A critical assessment of some biomarker approaches linked with dietary intake

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Contents

In this review many examples are given of the complexities involved in using some biomarkers in relation to assessing the effects of dietary exposure, when there is frequently a need to determine changes following long-term low level exposure to dietary components. These range from understanding why the biomarker might be valuable and how best it can be measured, to the pitfalls which can occur in the interpretation of data.

Analytical technique is considered in relation to folate and selenium, and flavonoid and carotenoid species are used to illustrate how the metabolism of a compound may alter the validity or adequacy of a marker. Vitamin A is discussed in relation to the difficulties which can arise when there are several biomarkers that may be available to assess exposure to one nutrient. Vitamin B_{12} is discussed in relation to the dietary choices made by individuals. Possible interactions and the role of measuring total antioxidant capacity is considered in some detail. In contrast to most nutrients, there is a marked lack of biomarkers of either exposure or effect for most non-nutrients. The role of biological effect monitoring is considered for dietary contaminants, fumonisins and polyhalogenated aromatic hydrocarbons. Aflatoxins are discussed to exemplify food contaminants for which the biomarker approach has been extensively studied. Finally some compounds which are deliberately added to foods and some which appear as processing contaminants are each considered briefly in relation to the requirement for a biomarker of exposure to be developed.

Biomarkers of exposure: Dietary intake: Nutrients: Non-nutrients

1. Introduction

This review is an assessment of the use of some biomarker approaches used for assessing dietary exposure and intake. Because many of the authors have analytical experience of the examples given, the review assesses some of the measurement techniques as well as the clinical appropriateness of the biomarkers chosen. It is not intended to be an exhaustive list of compounds or methods, but it is intended to offer an 'insight' into the current state of the art with regard to biomarkers of dietary exposure and effect.

2. Factors influencing the use of biomarkers of exposure and effect for nutrients and non-nutrients

Ideally, biomarkers should be specific, sensitive and not too invasive for human studies. These criteria are not always met and the authors decided to focus their review around the factors that might influence the use of a biomarker approach. Thus the effects of the analytical technique are considered in relation to folate and selenium. How appropriate or robust are the methods in current use? Biomarkers for flavonoid and carotenoid species are used to illustrate how the metabolism of a compound may alter the validity or adequacy of that marker. How consistent are the several biomarkers that may be available to assess exposure to a vitamin for example? Vitamin A is used to discuss this aspect whereas vitamin B_{12} is discussed in relation to the dietary choices made by individuals. The role of biological effect monitoring is considered for dietary contaminants, fumonisins and polyhalogenated aromatic hydrocarbons.

One of the reasons that specificity for a biomarker may be a problem is the number of possible interactions which may occur within the human body. This aspect and the role of measuring total antioxidant capacity is considered in some detail. In the final two sections, biomarkers for aflatoxins are used as an illustration of a food contaminant for which the biomarker approach has been extensively studied and the last section attempts to highlight some of the areas in which there is a significant lack of knowledge.

3. Effects of the analytical method

The analytical method used to determine a chosen biomarker may compromise the interpretation of the link between dietary exposure and the biomarker. A good example is the measurement of folate absorption and metabolism.

3.1. Folate

3.1.1. Absorption and metabolism. Dietary folates exist predominantly in a polyglutamated conjugated form. Since the folate carrier transports the monoglutamate form of the cofactor into cells, the folate polyglutamates must be hydrolysed to the monoglutamate form by the enzyme folylpolyglutamate hydrolase, also known as conjugase. After cell entry polyglutamylation occurs catalysed by the enzyme folylpolyglutamate synthetase. Under normal physiological conditions folates exist predominantly as the penta- and hexaglutamates. Once inside the cell, folates

can undergo interconversion into the various cofactor forms along with both reduction and oxidation. In general, studies on the distribution of the cofactor forms have shown that tetrahydrofolate, 10-formyltetrahydrofolate, and 5-methyltetrahydrofolate are the cofactor forms present in the highest quantities in tissues and cells. Circulating folates are monoglutamyl derivatives mostly in the form of 5-methyl tetrahydropteroylglutamate. Polyglutamate folates within the cell are incapable of cellular exit unless they are converted back into monoglutamyl derivatives. It is not possible at this time to predict the bioavailability of folate for a given diet. The relative bioavailability of synthetic polyglutamyl folates in many studies of widely differing design ranges from approximately 50 to 100 % (Gregory, 1997; Mullin & Duch, 1992; Selhub & Rosenberg, 1997).

The number of folate cofactor forms, variable polyglutamate chain lengths, and instability of many of the reduced derivatives have made separation of these complex mixtures extremely difficult (Mullin & Duch, 1992; Selhub & Rosenberg, 1997).

3.1.2. Food analysis. Since the polyglutamated forms of folate in the diet are hydrolysed to monoglutamates prior to transportation into the cell it is reasonable to analyse folate as the monoglutamates in food. Most high-performance liquid chromatography (HPLC) methods have been designed for the analysis of folate monoglutamates and several methods for chromatographic analysis of folic acid and its monoglutamate derivatives exist, especially reversed-phase HPLC methods. There does not appear to be any trend toward a common method. Many of the HPLC methods can only separate some of the folate monoglutamate derivatives. The choice of detection form is decisive. Several HPLC studies have been reported to separate standard solutions of different folate forms, whereas only a few methods deal with analysis of dietary folates. Besides, no standardised procedure for the hydrolysis of polyglutamate folates has been adopted, and the conjugase preparations differ in sources, activities, optimal pH and hydrolysis products (Mullin & Duch, 1992; Lucock *et al.* 1995; Wigertz & Jagerstad, 1995; Truswell & Kounnavong, 1997).

The paucity of information on the bioavailability of food folates stems principally from the complexity of dietary folates, their low levels in various foods, the presence of some dietary factors such as inhibitors of pteroylpolyglutamate hydrolase (PPH) that could affect folate bioavailability, and the inability to distinguish between newly absorbed folate and endogenous folates (Gregory, 1997; Selhub & Rosenberg, 1997). Analysis of native folates is complicated not only because the vitamin occurs in many different forms, but also because these folates are light- and heat-sensitive and are easily destroyed by oxidation (Wigertz & Jagerstad, 1995).

The bioavailability of added folic acid is greater than that of naturally occurring food folate. Folic acid does not occur naturally in significant quantities, but is the form of the vitamin added to foods during fortification, and it is very often used in studies measuring plasma folate in order to estimate folate status (Sauberlich *et al.* 1987; Gregory, 1997).

3.1.3. Choice of matrix for biomarker measurements. It should be stressed that analysing erythrocyte folate instead of plasma folate is to be recommended. Plasma folate varies much depending on recent intakes, changes in folate metabolism, and instability over time in plasma. Physiological and life-style variables, notably alcohol and tobacco use, are also important determinants of plasma folate. Plasma folate can be suitable if many other parameters are held constant, but it is not possible to use plasma folate in population studies (O'Keefe *et al.* 1995; Brussard *et al.* 1997; National Food Agency of Denmark, 1997; Selhub & Rosenberg, 1997; Stites *et al.* 1997).

Erythrocyte folate levels reflect body stores and are considered to be a measure of long-term status. Red blood cell folate content is about 20 times the concentration of plasma folate. There are some methodological problems with the analysis of erythrocyte folate, e.g. erythrocyte folate is reduced by vitamin B_{12} deficiency (Brussard *et al.* 1997; National Food Agency of Denmark, 1997; O'Keefe *et al.* 1995; Selhub & Rosenberg, 1997; Stites *et al.* 1997). Red cell folate is probably a good indicator to discriminate between normal folate status and deficiency. However, under therapeutic conditions or in the evaluation of bioavailability, there is a need for additional functional tests, sufficiently sensitive and specific, to reflect rapid changes of folate status. Elevated concentrations of homocysteine in serum or plasma is a test that seems promising (Jagerstad & Pietrzik, 1993; Ueland *et al.* 1993).

3.1.4. Poly- or monoglutamate form. The folate polyglutamates inside the cell are the true biological cofactors. Therefore, one could argue that considerable important biological data are sacrificed with the hydrolysis of polyglutamates to the monoglutamates in analysis of intracellular (e.g. erythrocyte) folate. However, there is not enough knowledge to link the different intracellular polyglutamates with the dietary exposure.

3.1.5. Analytical methods. Analysis of intracellular folates represents a complex analytical problem since eight potential parent species (folic acid, dihydrofolate, tetrahydrofolate, 10-formyltetrahydrofolate, 5-formyltetrahydrofolate, 5,10-methylenetetrahydrofolate, 5,10-methyltetrahydrofolate, 5-methyltetrahydrofolate) are present in variable states of polyglutamation. The digestion of these folate polyglutamates reduces all species to folate monoglutamates, which can then be analysed using methods similar to those used for food analysis (Mullin & Duch, 1992). The different forms of monoglutamate folates may have different biological activities in both micro-organisms and more importantly in humans, and it is therefore desirable to separate the different cofactor forms of folate.

Microbiological assays with the test organism *Lactobacillus casei*, in which growth response to a mixture of folates is measured turbidimetrically, can be used. Microbiological assays have limitations such as different biological activities of the monoglutamate folate forms in the microorganisms, and only an overall value of the folate content is gained, and there are great differences in the measured levels from one laboratory to another (Ek, 1983; Sauberlich *et al.* 1987; O'Broin & Kellecher, 1992; Cuskelly *et al.* 1996; Kelly *et al.* 1996; Selhub & Rosenberg, 1997; Truswell & Kounnavong, 1997).

For clinical purposes methods based on enzyme-linked immunoassay (ELISA) and commercial radioimmunoassay

(RIA) have replaced the microbiological method. These assays are simpler to perform than microbiological assays and are not affected by antibiotics. However, the affinities of different folate monoglutamates for the binding proteins vary considerably, making this assay method useful only for those tissues in which one form of folate predominates, e.g. serum or plasma. In addition their specificity for individual folate forms has been reported as being not uniform and the response to conjugated folates is also unclear (Jagerstad & Pietrzik, 1993; Wigertz & Jagerstad, 1995; Ortega *et al.* 1996; Quinn & Basu, 1996).

HPLC is the preferred way to measure folate, because HPLC provides quantitative information of the folate derivatives. However, HPLC methods can separate relevant folate forms, only in the form of monoglutamate derivatives (Lucock *et al.* 1995; Wigertz & Jagerstad, 1995). No standardised procedure for the hydrolysis of polyglutamate folates has been adopted, and the conjugase preparations differ in sources, activities, optimal pH and hydrolysis products (Wigertz & Jagerstad, 1995).

More complex affinity HPLC methods, which provide information of the distribution of glutamic acid chain length, are described, but these are not yet suitable for status assessment (Selhub, 1991; Selhub & Rosenberg, 1997) Improving ways to chromatographically speciate folyl-mono- (and poly)glutamates will open up a new frontier in understanding one-carbon metabolism in health and disease.

3.1.6. Conclusions. The link between dietary exposure and the biomarker can be compromised by several analytical and methodological problems. For folate the following should be considered.

- Choice of matrix is very important. It is better to measure erythrocyte folate than plasma folate.
- Standardisation of HPLC methods is needed.
- Standardised procedures for the hydrolysis of polyglutamate folates are needed.
- How many and which derivatives should be measured?
- How does HPLC compare with other methods which measure total folate?
- There is a great need for a better understanding of folate bioavailability and the factors that influence it.

3.2. Selenium

3.2.1. Importance of Se. Selenium (Se) is an element which has attention focused not only on its ability to behave as both an essential and a toxic element, but also on its putative role in disease and cancer prevention (Crews, 1998, 2001). Under ideal conditions, the essential and toxic effects should be controlled by appropriate dietary intakes of this element. However, it is not yet clear if the application of Se in disease prevention will require food fortification or dietary supplementation with elevated levels of specific chemical forms of this element. Studies with rodents indicate that Se supplementation, at levels above dietary requirements, is capable of lowering the incidence of tumorigenesis induced by chemical carcinogens or viruses (Diplock, 1993; Ip & Lisk, 1994). A recent human study indicated that certain cancers were prevented when daily

supplementation with yeast containing Se at 200 μg was given (Clark *et al.* 1996). This level is above both the United Kingdom Reference Nutrient Intake (70 and 60 μg/day for men and women, respectively) (Department of Health, 1991) and the United States National Research Council Recommended Daily Allowance (70 and 55 μg/day for men and women, respectively) (National Research Council, 1989). In order to understand how best to provide dietary sources for both essential and disease preventative functions, our knowledge of, and ability to measure, biomarkers of Se status needs some clarification. Diplock (1993) stated that two factors play an important part in forming a judgement about the reliability of blood or other tissue measurements of Se as an index of Se status. Firstly, the methodology for making the measurement of Se must be reliable and reproducible, and secondly, it must be established that the variable measured is directly related to the biochemical variables for Se activity *in vivo*.

3.2.2. Possible biomarkers of Se status. Selenium is essential for many biochemical pathways through a range of Se-containing proteins, hence there are many potential indicators of Se status (Arthur, 1999; Crews, 2001). Blood plasma or serum concentrations can indicate changes in status for populations exposed to low or moderate levels of the element and are commonly used in clinical practice. The levels can vary widely between populations and are related to dietary intake (Thomassen & Aaseth, 1989).

It seems reasonably well established that blood plasma Se will indicate an individual's status with regard to dietary Se intake but what of the status with regard to the known selenoproteins such as the glutathione (GSH; EC 1.11.1.9) peroxidases, Se-protein P, the iodothyronine deiodinases (EC 3.8.1.4) and sperm capsular protein? Arthur & Beckett (1994) point out that an important consequence of identifying the different glutathione peroxidases is the possibility that each has a different sensitivity to Se deficiency. For a better assessment of Se status, blood plasma Se concentration could be used in conjunction with a functional marker of effect, such as platelet GSH peroxidase activity (Crews *et al.* 1997).

However, functional effect assays using GSH have some limitations. For example, erythrocyte GSH peroxidase activity represents an easily accessible functional index of Se status although it offers poor sensitivity to modification. Increased activity was slow after supplementation of depleted subjects with 100–200 μg Se/day reflecting the long life span of red blood cells in circulation (Nève & Carpentier, 1989); and, platelet GSH activity can reflect recent changes in intake and body stores rather than long-term status (Whanger *et al.* 1988). In addition, for GSH peroxidase activity in plasma or blood cells there appears to be a plateau for the Se concentration in blood or plasma beyond which there is no increase in activity (Marchaluk *et al.* 1995). For erythrocyte GSH, the peroxidase activity plateaus at plasma Se levels between 0·76 and 0·89 μmol/l and in whole blood at 0·76 and 2·03 μmol/l in different studies (Nève, 1991; Marchaluk *et al.* 1995); for platelet GSH activity the plasma Se plateau has been found to be 1·39–1·71 μmol/l (Nève, 1991) and 1·25–1·45 μmol/l (Alfthan *et al.* 1991). In a recent human dietary intervention study, erythrocyte and plasma GSH peroxidase were found

not to be sensitive to large changes in Se intake over a six-week period whereas plasma Se concentrations determined after intakes of 25 and 425 µg/day for six weeks (mean plasma Se 64·6 and 103·8 µg/l, respectively) and platelet GSH peroxidase activity (for 425 µg/day intake, mean plasma Se 103·8 µg/l) were more sensitive indicators of change in Se status (Fox *et al.* 2000).

Since the Se content of GSH peroxidase and selenoprotein P in plasma can be saturated over the general nutritional range of intakes, it is not likely to respond to the wide range of intakes involved in long term Se supplementation (Janghorbani *et al.* 1999), which may be used in future disease prevention therapies. Conversely, on low Se intakes, adaptation may take the form of redistribution of Se among selenoproteins when supplements are given. Thus supplementation may result in Se in plasma being retained with albumin and not repleting critical pools of Se, because adaptation will have already ensured that dietary Se goes to the high priority protein-selenoprotein P (Finley *et al.* 1999).

A second major class of selenoproteins are the iodothyronine deiodinases which catalyse the conversion of thyroxine into its biologically active form, triiodothyronine and its inactive derivative reverse triiodothyronine (Arthur *et al.* 1997). Although a more thorough understanding of the mechanisms is required, it is possible that measurement of the T4:T3 values (ratio of thyroxine to the active hormone triiodothyronine) in human plasma may give an indication of iodothyronine deiodinase activity in otherwise inaccessible tissues (Olivieri *et al.* 1995; Arthur, 1999), providing a biomarker for a different aspect of Se biochemistry.

In summary, the following determinations have been suggested as useful in assessing functional Se status: plasma or whole blood Se concentrations, plasma GSH peroxidase activities, erythrocyte GSH peroxidase activities, Se-peroxidase activities in blood cells (platelets, lymphocytes and neutrophils), and thyroid hormone levels (Arthur, 1999). Given the wide range of functions of Se it is unlikely that any one biomarker will fit all requirements but direct measurement of Se in whole blood and plasma is currently the most common way of assessing Se status (Diplock, 1993).

3.2.3. Analytical considerations. Methods for determining total Se levels are generally well established and, with a number of certified reference materials now available, should enable the provision of reliable data. Most methods of determination (with the exception of purely instrumental methods such as neutron activation and X-ray fluorescence analysis) require the sample to be a dissolved mineralised state (Alt & Messerschmidt, 1988). For human tissues, wet ashing with acid (nitric and/or sulphuric and/or perchloric) is preferred. Extreme care and strictly controlled procedures are required to prevent losses of Se (Thomassen & Aaseth, 1989).

However, Reilly (1996) made the point that (in relation to the measurement of Se for reliable dietary intake data at both national and regional levels), 'in spite of the enthusiasm with which many investigators responded to this need for information, a good deal of what was published appeared to be contradictory and of doubtful reliability'.

In the same year, Alfthan & Nève (1996) critically reviewed published serum and plasma Se data using the TRACY protocol (an international project for producing reference values for concentrations of trace elements in human blood and urine). The TRACY criteria for assessing the information included the number of subjects and selection criteria, health status, diet, contamination control and sample storage and analytical treatment. Of the 36 papers covered (restricted to those produced in the previous 10 years), no paper received the maximum rating possible. It was noted that only two set out to determine reference values and one normal values. The authors write in their final paragraph: 'Analytical instruments have improved tremendously during the past decade. Unless they are applied critically and the results are presented with sufficient background information on the study population, analytical reliability and data treatment, published data may be of little use or even misleading'. Therefore, with regard to plasma Se as a biomarker of dietary exposure, the validity of the measurement may comprise the usefulness of the biomarker.

From an analytical viewpoint, the methods for some functional effect assays (GSH peroxidase activity in whole blood, serum, plasma and platelets), are less robust than total Se determinations. It should be noted that the methods are generally not standardised nor collaboratively tested (Crews *et al.* 1997), and variants of two generally accepted methods (Paglia & Valentine, 1967; Wendel, 1981) are used. The enzymic techniques for measurement of GSH activity can be difficult, having limited sensitivity, specificity and stability, as well as problems in obtaining a constant blank and defining a reference method (Faraji *et al.* 1987; Whanger *et al.* 1988; Huang & Åkesson, 1993). To further complicate the situation, there may be different homeostatic mechanisms for activity in different blood fractions (Reilly, 1996). Some radioimmunoassays have been developed for GSH serum and plasma for example (Huang & Åkesson, 1993; Huang *et al.* 1995). Commercial kits are available based on radioimmunoassay methods but these can be expensive, especially when considering population studies.

3.2.4. Conclusions. Ideally, if say three different but robust and well understood measurements of Se status could be made (e.g. plasma Se, platelet GSH peroxidase activity and perhaps an assessment of thyroid hormone activity?), then this 'battery' approach might enable individual Se status to be better defined and requirements for enhanced health better targeted. Such an approach would rely on a good understanding of the mechanisms of the various roles of Se and established and validated analytical procedures.

The latter part of this statement applies to all types of analyses and has added importance when data are used for informing policies for health and legislation. Selenium is currently of worldwide interest to the food, pharmaceutical and nutraceutical industries, to consumers, clinicians and academics. To make best use of the potential of Se to benefit human health, it is essential that accurate measurements of biomarkers for Se exposure and effect are conducted. Varo *et al.* (1994) commented that because the changes in disease rates result from a multitude of other factors, it will be

Table 1. Intakes of certain flavonoids in different population groups

Groups population	No. of subjects	Gender	Age (years)	Flavonol and flavone intake* (mg/day)	Reference
Zutphen (1993)	805	m	65–84	25·9±14·5	(Hertog *et al.* 1993)
Finland	5133	m/f	30–69	0–41·4	(Knekt *et al.* 1996)
USA	34789	m	40–75	7·1–40·0	(Rimm *et al.* 1996)
UK	1900	m	49–59	26·3±12·5	(Hertog *et al.* 1997)
Zutphen (1996)	552	m	50–69	23·5±7·6	(Keli *et al.* 1996)
Seven countries study	12763	m	40–59	2·6–68·2	(Hertog & Kromhout, 1995)

*Quercetin, kaempferol, myricetin, luteolin and apigenin.

difficult to determine whether an increased Se intake influences the health of a whole nation. Thus, conclusions on the role of Se in health must be based on experimental and clinical studies, on epidemiological studies with populations and on controlled clinical trials. Accurately measured and relevant biomarkers of Se status are an essential part of this process.

4. Effects of metabolism

4.1. Flavonoids

There are many examples of (iso)flavonoids in the diet, but the most data related to biomarkers of exposure are available for catechins (flavan-3-ols), quercetin derivatives (flavonols), genistein and daidzein (isoflavonoids). However, even the data for these compounds derive from only a few papers. Dietary flavonols and isoflavonoids are usually ingested as glycosides which can dramatically modify the biological properties. Further, there are several forms of catechin which include ester-linked galloyl groups ((+)-catechin (C), epicatechin (EC), epigallocatechin (EGC) and epigallocatechin gallate (EGCG)), and these modifications also change the absorption and biological properties.

4.1.1. Validity or adequacy of the biomarker. Further modification to the flavonoid is brought about by metabolism, which occurs by the intestinal mucosa/liver/kidney and by the gut microflora. Description of a detailed metabolic pathway for these compounds is not yet possible, but can involve deglycosylation in the small intestine (Day *et al.* 1998, 2000) or colon. Alternatively, breakdown in the colon by microflora and subsequent absorption of flavonoids or breakdown products such as benzoic and cinnamic acids can occur, followed by methylation, sulphation and glucuronidation of intact flavonoids or breakdown products in tissues such as liver and kidney. Most of the methods for measuring these metabolites are by HPLC, which especially with UV detection, is not sensitive enough to measure all of the metabolites. Further, the biological properties of the polyphenols are modified by metabolism (Day & Williamson, 2001), which makes prediction of effect difficult. Most methods so far use chemical or enzymic hydrolysis to convert some of the metabolites back into free aglycone.

Methods for analysis of polyphenols in urine and plasma employ gas chromatography, or HPLC with various detectors such as UV, mass spectrometric or electrochemical systems (Hackett *et al.* 1985; Lee Mao-Jung *et al.* 1995; Gross *et al.* 1996; Unno *et al.* 1996; Maiani *et al.* 1997; Nakagawa *et al.* 1997; Nakagawa & Miyazawa, 1997; Paganga

& Rice-Evans, 1997; Mauri *et al.* 1999). However, the results have not been validated between laboratories, and this is hampered by the lack of knowledge on the metabolites of most polyphenols in humans. It is also very difficult to obtain, use and quantify appropriate standards, especially of the metabolites. As a consequence of this, there is no reliable and comprehensive source of data for the content of phenolics in food and drink and often each country uses different tables. This leads to difficulties in estimation of the total intakes of phenolics.

There are some data on the intake of selected flavonoids in different population groups using compositional data (Hertog *et al.* 1993, 1997; Hertog & Kromhout, 1995; Keli *et al.* 1996; Knekt *et al.* 1996; Rimm *et al.* 1996), but there are no measurements of polyphenols in urine or plasma from the same studies to accompany this. The data show that typical intakes of selected flavonoids (quercetin, kaempferol, myricetin, luteolin and apigenin) are 0–68 mg per day (Table 1).

An approach has been developed using volunteer ileostomists, to avoid the complication of microbial fermentation and degradation in the colon which is usually a complicating factor in determination of metabolism of polyphenols. As an example, average absorption in the small intestine of quercetin (present as glycosides) from onions was 52 % of the total consumed, but most of this could not be accounted for in the plasma (Table 2) (Hollman *et al.* 1995). However, the method relies on subtraction of the amount found in the output from the small intestine from the total consumed.

There is more information on the uptake of isoflavonoids. As an example, uptake of genistein and daidzein was measured in 12 female subjects aged 19–41 years (Xia *et al.* 1994). The results given in Table 3 show that the concentrations of genistein and daidzein in the plasma were up to 2·2 μM, and in urine up to 14 and 6·2 μM, respectively, and that these levels were approximately proportional to intake.

4.1.2. Conclusions. Research to examine the effect of flavonoids *in vivo* in humans is limited compared to nutrients, and there is a need for further work. Future work could be to establish the half-life after ingestion of habitual dietary intake of these compounds in human, to establish the magnitude of the potential health effects in relation to other dietary components. It is also important to establish the baseline habitual level of polyphenols and metabolites in the plasma or urine in non-supplemented individuals. One of the most important areas is to elucidate the metabolism so that measurement of biomarkers can take account of this.

Table 2. An example of a study showing flavonoid (quercetin) uptake and metabolism using ileostomist subjects: nine healthy ileostomy subjects received a supplement of fried onions at breakfast (rich in quercetin glycosides) equivalent to 89 ± 14 mg quercetin aglycone; nine subjects received pure quercetin rutinoside, equivalent to 100 ± 5 mg; and nine subjects received 100 ± 5 mg of pure quercetin aglycone (Hollman *et al.* 1995)

	Ileostomy effluent*		Urine*	
Form of dietary flavonoid	Before (mg)	After (mg)	Before (μg)	After (μg)
Onion	1·8±1·1	37±11	1·2±1·9	275±129
Quercetin 3-rutinoside	1·3±0·9	72±15	3·6±5·1	73±190
Quercetin aglycone	1·7±1·3	66±9	1·0±1·1	115±75

* Amount of quercetin (as aglycone) over a time period of 13 h after ingestion.

4.2. Carotenoids

4.2.1. Metabolism of a compound may affect the validity of a biomarker.

This problem can be illustrated well by consideration of the carotenoids. More than 600 carotenoids exist in plants. Approximately 50 of them might be consumed in the human diet, yet only five to seven are found in serum in significant amounts: these are α- and β-carotene, β-cryptoxanthin, lycopene, lutein and zeaxanthin. Most of the available information concerns these compounds (Krinsky, 1993; Michaud *et al.* 1998), and β-carotene, which is the only one to figure in food composition tables. Yet some others may have interesting properties that would deserve a more thorough evaluation.

Biomarkers used to evaluate the dietary intake and/or the protective potential of carotenoids are either the serum carotenoid profile (Cooney *et al.* 1991) or, to a lesser extent, the amount of carotenoids in buccal mucosal cells (Peng *et al.* 1994). However, carotenoids undergo isomerisation or metabolism, and the resulting compounds, which are often not identified by usual analytical techniques, may differ in their biological effects.

4.2.2. Metabolism and isomerisation.

Some carotenoids (mainly α- and β-carotene; β-cryptoxanthin) are able to produce vitamin A by an enzyme-induced pathway in the intestine and/or the liver. Retinal is formed by central cleavage of the molecule and will be either reduced in retinol or oxidised in retinoic acid (Nagao *et al.* 1996). An eccentric cleavage pathway of provitaminic carotenoids may produce various apo-carotenals (Wang & Krinsky, 1997). The efficiency of this metabolism is not precisely known, yet it can affect most of the ingested carotenoid, which may then represent a large part of the vitamin A supply. Moreover, there is a high interindividual variability in this cleavage (Dimitrov *et al.* 1988). In consequence, the serum level of provitaminic carotenoids reflects only partly the dietary intake.

Carotenoids are susceptible to isomerisation, either in plants, or during storage and processing (Chen *et al.* 1994; Doering von *et al.* 1995). The absorption and subsequent distribution of these isomers in tissues vary. After ingestion of a mixture of β-carotene isomers, only the all-*trans* form is found in the blood, whereas 13-*cis* or 9-*cis* isomers may represent up to 20 % in some tissues. In contrast, the isomer pattern is similar in tissue and in serum after ingestion of a mixture of lycopene isomers (Chen *et al.* 1994; Doering von *et al.* 1995).

In addition, carotenoids such as lycopene or lutein are metabolised *in vivo* following oxidation or non-enzymatic dehydration (Khachik *et al.* 1995). Most of the routine HPLC methods for carotenoid analysis do not discriminate between these isomers or metabolites, and may not give an accurate estimation of the dietary carotenoid intake (Nelis & Deleenher, 1983; Bieri *et al.* 1985; Hart & Scott, 1995). More specific methods exist which allow the identification of carotenoid metabolites and/or isomers (Schmitz *et al.* 1994; Khachik *et al.* 1997; Lessin *et al.* 1997). However, none of these methods has been agreed as a reference method, nor have any been thoroughly validated.

4.2.3. Antioxidant role.

Beyond the difficulties that the metabolic pathways may add to the evaluation of dietary exposure to carotenoids, it may also hamper the correct estimation of the biological effects of the carotenoids, and especially of their antioxidant roles. The antioxidant potency of one single molecular species is difficult to assess unequivocally. The results of *in vitro* studies are conflicting and some authors suggest that, *in vivo*, the 9-*cis* isomer of β-carotene could be a more efficient antioxidant than the all-*trans* form (Schmitz *et al.* 1991; Schut *et al.* 1997). The interaction between the various dietary antioxidants should

Table 3. An example of a study showing isoflavonoid uptake and absorption in 12 female subjects aged 19–41 years (Xia *et al.* 1994)

Dietary intake (mg)		Urine after 12–24 h (μM)		Plasma after 6.5 h (μM)	
Daidzein	Genistein	Daidzein	Genistein	Daidzein	Genistein
24·7±3·66	19·3±2·9	4·5±3·1	0·93±1·55	0·79±0·04	0·74±0·44
45·9±6·80	36·2±5·4	11·5±10	3·89±6·07	1·22±0·67	1·07±0·63
70·7±10·5	55·7±8·2	14·2±12	6·18±9·77	2·24±1·18	2·15±1·33

not be underestimated when assessing the protective potential of carotenoids.

A better knowledge of the metabolism and physiology of carotenoids is required in order to correlate the level of serum or tissue carotenoids with their antioxidant potency. Accurate and validated analytical methods need to be developed and used in order to adequately compare the data obtained in different laboratories.

5. Consistency of information from several biomarkers for one component

5.1. Vitamin A

5.1.1. When assessing exposure to a nutrient or a non-nutrient, the information obtained via several biomarkers could be inconsistent. An adequate knowledge of the physiology and metabolism of the compound is needed to correctly interpret the data. Vitamin A status exemplifies the problem as it can be assessed by several biomarkers.

5.1.2. Retinol. The most frequently used biomarker is serum retinol, although it has a major drawback. The retinol concentration in serum is tightly regulated to be around $2\,\mu M$, and is not related to the food intake or to the level of vitamin A body stores (i.e. liver), except in extreme hypo- or hypervitaminosis A (Underwood, 1994). Another test estimates both the liver stores and the ability to mobilise these stores. This is the relative dose-response test (RDR) which measures the possible transient increase of serum retinol 5 hours after a physiological oral dose of vitamin A. Because serum retinol is regulated, it should not vary during this period, and, in normal conditions, the newly absorbed vitamin A is stored. However, if the subject's tissues urgently require vitamin A, the newly absorbed vitamin A will be immediately mobilised and this will lead to a limited and transient, but still detectable, increase in serum retinol (Amadee-Manesme *et al.* 1984).

5.1.3. Impression cytology. A third series of tests investigate vitamin A-dependent functions. Impression cytology refers to the role of vitamin A as a protector of epithelia. The fact that the eye is the organ studied does not mean that these tests have a link with the biochemistry of vision, although a severely altered conjunctiva will lead to impaired vision. This test stains for goblet cell presence in the eye conjunctiva after collection on a filter paper (Natadisastra *et al.* 1987).

5.1.4. Interpretation of two or more biomarkers. When two or more of these biomarkers are used on the same individual or in the same population, it can happen that the serum retinol is in the normal range whereas the RDR indicates a deficient or worse-than-deficient status, and results from impression cytology may or may not be in the normal range. Similarly, other combinations of status may be found (Ward *et al.* 1993; Azais-Braesco *et al.* 1995). These situations may be the result of the threshold values chosen to delineate the different measures of status. However, some discrepancies sometimes remain, even when thresholds are modified. To overcome these discrepancies, one should remember the physiological basis of the biomarkers. Impression cytology detects early functional symptoms of vitamin A deficiency. Serum retinol reflects only very low

or very high liver vitamin A stores, and gives little information about the functionality of these stores. The RDR estimates the adequacy of the liver stores to fulfil the tissue requirements and gives information about the metabolic ability of the organism to mobilise these stores. Retinol is secreted from the liver bound to retinol-binding protein (RBP), which has a short half-life of 12 hours. Because RDR addresses retinol secretion over a short period of time, it may detect a default in the synthesis or secretion of RBP, which cannot be seen with a simple measurement of serum retinol. This could be the case during liver diseases and protein malnutrition (Russell *et al.* 1983) or in elderly people (Bulux *et al.* 1992). Other pathophysiological conditions, such as infectious or inflammatory diseases, renal failure and diabetes alter RBP metabolism (Goodman, 1984) and may consequently modify the meaning of the results of the RDR test. In contrast, serum retinol could be decreased below normal levels in some situations, e.g. total parenteral nutrition (Howard *et al.* 1980), and retinoid therapy (Formelli *et al.* 1993), although liver vitamin A stores are likely to be adequate. In such situations, clinical symptoms of vitamin A deficiency might be observed.

The information given by one biomarker should therefore be carefully interpreted. This often requires additional data, not directly related to the compound, yet necessary to ensure that the considered biomarker is valid. Whenever possible the combined use of several biomarkers, should be favoured. Although vitamin A metabolism has been and is still being extensively studied, a non-ambiguous biomarker of vitamin A functional status is still awaited.

6. Effects of individual life-styles

6.1. Vitamin B_{12}

6.1.1. Variation in dietary intake due to different life-styles may influence the usefulness of the biomarker. This is exemplified by Vitamin B_{12} intake from the diets of vegetarian and non-vegetarians. All vitamin B_{12} found in nature is made by micro-organisms. The vitamin is absent from plants except when they are contaminated by micro-organisms (Herbert, 1997). Dietary deficiency of vitamin B_{12} can result from a strict vegan (all plant food) diet because vitamin B_{12} is not synthesised by any plant, nor does any plant need, use, or store vitamin B_{12}. Vegetarians who include animal food in their diets (e.g. milk and eggs) ingest adequate amounts of vitamin B_{12}, and if they develop vitamin B_{12} deficiency, it is for the same reasons that omnivorous subjects develop deficiency (Herbert, 1994). Human milk of women consuming strict vegetarian diets contains significantly less vitamin B_{12} than milk from women consuming omnivorous diets, and among women consuming vegetarian diets, milk vitamin B_{12} concentrations are inversely related to the length of time a vegetarian diet has been consumed (Specker *et al.* 1990; Ford *et al.* 1996). That vitamin B_{12} deficiency in some third-world vegan children and adults is delayed may in part be related to cleanliness. The less thoroughly hands are washed after defecating and the more frequently they suck their fingers, the more they protect themselves against vitamin B_{12} deficiency (Herbert, 1997).

6.1.2. Stages of negative cobalamin balance. When the supply of vitamin B_{12} is discontinued, four stages of negative cobalamin status are recognised: stage I, serum depletion and low holotranscobalamin II (holoTCII), stage II, cell depletion (low holohaptocorrin and low red cell vitamin B_{12} concentrations), stage III, biochemical deficiency (slowed DNA synthesis, elevated serum homocysteine and methylmalonate concentrations); stage IV, clinical deficiency (Herbert, 1994, 1997). Vitamin B_{12} deficiency can occur if ingestion (vegetarians), absorption and utilisation are inadequate and if requirement, excretion and destruction of the vitamin is increased. Depletion precedes deficiency by months to years. It is possible to diagnose negative balance at either of the two stages of depletion (serum and cell depletion) before the negative balance reaches the stages of deficiency (Herbert, 1994).

6.1.3. Indicators of depletion. Total serum vitamin B_{12} is the sum of vitamin B_{12} bound to the circulating delivery protein, transcobalamin II (TCII), and vitamin B_{12} bound to the circulating storage protein, haptocorrin. TCII delivers the vitamin to all metabolically active tissues. Total serum vitamin B_{12} concentration is a relatively late indicator of deficiency because normally about 80 % of total serum vitamin B_{12} is bound to serum holohaptocorrin, a late indicator, and only about 20 % is bound to the early indicator, serum holoTCII. Because TCII is depleted of vitamin B_{12} within days after absorption is stopped, the best screening test for early negative vitamin B_{12} balance is a measurement of vitamin B_{12} bound to TCII (holoTCII). Due to the very short half-life (6 min) of holoTCII, malabsorption of vitamin B_{12} rapidly produces a decrease in the amount of holoTCII (Das *et al.* 1991; Tisman *et al.* 1993; Herbert, 1994; Scott, 1997). Vegans with relatively low total serum vitamin B_{12}, but no evidence of biochemical or clinical deficiency have borderline low holoTCII ranging between 40 and 60 pg/ml (normal range: 200–900 pg/ml; mean: 450 pg/ml) (Herbert, 1994).

6.1.4. Indicators of deficiency. Vitamin B_{12} functions as a cofactor for several enzymatic reactions in cellular metabolism and, in particular, is needed for the conversion of methylmalonyl-CoA into succinyl-CoA. Deficiency of vitamin B_{12} inhibits this enzymatic conversion and results in an increase in metabolites such as methylmalonic acid (MMA). Urinary excretion of MMA in elevated amounts is one indicator of vitamin B_{12} deficiency (Specker *et al.* 1990; Specker, 1994). Besides, measuring urinary MMA levels makes it possible to distinguish between deficiencies of folate and vitamin B_{12}, since MMA excretion is not increased by folate deficiency (Sauberlich *et al.* 1974). Another indicator of vitamin B_{12} deficiency is low total serum or plasma vitamin B_{12} concentration (Dagnelie *et al.* 1989; Miller *et al.* 1991). Vitamin B_{12}-deficient subjects may also have increased levels of plasma homocysteine (Stabler *et al.* 1990; Hornstra *et al.* 1998).

6.1.5. Clinical deficiency symptoms. Vitamin B_{12} deficiency due to a lack of dietary intake of the nutrient is rare, but can occur among vegans. Such persons may have low serum levels of vitamin B_{12}, and may develop a sore tongue, paraesthesias, amenorrhoea, and other signs of a vitamin B_{12} deficiency. The classical symptoms of vitamin B_{12} deficiency are megaloblastosis, notably macrocytic anaemia, and/or progressive neurological degeneration (Sauberlich *et al.* 1974; Chanarin *et al.* 1985; Bover & Wald, 1995; Herbert, 1997). Since most cases of vitamin B_{12} deficiency are the result of an impaired absorption of the vitamin due to lack of the intrinsic factor in the gastric secretions, vitamin B_{12} deficiency is more a clinical consideration than a nutritional problem (Sauberlich *et al.* 1974). Case reports of breast-fed infants of strict vegetarian mothers, who developed vitamin B_{12} deficiency resulting in severe neurological damage, have been published (Specker, 1994).

6.1.6. Reabsorption. The enterohepatic circulation of vitamin B_{12} is very important in vitamin B_{12} economy and homeostasis. An efficient enterohepatic circulation and significant liver stores of vitamin B_{12} keeps the vegan from developing B_{12} deficiency disease for up to 20–30 years because even as the body store and daily bile vitamin B_{12} output fall, the amount of bile vitamin B_{12} reabsorbed rises to nearly 100 %. Inside the ileal cells reabsorbed, and food, vitamin B_{12} is bound to TCII and the resultant holoTCII is transported via the bloodstream to all the cells that need it (Herbert, 1994).

If daily vitamin B_{12} absorption continues to be less than daily vitamin B_{12} loss, negative balance will progress to stage II, depletion of stores. At this point, many vegans stabilise for years with depleted stores, because depleted stores trigger a more efficient absorption of the trace amounts of vitamin B_{12} from bacterial contamination of the small intestine and vitamin B_{12} secreted in the bile. Eventually, however, continuing slight negative balance will deplete vitamin B_{12} stores, after which stage III negative balance occurs (Herbert, 1994). The infant of a vegan mother, who has almost no enterohepatic vitamin B_{12} circulation because of deficient vitamin B_{12} liver stores will rapidly develop vitamin B_{12} deficiency (Herbert, 1994; Specker, 1994; Sanders & Reddy, 1994).

6.1.7. Analysis of vitamin B_{12}. For routine assessment of vitamin B_{12} nutritional status of individuals, measurement of serum or plasma vitamin B_{12} content is the first choice in most laboratories. The tiny quantity of vitamin B_{12} activity in human serum can be measured microbiologically or by radioassay. Radioassay has many advantages over microbiological assay. About one-third of the 'vitamin B_{12}' in serum is in fact not cobalamins, but other corrinoids, which cannot be used by humans but may be used by bacteria. Thus, many microbiological assays find normal vitamin B_{12} levels in vitamin B_{12} depleted people because the assay in fact measures noncobalamin corrinoids. To avoid this problem, most laboratories use competitive inhibition radioassays that measure only cobalamins. In addition, radioassays are technically simple, easily standardised and precise, and false low results do not occur if serum contains antibiotics or other substances that inhibit growth of microorganisms (van den Berg, 1993; Whitney & Rolfes, 1996; Herbert, 1997).

The earliest detectable serum marker of negative vitamin B_{12} balance is low serum holoTCII. The determination of holoTCII involves only one additional step in the radioassay for vitamin B_{12} (i.e. separation of TCII from haptocorrin by absorption onto a microfine precipitate of silica). Estimation

of the vitamin B_{12} content of the absorbed serum, giving the vitamin B_{12} content bound to haptocorrin, and subtraction of this value from the vitamin B_{12} content of the untreated serum, provides the vitamin B_{12} content bound to TCII (holoTCII) (Das *et al.* 1991; Tisman *et al.* 1993).

6.1.8. Conclusions. Variation in intake can influence the biomarker. Low intake of vitamin B_{12} (e.g. vegans) triggers the reabsorption of vitamin B_{12}, and no useful biomarker of exposure exists in this case. However, serum holoTCII concentration is a good marker of early depletion both in the case of insufficient absorption and intake.

7. Biological effect monitoring

7.1. Fumonisins

Fumonisin mycotoxins are structurally related compounds, produced mainly by *Fusarium verticillioides* (=*F. moniliforme* J. Sheld) and *F. proliferatum*, which occur largely in maize and maize-based food and feed (Shephard *et al.* 1996*a*). Fumonisins in home-grown maize have been associated with an elevated risk for human oesophageal cancer in Transkei, South Africa (Rheeder *et al.* 1992). These mycotoxins, due to their structural similarity with sphinganine (SA) and sphingosine (SO), intermediate compounds in the sphingolipid metabolism, cause the inhibition of sphingolipid biosynthesis through inhibition of the enzyme sphinganine (sphingosine) *N*-acyl transferase (also known as ceramide synthase) (Wang *et al.* 1991). The result of this inhibition is an accumulation of free SA and an increase in the ratio of free SA to free SO in tissues and biological fluids after exposure to fumonisins (Riley *et al.* 1994). The measurement of SA and SA/SO ratio in tissue and biological fluids is a biomarker for assessing exposure to these mycotoxins (Riley *et al.* 1994; Merrill *et al.* 1996). The sensitivity of this biomarker to fumonisins has been demonstrated in different animal species, e.g. pig, rat, horse, poultry, catfish, rabbit and non-human primates (Wang *et al.* 1991; Goel *et al.* 1994; Riley *et al.* 1994; Gumprecht *et al.* 1995; Shephard *et al.* 1996*b*). A positive correlation between fumonisin intake and SA/SO values in rat urine was observed with a correlation coefficient value of 0·99 (Solfrizzo *et al.* 1997*a*). Measurable concentrations of SO and SA have been reported from urine of women, whereas low levels or no measurable concentrations of SA were reported from urine of men (Castegnaro *et al.* 1996; Solfrizzo *et al.* 1997*b*). The SA/SO ratio has been determined in plasma and urine of rural populations in Africa consuming home-grown maize as their staple diet. No significant difference ($P > 0·05$) was found in the SA/SO ratios between populations living in regions where mean total fumonisin levels detected in randomly collected home-grown maize samples were 580 ng/g and <10 ng/g, respectively (van der Westhuizen *et al.* 1999). The need to validate the SA/SO biomarker on humans has been emphasised in a review on progress in the development of biomarkers to better assess human health risks linked with fumonisin exposure (Turner *et al.* 1999).

Sphinganine and SO can be measured by HPLC-fluorescence detection after precolumn derivatisation. The three published analytical methods for the determination of SA and SO in animal serum, urine and tissues and human urine, differ mainly in the clean-up step. In the first method, the extract is measured by HPLC soon after sample extraction with ethyl acetate (Castegnaro *et al.* 1996). In the other two methods, either chloroform is used for solvent extraction, followed by basic hydrolysis and several liquid–liquid clean-up steps (Riley *et al.* 1994) or a simple silica gel minicolumn is used to clean-up the sample before HPLC analysis (Solfrizzo *et al.* 1997*b*).

7.1.1. Biological effect. The SA/SO ratio is a good example of a biomarker of exposure that is also useful for monitoring the biological effect of fumonisins, as ceramide synthase is a key enzyme in the sphingolipid metabolism and sphingolipids have many important functions in cell membrane. The accumulation of SA caused by the exposure to fumonisins has been demonstrated to produce a mitogenic effect that can often affect cell transformation (Schroeder *et al.* 1994; Merrill *et al.* 1996). This effect has been proposed as a plausible molecular mechanism to explain the carcinogenicity of fumonisins (Schroeder *et al.* 1994).

7.2. Polyhalogenated aromatic hydrocarbons (PHAHs)

7.2.1. Introduction. Polyhalogenated aromatic hydrocarbons (PHAHs) are ubiquitous pollutants that are especially associated with aquatic food chains. Highest concentrations are found in those organisms at the top of the food chain such as fish-eating mammals (Murk *et al.* 1997) and thus humans are exposed to these contaminants via dietary intake. PHAHs are lipophilic persistent compounds and elicit a number of species specific toxic responses in laboratory and wildlife species. Relatively low residue levels are only permitted in certain food items (Bovee *et al.* 1998). Polychlorinated biphenols (PCBs), polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are present in the environment, not as individual congeners but as complex mixtures. The relative and absolute concentrations of the individual congeners are continually changing. This fact makes detection complicated and also makes it virtually impossible to accurately predict the biological and toxic potency of these mixtures to animals and humans. Consequently one problem that scientists face in the evaluation of risk to PCBs, PCDDs and PCDFs is the identification and quantification of toxic and/or bioactive congeners in biological samples. Although the present clean-up procedures and high resolution capillary gas chromatography–mass spectrometry (GC-MS) permit the separation, identification and quantification of individual PCDD, PCB and PCDF congeners, these analytical procedures require highly sophisticated equipment and training. They are also very costly and time-consuming, particularly when samples may theoretically contain more than 200 different PCB, more than 130 different PCDF and more than 75 different PCDD isomers and congeners.

Additionally, the concentration of these congeners in a given sample provides only part of the information necessary to evaluate their potential for biological/toxicological effects in humans and animals. Moreover, even if reliable congener-specific concentrations in samples can be determined, it is very difficult to predict their biological effects because the toxic potencies of the congeners can vary

significantly, and interactions among various congeners have exhibited synergism, additivity or antagonism. These interactions have generally not been considered when attempts have been made to predict the biological effects of mixtures. Therefore, from both a human and animal health standpoint, bioassays capable of detecting and estimating the relative biological or toxic potency of complex mixtures of PCBs, PCDDs and PCDFs would be extremely valuable (Brouwer *et al.* 1995).

7.2.2. Receptor-based assays. Numerous bioassays have been developed based on arylhydrocarbon-receptor (AhR)-dependent induction of cytochrome *P*450 1A1. They utilise either AhR-containing extracts, or mammalian cell cultures, to measure a specific biological response (Aarts *et al.* 1996). The majority of the AhR-dependent bioassays are based on measurement of the induction of gene expression. In these assays, the magnitude of induction by the mixture is expressed relative to TCDD (total chlorinated dibenzo-p-dioxins) and the calculated values are then expressed as TCDD-TEQs (toxic equivalents). Induction assays can be based on AhR ligand binding, DNA binding, P450 1A1(EROD) induction or induction of a reporter gene.

7.2.3. CALUX assay. Reporter gene assays have been developed to measure functional inducers via the oestrogen receptor (ER) as well as the Ah. Recombinant cell lines have been developed, which exhibit stable AhR- or ER-mediated firefly (*Photinus pyralis*) luciferase gene expression. The response by, respectively, dioxin or oestradiol in these so-called CALUX (chemical activated luciferase expression) assays is dose-dependent and very sensitive, with detection limits below 1 pM or 0·24 fmol (Legler *et al.* 1996; Murk *et al.* 1996). In addition to measuring the toxic potency of individual compounds or mixtures, the CALUX assay has been successfully applied to monitoring AhR- or ER-active compounds in a variety of matrices, such as human milk and blood plasma (Aarts *et al.* 1996; Murk *et al.* 1997; Bovee *et al.* 1998), cow's milk (Bovee *et al.* 1998), and growth promoting agents, veterinary drugs and environmental contaminants in food (Hoogenboom *et al.* 1999).

8. Effects of interactions

8.1. Interpretation of a biomarker is complicated by interactions

8.1.1. Interactions in the gut. These interactions may modify biomarkers of exposure of the external dose in the gut or the internal dose after uptake in the blood. The complex subject of gastrointestinal physiology and function is well reviewed in Salminen *et al.* (1998) in which it is stated that there is evidence for a strong interaction between the intestinal microflora, gut mucosa and gut-associated lymphoid tissue (part of the mucosal immune system). It is well known, but not well understood, that diet can alter the composition of gut microflora (Rowland, 1991). Similarly, the cellular and molecular events by which the digestive flora influences the immune system are poorly understood and therefore development and validation of biomarkers is not far advanced. Examples of other interactions in the gut are: the inhibition of resorption of nutrients by antinutritional factors such as protease inhibitors,

lectins or haemaglutinins and lathyrogens present in beans; the inhibition of toxicity of bile acids by binding to Ca-phosphate complexes, dilution and increased passage of bile salts by binding to fibre, and the metabolism affecting influence of bacterial enzymes in colon (Jacobson, *et al.* 1984; Potter, 1996; Rijnkels *et al.* 1997; Rijnkels, 1998; Salminen *et al.* 1998).

8.1.2. Polyphenols. The synergistic effects of polyphenols have been studied only rarely and the results are not conclusive (Bors *et al.* 1996; Terao *et al.* 1994; Negre-Salvayre *et al.* 1995). Ratty & Das (1988) have demonstrated that flavonoids interact in the polar surface region of the phospholipid bilayers. It is likely that flavonoids are localised predominantly near the surface of membranes where aqueous peroxyl radicals are trapped easily. They are thus accessible to chain-initiating peroxyl radicals more readily than α-tocopherol and may prevent the loss of α-tocopherol. In rats fed with highly peroxidable oils, the intake of tea catechins prevented a decrease in α-tocopherol (Nanjo *et al.* 1993).

8.1.3. Interactions between antioxidants. Dietary components may interact during their absorption and metabolism and this interaction complicates the interpretation of the related biomarkers The *in vitro* synergistic inhibition of oxidation by vitamins E and C has been widely reported, as well as the recycling of tocopheroxyl radical in tocopherol by ascorbic acid (Niki, 1996). Other studies using *in vitro* or animal systems have confirmed a cooperation among antioxidants such as vitamin C, vitamin E and β-carotene. Of outstanding interest is the co-operation and/or interaction of β-carotene and vitamin E. Palozza & Krinsky (1991) have reported that vitamin E and β-carotene have an additive effect in reducing oxygen radical initiated lipid peroxidation in lipid extract from rat liver microsomes. It has been suggested that vitamin E increases the beneficial effect of β-carotene by protecting β-carotene from autoxidation (Palozza & Krinsky, 1992). Until now, such a metabolic cooperation has not been demonstrated *in vivo*, either in animals or humans. Results obtained in clinical trials regarding the antioxidant cooperation are contradictory. In a well conducted study by Blot *et al.* (1993, 1995) a combination of β-carotene (15 mg/day), vitamin E (30 mg/day) and selenium (50 mg/day) markedly reduced the incidence of oesophageal and gastric cancer whereas other combinations of retinol/ zinc, riboflavin/niacin and ascorbic acid/molybdenum were not effective. One highly controversial study from Finland (Heinonen *et al.* 1994) showed that β-carotene (20 mg/day), with or without vitamin E acetate (50 mg/ day), increased the incidence of lung cancer in male subjects with a chronic smoking habit.

Some experimental studies on humans have reported that supplementation with one dietary antioxidant modifies the serum concentration of other natural dietary antioxidants. However, there is not yet a consistent trend in the results from various authors. When β-carotene (15–60 mg/day during 3 or 4 months) is supplied to volunteers, the serum vitamin E level is decreased in some studies (Xu *et al.* 1992), whereas it remains stable in others (Willet *et al.* 1983; Mobarhan *et al.* 1994; Heinonen *et al.* 1994; Pappalardo *et al.* 1997). A β-carotene supplementation (20 mg/day for 2 years) in patients with colorectal adenomas

enhances the serum levels of other carotenoids, such as lycopene or α-carotene (Walquist *et al.* 1994). The opposite result was obtained in healthy patients receiving β-carotene (30 mg/day) during 6 weeks (Micozzi *et al.* 1992). In one study, the ingestion of a diet rich in β-carotene, lutein and lycopene led to a decrease in serum vitamin E (Chopra *et al.* 1996). It should be pointed out that in epidemiological studies without supplementation, the plasma concentrations of β-carotene and α-tocopherol are not correlated (Ascherio *et al.* 1992).

8.1.4. Considerations when using biomarkers. The above examples show that interactions or cooperation between dietary antioxidants are likely. Yet, it is difficult to clearly establish the conditions in which they occur; most of the discrepancies observed between the studies, especially on humans, might be explained by the differences in the experimental setting: dosages, duration, sex, age, pathologies, precise control of all the dietary antioxidants. However, some hypotheses exist to explain the interactions observed. They may be due to:

(1) a competition in intestinal absorption (Kayden & Traber, 1993; Kostic *et al.* 1995; Parker, 1996);
(2) a competition in serum transport, especially for lipophilic compounds which have to be carried on by lipoproteins (Chopra *et al.* 1996);
(3) a possible sparing effect of one antioxidant by another one (Xu *et al.* 1992; Walquist *et al.* 1994);
(4) a cooperative regeneration of one antioxidant by another, e.g. vitamin E which is regenerated by vitamin C (Niki, 1996);
(5) a cooperation between the various antioxidant molecules, which have different efficacies, in different physio-chemical environments (aqueous vs. lipohilic, various oxygen pressures) towards different free radical species (Burton & Ingold, 1984; Niki, 1996);
(6) an antioxidant or pro-oxidant effect of one antioxidant present in high concentrations (Blot *et al.* 1993, 1995; Goodman *et al.* 1993; Heinonen *et al.* 1994; Chopra *et al.* 1996).

The following considerations should be remembered when the serum level of an antioxidant is to be used as a biomarker. Firstly, the validity of the serum levels of antioxidant dietary components is limited to physiological levels. Outside the physiological range, the interaction between the compounds may affect the meaning of circulating values. This is especially true for lipophilic molecules. Secondly, when considering the antioxidant status, it is recommended that all, or as many as possible of the dietary antioxidant molecules (i.e. vitamin E, vitamin C, major polyphenols, Se) are considered, rather than one in isolation.

8.2. Total antioxidant capacity (TAC)

Free radical mechanisms are likely to be implicated in the pathogenesis of many degenerative diseases such as various types of cancer, cardiovascular and neurological diseases, cataract and oxidative stress dysfunction (Halliwell, 1996). Free radicals could be scavenged by naturally occurring antioxidants in (target) tissues. Measurement of the TAC of biological fluids might be useful as a marker for the ability of antioxidants present in body fluids to prevent oxidative damage to membranes and other cellular components.

There is an increasing amount of data indicating that various dietary constituents or supplemental ingredients act as 'antioxidants'. These antioxidants are often implicated in the prevention of various diseases (Halliwell, 1987). For some, e.g. α-tocopherol, there are sound *in vivo* data whereas for others, e.g. wine flavonoids or carotenoids, data to support their effectiveness *in vivo* in humans are limited.

8.2.1. Non-specificity. Antioxidants cooperate in the defence against oxidative stress, therefore assessing one antioxidant in isolation from the others is mainly for mechanistic purposes. Interest has therefore been focused on measurement of TAC in biological matrices and effects of dietary factors. The antioxidant capacity of biological samples can be monitored by a variety of simple, non-specific, screenings assays. These assays are scavenging assays using relatively 'stable' synthetic free radicals (e.g. ABTS), or reactive 'natural' occurring radicals (e.g. $OH^{\bullet}$ or $O_2^{\bullet -}$). The TAC of biological samples can also be evaluated in clinical studies which measure endproducts of free-radical damage of endogenous compounds such as lipids or DNA. Changes from the baseline levels of these products could then be ascribed to changes in the antioxidant capacity of the diet. A general problem with these assays is that they are poorly validated, and therefore, their specificity as biomarkers is still under debate.

8.3. Scavenging assays using 'stable' synthetic free radicals

8.3.1. Trolox equivalent antioxidant capacity (TEAC). The TEAC assay is used for measurement of the TAC of biological matrices such as plasma and serum. It is a spectrophotometric detection method based on scavenging of long-lived radical anions ($ABTS^{\bullet -}$). All compounds, present in the serum or plasma, that are able to scavenge the radicals are detected as potential antioxidants. The greater part of the activity of serum or plasma (approximately 80 %) is ascribed to albumin and urate (Wayner *et al.* 1987), thus effects of antioxidant supplementation are relatively small. Differences in serum TEACs have been described in critically ill patients (Dasgupta *et al.* 1997), but effects of antioxidant supplementation on the assay are still controversial (Maxwell *et al.* 1994; Ryan *et al.* 1997).

8.3.2. Oxygen radical absorbance capacity (ORAC). The ORAC assay is a relatively simple, but sensitive method, and can be used for quantifying the oxygen radical absorbance capacity of antioxidants in biological tissues (Cao *et al.* 1993). In this assay, β-phycoerythrin is used as an indicator protein and 2,2′-azobis(2-amidinopropane) dihydrochloride as a peroxyl radical generator. This assay is unique because the reaction goes to completion so that both inhibition time and inhibition degree are considered in quantifying ORAC. The ORAC assay has high specificity and responds to numerous antioxidants (Cao & Prior, 1998). By utilising different extraction techniques in sample preparation, one can remove serum proteins making the ORAC assay more sensitive for antioxidant changes in serum or plasma.

8.3.3. 1-diphenyl-2-picrylhydrazyl (DPPH) free radical method. The relatively stable DPPH free radical is used for antioxidant activity measurements of lipid-soluble compounds. The disappearance of the radical can be followed spectrophotometrically and expressed as radical scavenging ability (Bondet *et al.* 1997). Interpretation of the assay could be complicated if the absorption spectra of the test compounds overlap with the DPPH spectrum (515 nm) as with compounds such as carotenoids (Noruma *et al.* 1997).

8.3.4. Ferric reducing ability (FRAP) assay. This is a simple, automated test measuring the ferric reducing ability of plasma. This assay has been presented as a novel method for assessing 'antioxidant power' (Benzie & Strain, 1996). Ferric to ferrous ion reduction at low pH causes a coloured ferrous-tripyridyltriazine complex to form. FRAP values are obtained by comparing the change in absorbance. This assay is simple and inexpensive but does not measure the sulphydryl group-containing antioxidants (Cao & Prior, 1998). The antioxidant capacity of an antioxidant against a free radical does not necessarily match its ability to reduce Fe^{3+} to Fe^{2+}. Interpretation of the assay could be complicated since the reaction of iron is also involved in free radical generation processes, e.g. the catalysed generation of $OH^{\bullet}$ from H_2O_2.

8.4. Scavenging assays with specific radicals

8.4.1. Hydroxyl and superoxide radical scavenging. Scavenging by the hydroxyl radical ($OH^{\bullet}$) and the superoxide radical ($O_2^{\bullet-}$) can be measured indirectly. In the $OH^{\bullet}$ scavenging assay, or the 'deoxyribose assay', $OH^{\bullet}$ radicals are generated from hydrogen peroxide, ascorbate and $FeCl_3$. Scavenging activity can be assessed by measuring competition between the test compound with deoxyribose for hydroxyl radicals (Halliwell *et al.* 1987). However, artifacts may occur with this test as some substances can rapidly react with hydrogen peroxide. Also powerful iron-chelators cannot be used in this assay as the compounds interfere with the measurement products.

In the superoxide radical scavenging assay, $O_2^{\bullet-}$ is generated from xanthine/xanthine oxidase (X/XO) and scavenging can be measured by reaction of $O_2^{\bullet-}$ with cytochrome *c* or nitroblue tetrazolium (Halliwell, 1985). Also this assay is sensitive to artifacts which include inhibition of xanthine oxidase or reduction of cytochrome *c* or nitroblue tetrazolium. High absorbance at 290 nm of the test compound(s), or radicals formed by the test compound after scavenging $O_2^{\bullet-}$ that reduces cytochrome *c* or nitroblue tetrazolium also interfere.

Scavenging of various free radicals, such as $OH^{\bullet}$ and O_2^-, can be assessed using highly sensitive electron spin resonance with a spin trap (Noda *et al.* 1997). Using this technique, radical scavenging activity of several body fluids can be measured by normalising the signals relative to the standard activity of vitamin C or a stable water-soluble analogue of vitamin E. This method is less prone to artifacts, but more laborious.

8.4.2. Other scavenging assays. The LDL oxidisability test, measuring LDL lipoprotein oxidation, is often used to assess antioxidant capacity (Esterbauer *et al.* 1992). LDL oxidation is initiated by free radicals. During this process, the rate of oxidation is dependent on endogenous antioxidants in LDL, accounting for the lag-phase of oxidation. LDL oxidation is efficiently inhibited by lipophilic antioxidants of which α-tocopherol appears to be the most important. Epidemiological studies suggest preventive effects towards atherogenic lesions to be associated with an increased uptake of lipophilic antioxidants such as vitamin E and carotenoids (Rimm *et al.* 1993). It is not clear whether this assay determines additional unidentified components and it is also suggested that the results are affected by the methods applied for isolating LDL (Diplock *et al.* 1998).

8.5. Damage assays

Instead of scavenging assays, damage to DNA, proteins and lipids can also be used for monitoring total antioxidant capacity.

8.5.1. Oxidative DNA damage. Oxidative DNA damage can be characterised *ex vivo* by measuring modified DNA bases as a result of severe reactive oxygen species (ROS) attack on DNA. The most exploited DNA lesion that is used as an index of oxidative DNA damage is 8-hydroxy-deoxyguanosine (8-OHdG) (Loft *et al.* 1994). This product is a result of free radical attack on guanine and may be a useful marker of damage on DNA since it is excreted in urine. For measurement of urinary 8-OHdG, liquid chromatography (HPLC) with electron capture detection (Shigenaga *et al.* 1994; Verhagen *et al.* 1997*a*) is the most frequently used method.

The validity of this urinary measurement is supported by the fact that the level of 8-OHdG is presumably not changed by the diet since nucleosides are not absorbed from the gut. Thus excretion is presumed to reflect integrated endogenous DNA damage. However, it is possible that some or all of the 8-OHdG excreted in the urine may arise from deoxyGTP (Diplock *et al.* 1998).

Analysis of 8-OHdG in DNA, most notably from lymphocytes, has been done but is hampered severely by the fact that artificial formation during sample clean-up overwhelms the background levels (some say by up to 2 orders of magnitude; Verhagen, 1998). New approaches are being developed for measuring 8-OHdG using immunoaffinity assays and liquid chromatography–tandem mass spectrometry.

8.5.2. COMET assay. Another technique for measuring antioxidant capacity by evaluating DNA damage is the 'COMET' assay (Duthie *et al.* 1996). This assay measures DNA damage, i.e. DNA breaks, in cells, e.g. lymphocytes (Betti *et al.* 1993; Anderson *et al.* 1994). DNA damage (i.e. breaks) can be visualised by staining and unwinding electrophoresis of the DNA. The assay can be quantified by scoring of fluorescence intensity of the tails. The sensitivity of this technique can be improved by the use of DNA restriction enzymes (e.g. endonuclease III) converting the oxidised bases into strand breaks (Duthie *et al.* 1996). Dietary studies, performed with the COMET assay, show a positive effect of antioxidative food components on the endogenous DNA damage (Collins *et al.* 1995).

8.5.3. Oxidative damage to proteins. Oxidative damage to proteins may also be of importance *in vivo* because it may affect receptor function, enzyme activity or transport mechanisms and may contribute to secondary damage to other biomolecules, such as inactivation of DNA repair enzymes.

Attack by various ROS on tyrosine might result in the production of 3-nitrotyrosine that can be measured immunologically or by HPLC or GC/MS (van der Vliet *et al.* 1996). Nitrotyrosine is excreted in urine (Ohshima *et al.* 1990), although the possible confounding effect of dietary nitrotyrosines and of dietary nitrate and/or nitrite is yet to be evaluated.

More use has been made of the carbonyl assay (Levin *et al.* 1995), a non-specific assay of oxidative protein damage to assess 'steady-state' protein damage in human tissues and body fluids. The carbonyl assay is based on the reaction of ROS with amino acid residues in proteins (particularly histidine, arginine, lysine and proline) giving carbonyl functions that can be measured after the reaction with 2,4-dinitrophenylhydrazine or by antibodies. Measurement of carbonyls in human plasma can be a useful marker in nutritional studies, but more work has to be done to identify the molecular nature of the protein carbonyls in human plasma.

8.5.4. Lipid peroxidation. A range of methods is available for measurement of markers of lipid peroxidation and products of lipid oxidation *in vivo*. 'Whole body' lipid peroxidation has been determined by measuring hydrocarbon gases (ethane, pentane) in exhaled air and urinary secretion of thiobarbituric acid reactive substances (TBARS) (Gutteridge & Thickner, 1978). Hydrocarbon gas exhalation has too many confounding variables to be applicable in free-living human subjects (Springfield & Levitt, 1994). Urinary TBARS is also not a suitable assay to assess whole-body lipid peroxidation in response to changes in dietary composition (Dhanakoti & Draper, 1987), because many artificial products could be generated. HPLC detection can be used to separate the 'true' malondialdehyde adduct.

Identification of peroxides formed as a consequence of *in vivo* oxidative stress can be assessed by a number of methods, such as the xylenol orange or iodometric methods (Jiang *et al.* 1992; Thomas *et al.* 1989).

Other products of lipid peroxidation that can also be measured are 4-hydroxynonenal (Douki & Ames, 1994) and probably the most specific product F_2-isoprostanes (Morrow & Roberts, 1994). F_2-isoprostanes can be measured in plasma and urine of healthy volunteers with GC-MS and radioimmunoassay (Basu, 1998) and are indicative of ongoing lipid peroxidation in healthy human subjects. This urinary marker of lipid oxidation as an indicator of whole body lipid peroxidation has to be further developed but is highly promising.

8.6. 'New' functional redox-controlled assays

8.6.1. Activation of transcription factors. Electromobility shift assay is a 'new' approach that could be used in assessing TAC. Transcription factors (e.g. AP-1 and NF kappaB) have been implicated in the inducible expression of a variety of genes, involved in inflammatory and immune responses, in response to oxidative stress (Sen & Packer, 1996). It has been found that activation of transcription factors are redox regulated and can be inhibited by antioxidants (Pinkus *et al.* 1996). Using human T-lymphocytes, basal activation of transcription factors can be determined and changes caused by dietary modulation can be measured as total antioxidant

capacity. Increased activation has already been shown in patients with leprosy (Zea *et al.* 1998), sepsis (Böhrer *et al.* 1997) and with type-1 diabetes (Hofmann *et al.* 1998).

8.7. Gaps in area of TAC

There is a great need for standardisation and validation of these simple screening assays. A clear extrapolation of these results to the normal physiological situation is not yet available, but the use of different assays instead of one might provide a better index of the antioxidant status. The same applies to the 'damage' assays, as overestimation might occur with these assays and lead to incorrect interpretation of the results. It should be kept in mind that all these assays reflect only a single aspect, i.e. scavenging of one type of radical or total antioxidant capacity. Combinations of the scavenging and damage assays might provide more information about the TAC than either one of them. 'New' assays on activation of transcription factors look promising, but they need further experimentation.

9. Biomarkers for aflatoxin exposure and effect

9.1. Introduction

Aflatoxins are naturally occurring mycotoxins produced mainly by *Aspergillus flavus* and *A. parasiticus*. Dietary contamination is ubiquitous in areas of the world with hot, humid climates, although humans can be exposed to these toxins everywhere due to the globalisation of food markets. Human hepatocarcinogenicity of aflatoxins has been definitely established by the International Agency for Research on Cancer and World Health Organization (IARC-WHO, 1993). Aflatoxins have been shown to produce liver cancer and acute hepatic toxicity in humans although they are also proposed as a risk factor for other diseases such as kwashiorkor (protein-energy malnutrition) and Reye's syndrome (Hall & Wild, 1994). Acute hepatic toxicity produced by aflatoxins causes histological changes in the human liver that consists of centrolobular lesions characterised by loss of retainable cytoplasm, with areas of liver cell necrosis, polymorphonuclear neutrophil infiltration and mild fatty changes in midzonal cells. These observations were made on the liver of a 15-year-old boy who died of acute hepatic toxicity linked to the consumption of mouldy cassava containing 1·7 mg/kg of aflatoxins (IARC-WHO, 1993).

These mycotoxins are among the few environmental carcinogens for which quantitative risk assessments have been attempted (Groopman *et al.* 1996). This goal was achieved by the use of specific and sensitive biomarkers in epidemiological studies. The development of molecular biomarkers for aflatoxins is based on the extensive research data available on the metabolism, macromolecular adduct formation, and general mechanisms of action of these toxins both in experimental and human studies (Eaton *et al.* 1994; Groopman, 1994; Hsieh & Wong, 1994; Groopman *et al.* 1996).

The rationale for molecular biomarkers can be exemplified by the relation between aflatoxin in the diet and liver cancer. External exposure can be measured by analysing foods for their aflatoxin content and by assessing individual

daily intake of each food by interview. Measurement of this exposure is particularly difficult. Thus, assessment of exposure based on food intake is problematic, even given newer, more specific, methods of aflatoxin measurement (Hall & Wild, 1994).

9.2. Biomarkers of exposure

Internal exposure to aflatoxins can be assessed by measuring aflatoxin M_1, (AFM_1) an oxidative metabolite of aflatoxin B_1 (AFB_1), in urine or in milk. AFM_1 is the 9α-hydroxy form of AFB_1 and is one of the most predominant aflatoxin metabolites in human urine. It is formed by the oxidation of AFB_1 by microsomal cytochrome $P450$ and is excreted in urine at an amount between 1·2 and 2·2 % of dietary AFB_1 (Groopman, 1994). A human study conducted in China with a total of 252 urine samples showed a reasonable correlation ($r = 0.65$) between total dietary AFB_1 intake and total AFM_1 excretion in urine during a 3-day period (Zhu *et al.* 1987). AFM_1 can also be measured in human milk. Estimates of the percentage of aflatoxin in the diet excreted as AFM_1 in milk ranged from 0·09 to 0·43 % (Groopman, 1994). The measurement of AFB_1 in blood also gives information on internal dose in humans although it reflects only recent exposure.

9.3. Biomarkers of aflatoxin exposure and effect

9.3.1. Biologically effective dose markers. The biologically effective dose represents a relevant interaction between a substance and a body component. It can be defined as the amount of material interacting with subcellular, cellular and tissue targets or with an established surrogate (van Poppel *et al.* 1997). Among the various markers of biologically effective dose of aflatoxins, the measurement of DNA adducts (e.g. AFB_1-N^7-guanine) is of primary interest because these adducts represent damage to a critical macromolecular target. AFB_1-albumin adducts are also very useful because they are surrogate measures of covalent binding to DNA. Because their half-life is longer than that of AFB_1-N^7-guanine, these markers can reveal exposure integrated over longer time period (Groopman, 1994). AFB_1 is metabolised in the liver by cytochrome $P450$ enzyme which produces, together with other metabolites, a highly reactive AFB_1-8,9-epoxide metabolite that can covalently interact with nucleophilic centres such as DNA and serum albumin to form AFB_1-N^7-guanine and AFB_1-albumin adducts (Groopman *et al.* 1992*a*). Both urinary AFB_1-N^7-guanine and serum AFB_1-albumin adducts appear to reflect DNA damage in the hepatocytes in an experimental system (Wild *et al.* 1986).

9.3.2. AFB_1-N^7-guanine. The primary AFB_1-DNA adduct is AFB_1-N^7-guanine which is removed from DNA rapidly (half-life 8–10 h) and is excreted exclusively in urine (Groopman, 1994). AFB_1-N^7-guanine is measured in urine samples using chromatography. Human urine samples are purified on Sep-Pak C18 cartridge, then purified using an immunoaffinity column and finally analysed by reversed phase HPLC with UV diode array detection (Groopman *et al.* 1992*a,b*, 1993). The use of urinary AFB_1-N^7-Gua adduct as a biomarker has been validated in the laboratory

and with human sample analyses, and provides a measure of acute exposure to aflatoxin B_1 (AFB_1) and reflects relatively short-term (24–48 h) exposure (Groopman *et al.* 1992*a,b*, 1993). In particular, a pilot study on humans showed that urinary AFB_1-N^7-guanine excretion and AFB_1 intake from the previous day had a correlation coefficient of 0·65 and $P < 0.000001$. The examination of the dose–response characteristics for AFB_1-N^7-guanine in urine of people living in China and the Gambia, with high incidence of liver cancer, showed the same excellent association of this biomarker with exposure (Groopman *et al.* 1992*a*, 1993; Qian *et al.* 1994). Although this measure is excellent for studies of acute exposure, it does not reflect chronic intake by individuals or by a populations (Hall & Wild, 1994).

9.3.3. AFB_1-albumin. AFB_1 binds quantitatively to peripheral blood albumin in rats in relation to dose. Upon repeated exposure, accumulation of binding occurs and the level of albumin binding parallels the binding to liver DNA (Wild *et al.* 1986). The major albumin adduct in rats has been characterised as an AFB_1-lysine residue (Sabbioni, 1990). The long half-life of albumin in humans (about 20 days) provides for a measure of human exposure to aflatoxin over the previous 2–3 months (Wild *et al.* 1996; Hall & Wild, 1994).

Complementary ELISA and HPLC–fluorescence techniques are currently used to assay the AFB_1-albumin adducts (Wild *et al.* 1990*a*). AFB_1-albumin adducts from serum are hydrolysed and aflatoxin residues are purified on Sep-Pak C18 cartridge and quantified by competitive ELISA against an AFB_1-lysine standard. For HPLC-based analyses, AFB_1-albumin adducts from serum are hydrolysed, purified on immunoaffinity column and the AFB_1-lysine adduct is quantified by reversed phase HPLC with fluorescence detection. The ELISA measures the total AFB_1-albumin adducts whereas HPLC measures only the AFB_1-lysine adduct. Therefore the ELISA results are 11-fold higher than HPLC results for the same tested sample (Wild *et al.* 1990*a*).

However, this biomarker provides some measurement of chronic exposure that is not available from other markers such as the AFB-N^7-Gua in urine. Moreover the measurement of serum AFB_1-albumin adducts provides a much better description of the average exposure avoiding daily fluctuations of results (Hall & Wild, 1994). The use of serum AFB_1-albumin adducts as biomarkers has been validated in experimental and human sample analyses (Wild *et al.* 1990*a, b*, 1992). In particular a study performed on 20 residents of Gambia showed a significant correlation ($r = 0.55$ $P = < 0.05$) between the dietary intake of aflatoxins and the level of albumin-bound aflatoxin at the end of the study (Wild *et al.* 1992). Other molecular epidemiological studies confirmed that serum AFB_1-albumin is a sensitive and specific biomarker for assessing exposure to aflatoxin in the population (Wild *et al.* 1990b; Groopman *et al.* 1996; Wang *et al.* 1996).

9.3.4. AF biomarkers in disease-aetiology. The use of serum AFB_1-albumin adducts and urine AFB_1-N^7-guanine biomarkers in epidemiological studies enabled the role of confounding factors in the aetiology of liver cancer, such as hepatitis B surface antigen (HBsAg), to be explained (Wang *et al.* 1996). These studies demonstrated that both

HBsAg and aflatoxin exposure are risk factors for developing human liver cancer and that people positive for HBsAg, and exposed to aflatoxins, are much more likely to develop liver cancer. In particular, results from the epidemiological studies where both aflatoxin biomarkers and HBsAg were analysed, showed that the relative risk for liver cancer was 3·4, 7 and 59 for individuals found positive for aflatoxin biomarker, HBsAg and aflatoxin biomarker+HBsAg, respectively (Ross *et al.* 1992; Qia *et al.* 1994).

An example of the early biological effect in humans exposed to aflatoxins is the mutation of the tumour suppressor gene *p53*, which is the most common mutated gene detected in human cancers (Groopman, 1994). Two independent studies of *p53* mutations in hepatocellular carcinomas occurring in populations exposed to aflatoxin revealed high frequencies of GT transversions, with clustering at codon 249, whereas the same study conducted in hepatocellular carcinomas from Japan, and other areas in which little exposure to aflatoxin occurs, revealed no mutations at codon 249 (Groopman, 1994).

9.4. Biomarkers of susceptibility

Along the whole path from exposure to disease, the level of exposure plays a role but also there is genotypic or phenotypic predisposition. A sensitivity/susceptibility marker determines an individual's inherited or acquired trait that may predispose them to an increased risk of developing disease (van Poppel *et al.* 1997). Cytochrome *P*450 3A4 is a major hepatic enzyme involved in the bioactivation of AFB_1, benzo[a]pyrene diol epoxide and several other carcinogens. Liver samples with increasing levels of this enzyme produced higher amounts of AFB_1-N^7-guanine in DNA *in vitro*. A biomarker for the phenotypic determination of cytochrome *P*450 3A4 has been reported. In particular, the ratio of 6β-hydroxycortisol to cortisol is a marker used to assess the activity of cytochrome *P*450 3A4 (Groopman, 1994). This is a useful biomarker for assessing individual susceptibility to AFB_1 as well as to many other carcinogens. The liver is the usual target organ for both acute and chronic toxicity of aflatoxins. An immunosuppressive effect of these compounds has been demonstrated in laboratory animals which suggests that examination in more detail of putative immunosuppressive effects of aflatoxins in humans is required (Hall & Wild, 1994). The long-term goal of the research in the field of aflatoxin biomarkers is the application of biomarkers to the development of preventive interventions for use in human populations at high risk for cancer (Groopman *et al.* 1996).

10. Development of biomarkers – what are the gaps and why is the information needed?

10.1. Introduction

In contrast to most nutrients, there is a marked lack of biomarkers of either exposure or effect for most non-nutrients. For classes of non-nutrients which contain a large number of examples, such as polyphenols or glucosinolates, there are data for only a very limited number of representatives (Mennicke *et al.* 1983, 1988; Chung *et al.* 1992*a*; Jiao *et al.*

1994), and there are large numbers of (potentially) important dietary compounds for which no biomarkers exist at all. Such biomarkers are essential to examine the predicted effects (both positive and negative) on health of non-nutrients, which have been implied from a range of epidemiological, toxicological, animal and cellular studies (Zhang *et al.* 1992; Sharma *et al.* 1994; Zhang & Talalay, 1994; Ozierenski, 1994, 1995; Tawfiq *et al.* 1995). Interpretation of biomarkers of effect are hampered by lack of knowledge on the metabolism of most non-nutrients and their mechanisms of action in humans *in vivo*. Although some non-nutrients are predicted to have positive health benefits at dietary concentrations, at higher levels there may be toxicity or antinutritional effects (as seen for some studies on β-carotene, see above). For ethical reasons, therefore, it is not possible to use high amounts of purified non-nutrients with humans, although most animal experiments have been performed at relatively or very high levels (Ioannou *et al.* 1984). Some studies have been performed using whole food (Bogaards *et al.* 1994; Duncan, 1997), but the complexity of the material and the presence of other components makes it difficult to interpret the results.

There is a wide variation in the contents of non-nutrients in foods, especially where the compounds in question are derived from processing or cooking of the food, or where storage of the food affects the components. Reliable tables of compositional data either do not exist or are not possible to construct. These factors make it difficult to conduct epidemiological or intake studies in populations.

There are only a few studies to date which have addressed bioavailability of most non-nutrients. Of these, many do not measure dose–response, and so a validation of the link between the measured biomarker and the dietary intake is not established. For many of the non-nutrient compounds, there is a real lack of information on how much is absorbed, the amount that enters target cells and tissues and in what form (e.g. free, protein-bound, conjugated or glycosylated), and the existence of possible interactions (additive or synergistic) between compounds. For these reasons, there is very little information reflecting long term exposure, especially for substances which give rise to highly reactive metabolites, since in these cases, only stable endproducts in plasma or urine can be measured.

There is a lack of standardisation of methodology for the non-nutrients with few accredited or interlaboratory methods. Often the detection limits are not sufficient for studies which employ realistic dietary intakes as opposed to high levels.

10.2. Glucosinolates

Glucosinolates are a group of secondary plant metabolites found in *Brassicaceae* (e.g. cabbage, broccoli, Brussels sprouts, cauliflower, mustard, horseradish, watercress) and are β-thioglycosides of which the aglycone is a (Z)-*N*-hydroximine sulphate ester. There is a wide range of side-chains which give rise to a large number of individual glucosinolates. During processing, cooking and eating, these compounds are degraded to an even larger number of compounds, such as isothiocyanates, nitriles, epithionitrile and indoles, many of which are bioactive (Plumb

Table 4. Summary of the results of case-control studies concerning an association between consumption of brassica vegetables and cancer development; data shown for different vegetables (adapted from Verhoeven *et al.* 1996)

Type of brassica	Number of studies showing an association (of which statistically significant)			Number of studies showing a different association by gender	Total number of studies
	Inverse association	No association	Positive association		
All types	39 (20)	4	9 (1)	1: inv./pos. 1: no/pos. 4: pos./inv.	58
Cabbage (excl. Chinese cabbage)	17 (7)	5	2 (1)		24
Broccoli	10 (7)	3	1 (0)	1: inv./no 2: no/inv. 1: pos./no	18
Cauliflower	8 (2)	1	2 (0)	1: pos./no	12
Brussels sprouts	2 (0)	2	2 (0)	1: pos./inv.	7

inv., inverse; pos., positive; no, no association.

et al. 1996; Fenwick *et al.* 1983, 1989). There are biomarkers of exposure only for certain isothiocyanates (allyl-, benzyl- and phenethyl-) (Brusewitz *et al.* 1977; Mennicke *et al.* 1983, 1988; Ioannou *et al.* 1984; Eklind *et al.* 1990; Chung *et al.* 1992*a*; Jiao *et al.* 1994; Duncan, 1997). The potent bioactivities (measured in animal models or at the cellular level) of many of these breakdown products need to be demonstrated *in vivo*; therefore, biomarkers of both exposure and effect are required but interpretation is complicated by the existence of large numbers of breakdown products and their metabolites.

Biomarkers of exposure include *N*-acetyl cysteine conjugates of isothiocyanates in urine. However, the methods used have not been validated or standardised between laboratories, and were on a small number of volunteers. There is a need for development of methods which would be capable of measuring biomarkers of exposure to several glucosinolates simultaneously, such as estimation of mercapturic or hippuric acid derivatives. There are very few data on the bioavailability of these compounds, and in particular information is needed on the correlation between the biomarkers and the dietary intake.

Interest in biomarkers for glucosinolates arises from the proposed biological effects of the breakdown products of these compounds. The putative beneficial effect of cruciferous vegetables in particular is supported by the results of many case-control studies (Tables 4 and 5), unlike cohort studies in which this effect has not (yet?) been established (reviewed in Verhoeven *et al.* 1996, 1997*a*). Mechanisms

involved in chemopreventive action of the glucosinolate breakdown products indoles and isothiocyanates are anti-oxidant activity, modulation of biotransformation enzymes, and modifying binding of carcinogens to DNA. In addition, indoles may affect the development of hormone-related cancers by modulation of the metabolism of oestrogen (reviewed in Verhoeven *et al.* 1997*b*). There are ample data on the effects of cruciferous vegetables, glucosinolates and their breakdown products in *in vitro* studies and in studies with experimental animals. In contrast, the majority of human data are from epidemiological studies and the indications towards the chemopreventive potential of cruciferous vegetables in humans is strong (see above), but the number of experimental studies on cruciferous vegetables in humans is very limited. In principle, biomarkers of the effect of glucosinolate breakdown products are closely related to the following mechanisms (see Table 6).

(1) Induction of (detoxification) biotransformation enzymes by Brussels sprouts, e.g. glutathione-*S*-transferase (Bogaards *et al.* 1994; Nijhoff *et al.* 1995).
(2) Inhibition of oxidative damage to tissue macro-molecules by Brussels sprouts (e.g. levels of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) in urine (Verhagen *et al.* 1995).
(3) Inhibition of carcinogen activation and carcinogen–DNA formation (e.g. inhibition of the oxidative metabolism of the lung carcinogen NNK (4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone)) (Hecht, 1996*a*)

Table 5. Summary of the results of case-control studies concerning an association between consumption of brassica vegetables and cancer development; data shown for different cancer types (adapted from Verhoeven *et al.* 1997*b*)

Cancer site	Number of studies showing an association for one or more brassica vegetables (of which statistically significant)		Total number of studies
	Inverse association	Positive association	
Colon	11 (6)	4 (0)	15
Stomach	8 (5)	3 (1)	11
Rectum	8 (4)	3 (0)	10
Lung	9 (6)	2 (0)	9

Table 6. Non-nutritive chemopreventive substances: sources and possible mechanisms of action (adapted from Verhagen *et al.* 1997*b*)

Chemo-preventive agents	Primary sources	Possible mechanism(s)*							
		1	2	3	4	5	6	7	8
Carotenoids	Fruits, vegetables, cereal					x		x	x
Chlorophyllin	Leafy vegetables				x	x		x	
Coumarins	Vegetables, citrus fruits					x			x
Diallyl sulphides	Onion, garlic					x			
Dietary fibre	Fruits, vegetables, legumes, seeds	x							
Flavonoids	Fruits, vegetables, tea		x		x	x	x	x	x
Indoles	Cruciferous vegetables				x	x		x	x
Terpenoids	Citrus fruits					x			x
Isothiocyanates	Cruciferous vegetables				x	x			
Phenolic acids	Fruits, vegetables, nuts, tea, coffee.	x	x	x	x	x	x	x	x
Phytic acid	Legumes, cereals							x	x
Plant sterols	Vegetables					x		x	x
Protease inhibitors	Seeds, legumes, grains						x	x	

* Mechanism(s): 1, Prevention of formation/uptake of carcinogens; 2, scavenging of (activated) carcinogens; 3, shielding of nucleophilic sites in DNA; 4, inhibition of formation of DNA/carcinogen complex; 5, modifying the activities of biotransformation enzymes; 6, modifying the activities of other enzymes; 7, antioxidant activity; 8, other.

In summary, indications for the chemopreventive potential of cruciferous vegetables, glucosinolates and their breakdown products in *in vitro* studies and studies with experimental animals are quite strong. These data support the results of many epidemiological studies showing beneficial effects of cruciferous vegetables. In contrast, human data on particular chemical entities in these vegetables (their actual exposure as well as their effects) are scarce.

10.3. Polycyclic aromatic hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons (PAHs) always exist as mixtures in the environment, and are present in smoke, char-grilled food, exhaust gases from engines and from several industrial processes. Workers from industrial settings with high airborne PAH-levels such as gas works, coke works and primary aluminium industries show excess rates of lung cancer. More than seven PAHs are known to be carcinogenic (Mumtaz *et al.* 1996).

PAHs are present in food due to environmental pollution or from heat processing. Most leafy vegetables take up PAHs from the air, especially when the vegetables are grown in the neighbourhood of emission sources. Uptake of PAHs by plants can also occur from the soil. Certain seafoods, such as mussels and oysters, can contain high levels of PAHs, and char-grilling, grilling and smoking of meat and fish increases the PAH level. Daily intake is estimated to be 3–16 μg/day (de Vos *et al.* 1990).

Although more than 100 different PAHs are known, extensive knowledge is only available on the metabolism of just one of the carcinogenic PAHs, benzo(a)pyrene. The intermediate epoxide-benzo(a)pyrene and benzo(a)pyrene diol epoxide covalently bind to nucleophilic sites in DNA to form benzo(a)pyrene-DNA adducts. These type of metabolites are carcinogenic intermediates of benzo(a)-pyrene and, in general, of the PAH.

A biomarker of exposure measures either the internal dose in body fluids or measures the dose at the target site in an exposed individual. Since PAHs are a group of compounds, two approaches can be followed. The biomarker can be a marker of the whole group, or a marker specific for a single compound. A large variety of approaches and techniques have been tested, e.g. PAH levels in blood and urine, reduction of PAH levels in urine, urinary thioethers, mutagenicity in urine and DNA–PAH adducts in white blood cells, using a wide range of different techniques such as methods involving bacteria, UV- and fluorescence spectrometry, immunochemical methods, GC-MS and liquid chromatographic methods. The composition of individual PAHs in different diets varies, and so when a single compound is used as an indicator or marker, this is not necessarily a good indicator of total intake. Thus there is a need to have a good overall biomarker of effect representing the changing PAH-content, especially of the carcinogenic representatives (Table 7).

10.4. Heterocyclic amines (HAs)

Proteins in cooked meat can be the source of many substances with mutagenic or carcinogenic potential. Reaction products between amino acids and sugars give rise to Maillard reaction products that contribute to taste and flavour, but at prolonged very high temperatures give rise to more toxic substances. The food-derived heterocyclic aromatic amines (HAs) comprise a group of about 20 compounds that have been isolated from cooked animal protein (meat and fish) and from the pyrolysis of proteins and individual amino acids. HAs have been recognised as potent mutagens in the *Salmonella*-microsome assay (Ames-test) and as carcinogens in long-term rodent experiments. Circumstantial evidence suggests that these compounds are also likely to elicit carcinogenesis in humans (Sugimura, 1997, for review).

The types and levels of HAs in foods are dependent on multiple factors such as type of meat (beef, chicken, fish), cooking techniques and temperature, degree of cooking and browning of the surface. The HAs from dietary sources which are most relevant to human health are: IQ, 2-amino-3-methylimidazo(4,5-f(quinoline)); MeIQ, 2-amino-3,4-dimethylimidazo(4,5-f(quinoline)); MeIQx, 2-amino-3,8-dimethylimidazo(4,5-f(quinoxaline)); and PhIP, 2-amino-1-methyl-6-phenylimidazo(4,5-b(pyridine)).

Table 7. Characteristics of available biomarkers of exposure to PAH (from Jongeneelen, 1997)

Parent agent	Biomarker
Total PAH as a mixture	Aromatic PAH-adducts in white blood cells
Total PAH as a mixture	Urinary thioethers
Total PAH as a mixture	Mutagenicity in urine
Specific PAH	PAH in urine after reduction
Specific PAH	Hydroxylated PAH
Benzo(a)pyrene	Adduct DNA-benzo(a)pyrene in white blood cells
Benzo(a)pyrene	Adduct haemoglobin-benzo(a)pyrene
Benzo(a)pyrene	3-Hydroxy-benzo(a)pyrene
Phenanthrene	Hydroxyphenanthrenes in urine
Pyrene	1-Hydroxypyrene in urine

HAs require metabolic activation, in particular *N*-hydroxylation of exocyclic amino groups, by CYP1A2, and subsequent esterification of the *N*-hydroxylamine, to form the ultimate highly reactive ester derivatives. The genotoxic *N*-hydroxy metabolites or their *N*-sulphate and *N*-acetoxy esters can react with electrophilic sites in protein and DNA. Individual differences in metabolic constitution such as levels of cytochrome *P*450 and *N*-acetyltransferase will influence individual susceptibility towards these compounds. Genotoxic activity results in various precarcinogenic effects such as mutations in oncogenes and tumour suppressor genes (H-*ras*, *p53*, *apc*), sister-chromatid exchanges and chromosomal aberrations. In addition to measuring levels of HAs in the human diet, there are other methods to measure human exposure to these compounds: protein adducts, DNA-adducts and urinary metabolites.

Measurement of adducts to proteins such as haemoglobin and serum albumin have the advantage of abundance, long life and ease of accessibility, but the data for HA protein adducts are very limited, even for *in vitro* and animal studies (Turesky & Markovic, 1995). Only a handful of studies are available for DNA adducts with several HAs (particularly at the C8 position of guanine) in animals and humans (Turesky & Markovic, 1995; Schut *et al.* 1997; Nerurker *et al.* 1996). Parent compounds, their metabolites (conjugates with glucuronic acid, sulphate, and glutathione) and DNA-adducts (upon excision repair) have been reported to be excreted in the urine of animals and humans, but again the data are very limited (Ji *et al.* 1994; Turesky & Markovic, 1995; Singh & Rothman, 1997; Snyderwine *et al.* 1997).

In summary, HAs are well recognised as relevant dietary risk factors for humans. Their potential as mutagens and carcinogens is established, and the bioactivation mechanism underlying their genotoxic potential is understood. In contrast, only few data are available to monitor exposure to these compounds. Animal data on metabolism are limited and there are even less human data. Future research should focus on the monitoring of reactive metabolites (protein- and DNA-adducts) and urinary excretion products.

10.5. Other non-nutrients

The subject area of food additives is vast and includes both natural and synthetic substances covering a large range of chemical entities, all of which must be subjected to extensive toxicity testing prior to approval. It is a heavily regulated area and good biomarkers of exposure and effect would enable realistic risk assessment and achievable regulations to be set. This is vital for EU and world trade considerations. Generally, animal studies *in vivo* are used to confirm that the food additive does not exhibit any undue toxic effects and to set a level of exposure, the acceptable daily intake which is associated with negligible risk for human consumption. EU Member States are required to establish additive consumption monitoring surveys (EC, 1994*a,b*, 1995). Biomarkers offer the means by which more direct, accurate and relevant (i.e. human health-related) data on food additive intake may be obtained complementary to established procedures such as per capita estimates, total diet studies and chemical analysis, thereby allowing better assessment of the risks associated with intake (Tennant, 1995).

Some examples illustrating the advantages and difficulties of biomarker measurements for food additives are given below.

10.5.1. Deliberately added compounds

Saccharin and acesulfame-K. The artificial sweeteners saccharin and acesulfame-K can be consumed at high levels, especially by children, diabetics and slimmers (ca. 35–50 % of the acceptable daily intake). Both compounds are excreted in the urine unmetabolised. Suitable methods of analysis have been developed as a means of estimating intake (Wilson and Crews, 1995, Wilson *et al.* 1999). The data showed good general agreement between calculated and measured intakes with urinary measurements providing additional information on exposure to sources not revealed by the questionnaire approach, e.g. drugs, toothpaste. This type of anomaly can be countered by good questionnaire design.

Azo dyes. Consumption of some of the azo dyes used as artificial food colorants has been linked to allergenic response, notably hyperactivity in children. Both the parent compounds and the products of metabolic microbial or hepatic azo-reduction (i.e. sulphonated aromatic amines) have been found in urine and faeces, and are promising candidates for biomarkers of exposure. Although most data are from animal studies (Jones *et al.* 1964; Phillips *et al.* 1987*a–c*; Chung *et al.* 1992*b*; Singh *et al.* 1993), some human studies are attempting to establish exposure/excretion relationships, which is complicated by the occurrence of metabolites common to several azo dyes thus limiting

biomarker specificity. In addition, little or none of the parent compound may be found at detectable levels in urine (Wilson and Crews, personal communication, 1999)

Hydrocarbon fractions from refined mineral oils and waxes. Various animal studies have shown that specific fractions of hydrocarbon material from refined mineral oils and waxes are preferentially absorbed and transported intact to tissues with the major portion excreted in the faeces. Bioaccumulation is affected by the physicochemical properties of the hydrocarbons concerned (molecular weight, viscosity, steric factors) and has been associated with increased organ weights (e.g. liver, kidney). Microscopy has revealed the presence of liver granulomas and other clinical changes have been observed such as lesions on the cardiac mitral valve (Baldwin *et al.* 1992; Firriolo *et al.* 1995; Miller *et al.* 1996; Nash *et al.* 1996; Smith *et al.* 1996). However, such obvious markers of exposure and effect are not suitable for human studies because of the necessary invasiveness required for sample collection. Hence, better biomarkers need to be identified (i.e. urinary, faecal, plasma) so that the links between dietary exposure, accumulation and excretion can be established. Only limited animal and human data are available on metabolic products of relatively short-chain hydrocarbons (Fedtke & Bolt, 1987*a,b*).

Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). The synthetic phenolic compounds butylated hydroxyanisole and butylated hydroxytoluene have widespread use as lipophilic antioxidants. Some individuals show allergic reactions to butylated hydroxyanisole and it is carcinogenic in the forestomach of rodents (Verhagen *et al.* 1990*a*). It is a strong inducer of oxidative DNA damage in the epithelial cells of the glandular stomach in rats (Schilderman *et al.* 1995*a*) and has been implicated in the *in vitro* induction of oxidative DNA damage and enhancement of cell proliferation in human lymphocytes (Schilderman *et al.* 1995*b*) monitored by increased levels of the biomarker 8-oxodG. Conjugated urinary metabolites of butylated hydroxyanisole (i.e. sulphates, glucuronides) have been identified as biomarkers of exposure. Low levels may be determined but methods have yet to be validated (Verhagen *et al.* 1989, 1990*a,b*, 1991*a,b*). Butylated hydroxytoluene is known to inhibit tumour formation by several chemical carcinogens, including aflatoxin B$_1$, by induction of hepatic glutathione-S-transferases (Allameh, 1997). However, the links between dietary exposure of butylated hydroxyanisole and butylated hydroxytoluene and health are not relevant at dose levels below the respective acceptable daily intakes.

Although many other food additives have been of recent toxicological interest, valid biomarkers have yet to be identified, e.g. the intense sweeteners aspartame and cyclamate, caffeine, gallate ester antioxidants, the flavour enhancer monosodium glutamate, sulphites, nitrate and nitrite preservatives, the dough improver potassium bromate, carbohydrate polyesters, starch hydrolysates and smoke condensates.

10.5.2. Contaminants and processing artifacts. This is a vast area hence only a few examples are discussed.

Phthalates and related compounds. Di-2-ethylhexyl phthalate (DEHP), a peroxisome proliferator, has proved to be a non-genotoxic carcinogen in rodent studies. The toxicology of other phthalates is less well investigated.

Phthalates also demonstrate oestrogenic activity and have been associated with polycystic ovaries and testicular atrophy in experimental animals (Medical Research Council, 1995). DEHP is reported to have no effect on sulphotransferase expression (Witzmann *et al.* 1996). The metabolism of phthalate esters has been studied widely (Thomas & Northup, 1982; Egestad & Sjoberg, 1992). The parent diester phthalate is not excreted in the urine but is rapidly de-esterified to form the corresponding phthalate monoester, a proportion of which is further metabolised. There are very few detailed metabolic studies on phthalate esters in humans and those that are available focus on di-2-ethylhexyl phthalate. In general between 10 and 50 % of an administered dose is recovered in the urine within 24 hours as metabolites. The fate of di-2-ethylhexyl phthalate following oral ingestion of 30 mg has been examined and it was shown that the monoester derivative accounted for about 10 % of the total metabolites excreted in the urine in 24 hours (Schmid & Schlatter, 1985). In a similar study, a somewhat higher figure of 20 % has been reported (Albro *et al.* 1982). In a reported case of occupational exposure, mostly airborne, the monoester derivative accounted for 26 % of total metabolites (Dirven *et al.* 1993). Gas chromatography–mass spectrometry (GC-MS) has been used to analyse alkylated monoesters following extraction from urine (after deconjugation with β-glucuronidase) using solvent partition and solid-phase clean-up. HPLC with UV detection has also been used to analyse phthalate monoesters but is not as sensitive as GC-MS. The mass spectrometric characterisation and quantitation of both diester- and monoesterphthalates have been reported using electron impact (Dirven *et al.* 1993) and chemical ionisation (Muszkat *et al.* 1997). Thermospray HPLC-MS has similarly been used (Baker, 1996). These data strongly suggest that the monoester derivatives of diester phthalates are likely to be promising candidates as urinary biomarkers of exposure. However, the available data only relate to 2-di-ethylhexyl phthalate and have been generated using either extreme exposure levels or airborne, rather than oral, administration. As a consequence they cannot be utilised to quantitatively relate urinary levels of the metabolite to dietary exposure of the parent phthalates. Before such a biomarker-based approach is adopted, the relationship between dietary exposure and urinary excretion needs to be established. Urinary metabolite measurements have been used to confirm estimated daily intakes of di-2-(ethylhexyl), adipate a plasticiser found in PVC-based food wrapping materials (Massey, 1995).

BADGE. Bisphenol A diglycidyl ether (BADGE) and its hydrolysis products, such as bisphenol-A, possess oestrogenic activity (Sumpter & Jobling, 1995). The oestrogen receptor-binding affinities have been examined (Waller *et al.* 1996). There are significant gaps in the knowledge especially with regard to vitellogenesis. The quality and quantity of biological data for relevant biomarkers of toxicity and hormonal responsiveness are limited and biomarkers are needed. Susceptible (genetic) risk groups may include low age groups and males

Nitrosamines. The class of nitrosamines consists of many compounds (volatile and non-volatile). The different nitrosamines are implicated in various health diseases (Wishnok *et al.* 1993; Lockwood *et al.* 1994; Monarca

et al. 1996) and all are known to produce carcinogenic tumours (lung cancer, stomach cancer, colon cancer). Biomarkers of exposure include urinary biomarkers, increased cytosine DNA-methyltransferase activity and DNA modifications. For certain nitrosamines such as those occurring in tobacco smoke, biomarkers have been characterised (Chang *et al.* 1994; Falter *et al.* 1994; Kresty *et al.* 1996; Hecht, 1996b; Pirier & Weston, 1996). The usefulness of the various candidate biomarkers is complicated by the multiplicity of nitrosamine compounds present in a wide variety of foodstuffs. High consumption of foods containing nitrite and/or nitrate may possibly increase exposure to nitrosamines and susceptibility to disease.

Chloropropanols. Chloropropanols are formed from protein hydrolysates which are used widely as seasonings and savoury flavours, by the reaction of hydrochloric acid with residual lipids. In a study of chronic effects in F344 rats, high doses of 3-monochloro-1,2-propanediol (3-MCPD) produced benign renal tumours in both sexes and Leydig-cell and mammary tumours in males. 3-MCPD is genotoxic *in vitro*, but there is no evidence of genotoxicity *in vivo*. The non-genotoxic mechanisms associated with rat studies and the lack of carcinogenicity of 3-MCPD in mice and Sprague–Dawley rats, have been concluded to have developed as a result of non-genotoxic mechanisms and are considered not to be relevant to humans exposed to trace amounts of 3-MCPD (Lynch *et al.* 1998). Very low levels have been found in foods and food ingredients, formed possibly as a result of processing or storage. Theoretically, small amounts of 3-MCPD could pass from some food contact materials (e.g. epichlorohydrin resins) into food. Currently there are no biomarkers for exposure to 3-MCPD in humans.

Xylenes. Xylenes (*o-*, *m-* and *p-*) are widely used in industry and medical technology as solvents. In addition, several food components are known to degrade during processing to produce these and similar compounds. For example, at relatively high (>70°C) temperatures, carotenoids can undergo a series of degradation reactions to produce a range of products including aromatic compounds, principally xylene and toluene. The main aromatic thermal degradation product of the apocarotenoid colouring component of annatto (E160b) has been identified as *m*-xylene (McKeown, 1963, 1965; Scotter, 1995). *m*-Xylene has been found in commercial annatto food-colouring preparations up to a level of 200 mg/kg (Scotter *et al.* 2000) and has been detected in the headspace of heated foodstuffs containing annatto (Scotter, unpublished results). Recently, the amount of the major metabolite of xylene, methylhippuric acid, in urine has been recommended as an indicator of exposure. Of the xylene absorbed, about 95 % is metabolised in the liver to methylhippuric acid and 70–80 % of metabolites are excreted in the urine within 24 hours. No studies have been made on chronic exposure to xylene(s) from dietary sources. Animal studies have failed to provide convincing evidence that xylene is carcinogenic or has significant genetic or reproductive effects but they have confirmed that xylene has effects on many organ systems, including the CNS, liver, kidney, haemopoietic tissues and respiratory tract (Langman, 1994). Metabolites of xylene(s) in untreated urine have been readily characterised and measured by HPLC (Tamburini *et al.* 1995; Moon *et al.* 1997). These data strongly suggest that isomers of methylhippuric acid are likely to be promising candidates as urinary biomarkers of exposure to xylenes. However, the available data have only been generated using either extreme exposure levels or airborne, rather than oral, administration. As a consequence they cannot be utilised as they stand to quantitatively relate urinary levels of the metabolite to dietary exposure of xylenes. Before such a biomarker-based approach is adopted, the relationship between dietary exposure and urinary excretion needs to be established.

11. Conclusions

Biomarkers have been defined as indicators of actual or possible changes of systemic, organ, tissue, cellular and subcellular structural and functional integrity which can be used, either singly or in batteries, to monitor health and exposure to compounds in populations and individuals (Koletzko *et al.* 1998). In the preceding sections we have given many examples of the complexities involved in using some biomarkers in relation to assessing the effects of dietary exposure, from understanding why the biomarker might be valuable and how best it can be measured, to the pitfalls which can occur in the interpretation of data. The two other reviews in this issue of the *British Journal of Nutrition* discuss the use of biomarkers in epidemiological studies (Wild *et al.*) and the role of biomarkers in disease and health with special reference to cancer, coronary heart disease and osteoporosis (Hanley *et al.*).

Many of the difficulties cited in all three reviews arise from the need to determine changes following long-term low-level exposure, or absence of exposure, to dietary components. Intake measurements based on food analysis and dietary intake tables have been proven to be a useful first step but their value is limited by their accuracy. Solving some of these difficulties would involve, ideally, long-term studies of human subjects and their diets but the resources required for such undertakings are frequently prohibitively expensive. The aim for biomarker research in health and disease should be the application of biomarkers to the development of *preventive* interventions for use in human populations. Disease due to factors other than essential nutrient deficiency is unlikely to be radically improved by dietary intervention alone. However, overall health and well-being can be improved by dietary intervention alone but this goal requires considerable advances in the understanding of the functions of dietary components and further improvements in the analytical techniques to measure and study these processes.

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