. Biomarkers that are very sensitive to intake and change almost immediately with consumption might be beneficial to investigate short-term dietary changes, but, in particular in nutritional epidemiology, chronic nutrient exposure is of more interest. It is therefore important to establish the relationship between biomarker, intake and time. It might thus be necessary to establish pharmacokinetic parameters such as the half-life and investigate different specimens such as 24-hour urine samples, red blood cells (RBC), adipose tissue or hair. A biomarker with a high sensitivity to recent intake might still be useful if the assessment is repeated frequently, but this has to be established in validation studies.

The relationship between biomarker and intake is often not linear, but follows a more complicated function. It is therefore of great importance to investigate this relationship, in particular whether the biomarker shows a floor or ceiling effect. For example, plasma vitamin C concentration does not increase proportionally with very high vitamin C intake.

Long-term repeatability of a biochemical marker of intake is usually a good indicator that the marker can be used as an estimate of long-term intake. For this reason, it is important to investigate the intra-person variability of nutritional biomarkers over a longer period of time.

Free-living individuals

Biomarker validation studies are usually always conducted in participants with a controlled diet designed to contain different amounts of the respective foods. While it is important to investigate this relationship, the tightly controlled nature of these diets makes it difficult to compare results to free-living individuals. However, in nutritional epidemiology it is the diet of free-living individuals that is most important, and biomarkers should therefore be validated in a similar population. One method for addressing these difficulties is to conduct dietary intervention studies where participants live under controlled conditions but receive their habitual diet. This allows a detailed analysis of their dietary intake while still obtaining the same dietary variability as in a free-living population.

Validation without intervention studies

While detailed validation studies are desirable, it is often not feasible to conduct them and biomarkers have to be validated using specimen and dietary data from existing studies. A high variation of a biomarker in a cross-sectional study is often a good indicator of a relationship with intake, although other factors can explain this association as well. The quality of a biomarker is often assessed using either the intra-class correlation between self-reported and biomarker data or Cohen's kappa statistic. However, these results only show the agreement between different analytical methods, not the association with true intake. Thus, a biomarker might agree well with self-reported intake and be considered a good biomarker of intake while the association with true intake is unexamined and poor.

6.7 Interpretation of results

Limitations and other considerations

Biochemical markers of intake are often considered an *objective* and more reliable dietary assessment method. However, while biomarkers are less subject to bias introduced by self-reporting, they still can introduce bias and measurement error and careful consideration when interpreting the results is important. Unless detailed data

from dietary intervention studies are available, a relationship between biomarker and intake is often only assumed based on self-reported data.

A nutritional biomarker is in most cases essentially a marker of bioavailability (except for markers detected in faeces), as only compounds that enter the body can be detected as biochemical markers. This can be advantageous when the bioactivity of specific nutrients is of interest, as the biomarker is often a better measure of exposure than dietary information. However, this is disadvantageous when dietary intake is the primary interest, for example when investigating food choice or the effect of dietary patterns. Biomarker bioavailability can be affected by a number of different factors, for example dietary composition, health, gastrointestinal microbiome or genetic factors. Biochemical markers are therefore ideally suited to investigate associations with specific compounds – or to some extent to specific foods – but are less well suited to investigating dietary patterns or the wider context of associations between diet and health.

Biomarker concentrations are also affected by other factors such as physiological status, disease state and malnourishment. The application of most biomarkers makes the assumption that the body is in homoeostasis, but this is not the case in a number of people, for example pregnant women, people with (long-term) illness or those still growing; malnourishment can also have an impact on the metabolism and thus biomarkers. It is therefore important to take these aspects into account when using biomarkers in human studies.

A further aspect to consider is the metabolism of compounds used as biomarkers. Most analytical methods are developed for individual compounds, and therefore inter-individual differences in metabolism can affect the outcome. This might be less of a problem when bioactive metabolites are used as biochemical markers, but in many cases the individual bioactivity of metabolites is not known. As the metabolism can be influenced not only by genetic factors but also by background diet, this is an additional source of variation that must be taken into consideration.

Correlation between intake and biomarker

The association between dietary intake and biomarker is often given as a correlation coefficient. However, a statistically significant correlation does not imply that the correlation is biologically relevant, and a lack of significance does not imply the opposite. A strong and significant correlation is often due to a high variability of biomarker and intake. Therefore, nutrients with a high variability of intake are likely to show a stronger correlation than nutrients that are ubiquitous in most foods and where the variability is therefore much lower. It is thus important to conduct controlled dietary intervention studies with a wide range of intake to validate candidate biomarkers properly and establish a correlation.

Biomarkers to improve measurement

Biochemical markers of intake are often used to validate other dietary assessment methods, in particular food frequency questionnaires (FFQs) and to a lesser extent diaries. This approach has the advantage that the two assessment instruments used, self-reporting and biomarkers, are less likely to have correlated errors.

For validation of an assessment intake, a true reference value is normally required. However, in nutritional epidemiology, such a value is usually not available unless dietary intake has been observed directly. Validation in this context is therefore the evaluation of the measurement error, which can then be applied to values obtained from the instrument. The relationship between two measurements is usually determined by calibration. In nutritional epidemiology, this is the relationship between a measurement – that is, self-reported data or a biomarker – and the habitual intake; however, the latter cannot be observed directly. For this reason, calibration in this context can often be assumed to be the estimation of a correction factor that can be applied to a dietary measurement to determine the habitual diet. *Recovery biomarkers* are most suited to method validation and calibration, as they allow a direct estimation of intake and therefore permit the determination of a correction factor, but *concentration biomarkers* have also been used for measurement error correction. Urinary nitrogen – as a recovery marker of total protein intake – has been used in the OPEN study as a reference instrument to investigate the measurement error structure of self-reported dietary assessment instruments, FFQs and 24-hour recall.

The measurement error introduced by the inaccuracies of dietary assessment methods affects the observed association between diet and disease, the so-called regression dilution bias. Information obtained using nutritional biomarkers can be used to correct observed associations for this bias. A commonly employed method is *regression calibration*, where the true value of thebintake is predicted using a measurement – such as biomarkers – and relevant confounders (Freedman *et al.* 2010). More information about the statistical methods used for measurement error correction can be found in the references and further reading.

Biomarkers to monitor compliance

Biochemical markers of intake can also be used to monitor compliance in dietary intervention studies. The choice of biomarker depends largely on the type of intervention and the length of the study: for specific intervention

foods – such as cruciferous vegetables – a single biomarker such as glucosinolates (or their *in vivo* metabolites) might be suitable, whereas for more complex dietary interventions a battery of biomarkers might be more appropriate (for example vitamin C, carotenoids and polyphenols for a diet with increased fruit and vegetable intake). In short-term studies, it is often sufficient to conduct spot checks and collect samples at random time points to ensure compliance. In these instances, the nature of the biomarker, long or short term, is less important. For longer-term and chronic studies, however, a closer monitoring of compliance is often desirable. This can be achieved either by the regular collection of samples at intervals determined by the physiological half-life of the compound, or the collection of specimens that provide long-term dietary information.

6.8 Outlook

Nutritional biomarkers of intake are an important tool in nutrition research, in particular in nutritional epidemiology. They are an established method to improve dietary assessment methods, but there is still a paucity of properly validated biomarkers, in particular for specific foods or food groups. Future research will therefore focus on the development of more complex markers of intake, employing methods similar to those used in metabonomics.

Another important field of biomarker research is the development of biomarkers of long-term intake. While multiple sampling can provide information about long-term intake and repeatability, this is often not feasible in large observational studies and it is not possible to conduct retrospectively.

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