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Review

Chromatographic techniques for the determination of putative dietary anticancer compounds in biological fluids

E.J. Oliveira, D.G. Watson*

Department of Pharmaceutical Sciences, University of Strathclyde, Strathclyde Institute of Biomedical Sciences, 27 Taylor Street, Glasgow G4 ONR, UK

Abstract

Although a great number of papers demonstrate an association between high intake of fruits and vegetables and reduced risk of certain types of cancer, the epidemiological evidence is not conclusive. The identification and quantification of specific dietary anticancer compounds in plasma, urine and tissues is an important aspect of this research. We surveyed the recent literature for original papers which involved the use of separation techniques for the detection and quantification in biological fluids and tissues of putative anticancer compounds which are present in the diet. The compounds included in this review are flavonoids, phytoestrogens, carotenoids, retinoids, vitamin E and ascorbic acid. The review covers papers published in the last 3 years. For each class of compounds we discuss the sample preparation, chromatographic conditions, and validation of the methods used, in order to identify current trends in the bioanalysis of each class of these substances.

Keywords: Reviews; Flavonoids; Phytoestrogens; Carotenoids; Vitamin E; Retinoids; Ascorbic acid

Contents

1. Introduction 4
2. Flavonoids and phytoestrogens 4
   2.1. Sample preparation 5
   2.2. Chromatographic conditions 7
   2.3. Detection 8
3. Carotenoids, retinoids, tocopherols, tocotrienols and their oxidation products 9
   3.1. Sample preparation 10
   3.2. Chromatographic conditions 17
   3.3. Detection 18
4. Ascorbic acid, dehydroascorbic acid and related compounds 20
   4.1. Sample preparation 20
   4.2. Chromatographic methods 22
   4.3. Detection 23
5. Nomenclature 23
References 23

*Corresponding author. Tel.: +44-141-548-4689; fax: +44-141-552-6443.
E-mail address: d.g.watson@strath.ac.uk (D.G. Watson).

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1. Introduction

There is compelling experimental evidence for the ‘‘in vitro’’ anticancer effect of several classes of dietary substances, including polyphenols and carotenoids. There is also epidemiological evidence of an inverse association between consumption of diets rich in fruit and vegetables and the incidence of certain types of cancer, including cancers of the stomach, colon, lung and oesophagus [1,2]. However, this evidence is sometimes weak and many studies have failed to confirm it, especially in hormone-related tumours such as breast cancer [3]. Part of the apparent inconsistencies in these results may be related to the fact that many published studies have relied on food frequency questionnaires in order to estimate the intake of dietary anticancer compounds, which can lead to errors. An important aspect of research that aims to elucidate the role of diet in the prevention of cancer is the analysis of specific dietary anticancer compounds in biological fluids, which can unequivocally be correlated with dietary intake.

This review surveys the current chromatographic techniques which are being used for the determination of selected dietary anticancer compounds and covers papers published in the last 3 years. The papers were selected through a computer search of BIDS Embase and Web of Science (ISI). All the compounds selected for this review have been shown to protect against oxidative damage ‘‘in vitro’’ and have been implicated in the prevention of certain types of cancer. However, since there is as yet no definitive and convincing epidemiological evidence for their role in cancer prevention ‘‘in vivo’’, we think they would be better described as putative anticancer compounds.

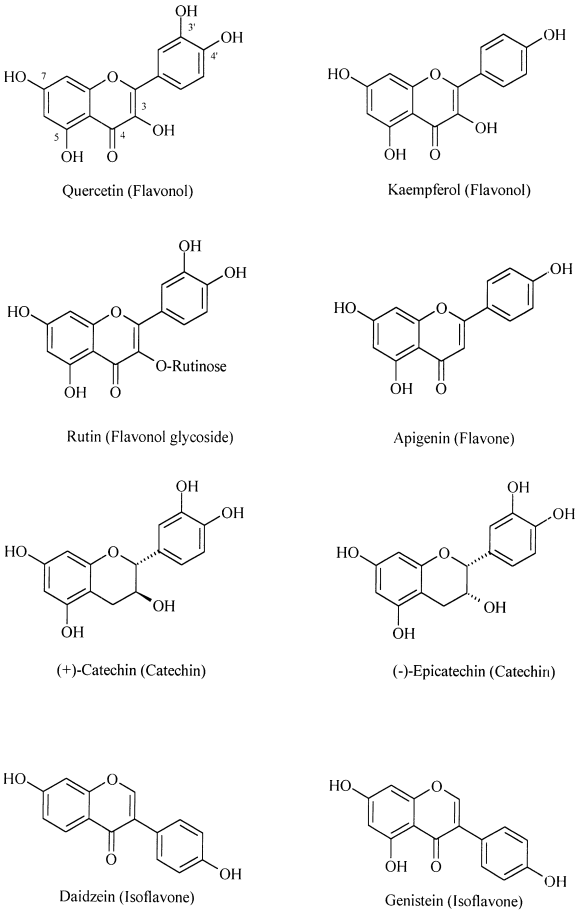


Fig. 1. Structure of some biologically important flavonoids.

2. Flavonoids and phytoestrogens

Flavonoids are a group of naturally occurring polyphenolic compounds (Fig. 1) which are present in most edible fruits and vegetables. The main dietary sources of flavonoids include onions, wine, tea, broccoli, and chocolate [4–6]. During the last decade an increasing number of studies have appeared in the literature concerning the protective role of this class of compounds in conditions such as coronary heart disease and cancer [7–9]. In addition, isoflavones (Fig. 1) have estrogenic activity and may have a role in prevention of hormone-dependent cancers. Although there has been a vast amount of work directed towards the study of the biological properties of flavonoids, most of these studies were done ‘‘in vitro’’, and thus do not take into account the poor bioavailability and extensive metabolism to which flavonoids are subjected ‘‘in vivo’’. Currently this deficiency is being addressed, and central to the study of flavonoid bioavailability and metabolism is the development of analytical techniques which are both sensitive and selective enough to allow the identification and quantification of flavonoids in the

low concentrations found in biological matrices. A recent comprehensive review of the analysis of flavonoids in foods can be found elsewhere [10].

2.1. Sample preparation

Sample preparation in order to carry out analysis of flavonoids in biological matrices usually involves a hydrolysis step prior to extraction, since polyphenols circulate in biological fluids mainly as glucuronic acid and sulphate conjugates. Exceptions to this found in the literature include a method for the determination of apigenin in plasma by HPLC with UV detection [11] in which plasma samples were directly analysed after protein precipitation with methanol and a study of the epithelial transport of chrysin by Caco-2 cells by Walle [12], in which the flavonoid was analysed in the buffer used in the experiment without further purification. More frequently flavonoids are first hydrolysed with β-glucuronidase, sulphatase or a mixture containing both enzymes. To determine specifically the amount of glucuronides or sulphates, preparations of β-glucuronidase free of sulphatase activity or sulphatase preparations free of β-glucuronidase activity are used. These enzymes are commercially available. For high precision in the estimation of metabolites, it is important to include a control compound to monitor for residual enzymatic activity. Thus, when β-glucuronidase hydrolysis is carried out, 4-methylumbelliferone sulphate is usually included to check for absence of residual sulphatase activity, and when β-glucuronidase is being used, phenolphthalein glucuronide is employed to check for absence of residual β-glucuronidase activity. The released aglycones are then extracted using liquid–liquid extraction or solid-phase extraction. Thus, Erlund [13,14] used solid-phase extraction with Bond Elut® C_{18} cartridges in the preparation of human plasma samples for the analysis of quercetin following hydrolysis with β-glucuronidase. A similar solid-phase extraction method was used by Young [15] in a study which evaluated the effect of fruit juice intake on urinary levels of quercetin. When sample preparation involves solid-phase extraction, it is important to control pH in the conditioning and washing steps by using an acidic modifier or a buffer in order to improve recovery by suppressing ionisation of flavonoids and increasing their retention. When liquid–liquid extraction is used, common solvents used for extraction of flavonoid aglycones are ethyl acetate and methylene chloride. With gas chromatography, extraction is often followed by conversion of flavonoids into their TMS derivatives. Examples are the determination of catechin and its metabolites in human plasma by GC–MS (Fig. 2), in which catechin and 3′-methyl catechin were derivatised with BSTFA [16], and a study of the effect of red wine on the human plasma levels of (+)-catechin [17]. Table 1 summarises the conditions used for sample preparation and chromatographic conditions used for the analysis of flavonoids in biological matrices in the cases where there was some degree of methodo-

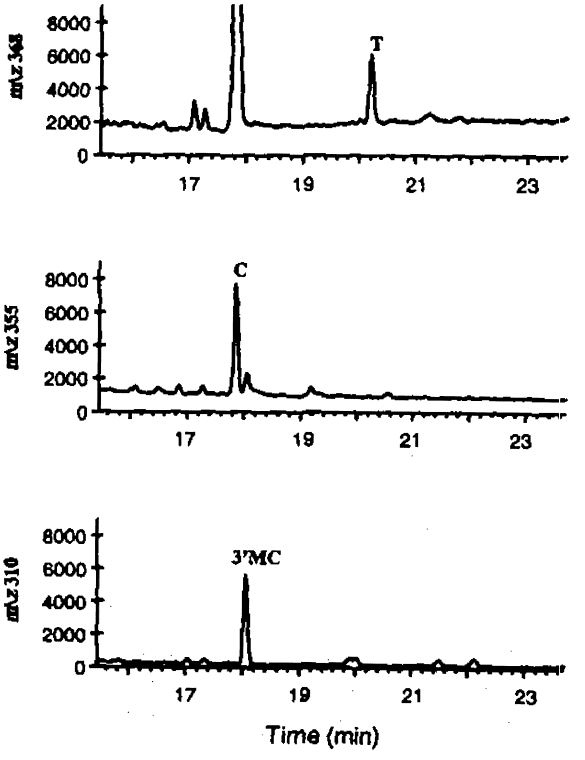


Fig. 2. GC–MS chromatogram of a human plasma sample after consumption of red wine showing the detection of catechin as TMS derivatives. (C), (+)-catechin; (3′MC), 3′-*O*-methyl-catechin; (T), taxifolin (internal standard). Reproduced from Ref. [16] with kind permission of American Society for Nutritional Sciences.

Table 1
Chromatographic methods for the determination of flavonoids and phytoestrogens in biological fluids

Compound/reference	Matrix/sample preparation	Technique and chromatographic conditions	Detection/validation/comments
Apigenin [11]	Plasma (0.5 ml)+MeOH (0.8 ml), centrifuged, and supernatant dried and redissolved in MeOH	HPLC. Column: SG C_{18} (150×3 mm, 7 μm). Mobile phase: 2% formic acid–MeCN–MeOH (40:35:25, v/v). Flow rate: 1 ml min^{-1}	UV detection at 349 nm. Method validated. Precision: 0.5–6%. Range: 0.5–200 μmol l^{-1}. LOD: 0.1 μmol l^{-1}
Flavonoid aglycones and glycosides (several) [75]	Urine (3 ml aliquot of 24-h urine samples) was filtered (0.2 μm filter) and to 2 ml of the filtered sample 250 ng of 5,7,8-trihydroxyflavone (I.S.) were added, samples were hydrolysed with β-glucuronidase+sulphatase and samples were evaporated to dryness under vacuum. Prior to HPLC samples were redissolved in 10% aqueous MeOH and 25 μl of morin dissolved in DMSO (10 ng ml^{-1}) added as an additional I.S. Sample was centrifuged and the supernatant injected	HPLC. Column: Zorbax 300 SB-C3 (50×4.6 mm, 3.5 μm) with a guard cartridge (4×4 mm, 5 μm) was used as column 1, and a Zorbax C_{18} (150×4.6 mm, 5 μm) as column 2. A column-switching technique was used with a gradient of 0.5% aqueous formic acid, acetonitrile, and methanol as mobile phase delivered at 0.8 ml min^{-1}. A six-port injection valve was used. Column 1 was used as clean-up column, and a flavonoid-rich fraction was injected from column 1 to column 2	UV detection at 260 and 350 nm and scans acquired between 210 and 600 nm in 2-nm steps. Mass spectrometry detection was used in series in APCI negative ionisation mode. The capillary voltage was 2.5 kV, corona current 20 μA, vaporizer temperature 500°C, drying gas temperature 300°C fragmentor voltage: 140 V. LOD: 0.25–2.5 ng ml^{-1} urine. Linearity from 25–1000 ng ml^{-1} urine
Quercetin and kaempferol [19]	Urine (1 ml) was analysed untreated or after hydrolysis with β-glucuronidase (100 units), sulphatase (10 units) or acid (0.5 ml of 3 *M* HCl). Deuterated analogues of analytes (200 ng) were added as I.S. and the flavonoids extracted using SPE (Isolute ENV^+, 100 mg). Extracts were dried under a stream of N_2 and derivatised with BSA before GC–MS	GC–MS. Column: Restek RTX-5 capillary column (30 m, 0.32 mm I.D., 0.5 μm film thickness), helium as carrier gas at 68.9 kPa. Injector temperature: 250°C, and transfer line at 280°C. Oven temperature program: 160°C (1 min), then at 20°C/min to 290°C, and to 320°C at 5°C min^{-1}	Negative ion chemical ionisation in SIM mode was used for quantification. Methane was used as reagent gas to give an ion source pressure of ca. 133 Pa. Recoveries were 76.6±10.5% and 73.0±9.8% for kaempferol and quercetin, respectively. Precision: C.V. for a single sample analysed as six aliquots: 9.40% for kaempferol and 7.34% for quercetin. LOD: 10 pg on column.
Catechin and catechin metabolites [16,17]	Plasma (either untreated or hydrolysed with β-glucuronidase+sulphatase) was extracted with CH_2Cl_2 (1 ml) and water (500 μl). Aqueous phase extracted 2× with EtOAc (2 ml, 1.5 ml). Combined organic layers dried over Na_2SO_4, solvent dried under stream of N_2, and the residue derivatized with 30 μl of BSTFA	GC–MS. Column: DB-5 capillary (30 m×0.25 mm I.D., 0.25 μm), helium as carrier gas. Oven: 100°C at 30°C min^{-1} hold for 30 min (+)-taxifolin used as internal standard	Mass spectrometry used with SIM Precision 5%. LOD 0.7 nmol l^{-1} (at $S/N = 3$)
Quercetin [13,14]	Plasma (1 ml) hydrolysed with β-glucuronidase+sulphatase and after dilution with phosphate buffer (pH 2.4) extracted with BondElut SPE cartridges	HPLC. Column: Inertsil ODS-3 (250×4.0 mm, 5 μm) with a pre-column filter. Mobile phase: 59% MeOH in phosphate buffer (70 m*M*, pH 2.4). Flow rate: 1 ml min^{-1}	ECD (electrode at +100 mV). Method validated. Precision 7.9% (inter-day) and 4.3% (intra-day). Recovery was 70–71%, LOD: 0.63 μg l^{-1} at $S/N = 3$ or more
Catechin [76]	Plasma (200 μl) was hydrolysed with β-glucuronidase+sulphatase, deproteinated with MeCN and centrifuged. Supernatant was treated with 1 ml alumina, suspended in 50 m*M* Tris buffer pH 7.0. Alumina was washed with 50 m*M* Tris buffer pH 7.0 followed by MeOH and supernatants were discarded. Alumina was dried under N_2 and 250 μl of 0.25 *M* perchloric acid added. Aqueous phase was separated and supernatant filtered before HPLC	HPLC. Column: RP C_{18} (150×4.6 mm, 5 μm) with a C_{18} guard column. Mobile phase: 40% MeOH–60% 50 m*M* NaAc pH 5.8 (A) and 7% MeOH–93% 100 m*M* NaAc pH 5.2 (B). For single electrode detection: isocratic at 60% A or gradient — 0 min 80% B, 1 min 60% B, 3.5 min 20% B, 20 min 20% B, and 30 min 80% B. For multielectrode detection: gradient was 0 min 80% B, 1 min 60% B, 3 min 60% B, 7 min 20% B, 9 min 0% B, 15 min 0% B, 18 min 20% B, 20 min 40% B, 23 min 60% B, and 25 min 80% B. Flow rate: 1 ml min^{-1}	ECD with single electrode (detection cell at +400 mV) or multielectrode detection (potentials −50, +150, +185, +200, +250, +300, +700, and +800 mV). Method validated. Recovery between 70 and 90%, LOD: 20 pg

Table 1. Continued

Compound/reference	Matrix/sample preparation	Technique and chromatographic conditions	Detection/validation/comments
(−)-Epicatehcin and metabolites [77]	Plasma, urine, and bile (0.5–2.0 ml) were extracted with EtOAc, extracts dried under reduced pressure and redissolved in mobile phase	HPLC. Column: ODS Capcell-pak C_{18} A6 (250×4.6 mm). Mobile phase: MeCN–EtOAc–0.05% phosphoric acid (12:0.6:90, v/v). Flow rate: 1.0 ml min^{-1}	UV detection at 280 nm with PDA spectra acquired between 230 and 450 nm. Method not validated
Catechin [78]	Urine (50 μl) was hydrolysed with β-glucuronidase and sulphatase and released aglycones extracted with EtOAc. Extracts were dried and redissolved before HPLC analysis	HPLC. Column: NBS C_{18} (150×4.6 mm, 5 μm). Mobile phase: (A) 30 m*M* NaH_2PO_4 with 2.37% MeCN and 0.12% THF pH 3.35; (B) 30 m*M* NaH_2PO_4 with 40% MeCN and 6.65% THF pH 3.45. Gradient: 0 min 96% A, 24 min 76% A, 35 min 5% A. Flow rate: 1.0 ml min^{-1}	EAD with electrode potentials at −90, −10, 70, and 150 mV. Method originally validated by Lee et al. [79]
Quercetin, isoquercitrin, rutin, ferrulic acid and chlorogenic acid [80]	Urine. Urine samples (0.9 ml) were analysed for quercetin and ferrulic acid either untreated or after hydrolysis with β-glucuronidase. These compounds were isolated from urine (0.9 ml) by extraction with CH_2Cl_2 (10 ml). Rutin, isoquercitrin, and chlorogenic acid were extracted from urine by SPE using BondElut C_{18} cartridges	HPLC. Column: Nova-Pak C_{18} (250×4.6 mm, 4 μm) kept at 30°C. Mobile phase was (A) 20% aqueous methanol in 0.1% HCl and (B) MeCN. Flow rate: 0.8 ml min^{-1}. Gradients: quercetin and ferrulic acid: 0 min 5% B, 10 min 5% B, 45 min 50% B, 55 min 5% B. Chlorogenic acid: 0 min 100% B, 10 min 100% B, 25 min 50% B, 30 min 100% B. Rutin and isoquercitrin: 0 min 100% B, 20 min 100% B, 45 min 50% B, and 55 min 100% B	Detection by UV. Data acquired at both 280 and 320 nm. No validation data available
Daidzein [81]	Plasma samples (100 μl untreated or pretreated with β-glucuronidase+sulphatase) were mixed with 10 μl I.S. (biochanin A, 5 μg ml^{-1}, 75 μl 1 *M* NH_4Ac buffer pH 7 and 83.3 μl 1 *M* triethylammonium sulphate buffer pH 7 and heated at 60°C for 10 min. The samples were then extracted using SPE (Lichrolut RP18, 0.5 g). The methanolic eluate was dried under a stream of N_2 and redissolved in MeOH–1% AcOH (40:60, v/v). Tissue samples were homogenized, hydrolysed with β-glucuronidase+sulphatase and extracted with EtOAc	HPLC. Column: Phenomenex Luna C_{18}(2) (150×1 mm, 3 μm) with a guard column (30×1 mm) with same stationary phase. Mobile phase: (A) 10% MeCN and 1% acetic acid in water. (B) MeCN. A gradient of A and B was used. For free daidzein (untreated plasma): 0–5 min 25% B, 5–20 min 25–70% B, 20–40 min 70% B, 40–50 min 25% B. Flow rate was 40 μl min^{-1} (reduced from 260 μl min^{-1} with a splitter). For total daidzein in plasma and tissues: 0–5 min 25% B, 5–25 min 25–50% B, 25–40 min 50–100% B, 40–41 min 100–25% B, 41–50 min 25% B	UV detection at 250 nm. Method is a modification of a previously published one [82] and was not revalidated. LOD was 25 ng ml^{-1} for daidzein plasma

logical development in comparison with methods which were published previously. The table includes papers published in the period 1998 to early 2001.

2.2. Chromatographic conditions

By far the most frequently employed technique for analysis of flavonoids in biological fluids is HPLC (see Table 1). Reversed-phase columns such as C_{18} are the most frequently used. Mobile phases usually consist of a combination of an acidic buffer (or an aqueous phase with an acid as additive) and an organic solvent component, usually methanol or acetonitrile. The use of an acid modifier is important to suppress ionisation of the more acidic phenolic groups and interactions of these groups with residual traces of metals in the stationary phase that are detrimental to peak shape. Usually the best peak shape is obtained with trifluoroacetic acid, although formic, acetic and phosphoric acid are widely used. When interfacing of liquid chromatography with mass spectrometry is required, the correct choice of the acidic modifier is crucial for the ionisation of polyphenols. For example, trifluoroacetic acid is not suitable for the analysis of flavonoids in negative ion electrospray mode, since it suppresses ionisation of the analytes. We found that acetic acid is a good acid modifier for the detection of flavonoids and flavonoid glucuronides in biological matrices using electrospray ionisation in negative ion mode [18].

When gas chromatography is combined with mass spectrometry it is possible to achieve excellent selectivity and sensitivity. Recent examples include the use of GC–MS for the quantification of quercetin and kaempferol as TMS derivatives in human urine (LOD ca. 10 pg on column) [19], and the determination of catechin and its metabolites in human plasma (LOD of 0.7 nmol l^{-1}) after consumption of red wine [16].

Another technique that has been applied to the analysis of flavonoids is capillary electrophoresis. This technique has high separation efficiency and is potentially well suited for interfacing with mass spectrometry. Recent papers on CE of flavonoids include the optimisation of parameters for the separation of flavonoids by micellar electrokinetic chromatography (MEKC) [20,21] and the use of capillary zone electrophoresis and MEKC for several natural products including flavonoids [22]. Rodríguez-Delgado et al. [21] found that intermediate concentrations of SDS (50 m*M*), low concentrations of MeOH (5%), and high electrolyte concentration (150 m*M*) produced the best results in MEKC for a series of 12 flavonoids including flavonols, catechins, flavonol glycosides and phenolic acids. One factor that limits the application of capillary electrophoresis for xenobiotic detection in biological fluids is its low sensitivity compared with HPLC. In a recent comparison of HPLC and CE for the analysis of catechins and theaflavins, Lee and Ong [23] found that the HPLC method was five times more sensitive than CE. The authors used UV detection at 205 nm instead of 275 or 375 nm at which catechins are usually measured. At this wavelength the specific absorbance of the catechins is generally higher and lower detection limits can be achieved.

2.3. Detection

Flavonoids extracted from biological matrices are usually monitored with UV or fluorescence detection. Detection by UV is frequently used, but for determination of free aglycones in plasma or urine, more sensitive methods are usually required. One alternative is fluorescence detection. A number of papers appeared in the literature in the last 2 years that made use of fluorescence detection. They are all applications or modifications of a method originally published by Hollman [24] which uses post-column derivatisation of the flavonoids with aluminium nitrate to form a fluorescent complex of aluminium. This method has a detection limit of 0.15 ng ml^{-1} for quercetin and 0.05 ng ml^{-1} for kaempferol [24]. Recent applications of this technique include papers investigating the bioavailability of rutin [25], bioavailability and metabolism of quercetin [26], influence of the sugar moiety on bioavailability of flavonoid glycosides [27] and the absorption and antioxidant effects of flavonoids from an onion meal [28]. One drawback of the method is that increasing the amount of water in the mobile phase decreases the response of the fluorescence detector to the flavonoid–aluminium complex. Thus, the detector response is maximum when the content of water in the mobile phase is kept at 10%, which is not enough to provide adequate retention of flavonoids in reversed-phase systems. With a water content in the mobile phase of 40% the detector response drops to about 37% of the maximum possible value, but the sensitivity is still greater than that obtained with UV detection.

Electrochemical detection is another sensitive and selective strategy for quantifying flavonoids in biological matrices. Electrochemical detection has been applied to the detection of flavonoids in biological fluids and tissues with limits of detection at the nanogram level. The use of a serial array of coulometric detection cells further increases the selectivity of electrochemical detection. Recent examples include the analysis of isoflavones and lignans in human serum by Gamache et al. [29]. The authors analysed the levels of the soya isoflavones genistein and daidzein and the lignans enterolactone and equol in unsupplemented human serum. Serial detectors with potentials set at 340, 470, 500, 530, 560, 620, 680 and 760 mV were used and the limits of detection were 0.8 ng ml^{-1} (daidzein), 1.2 ng ml^{-1} (enterolactone), 0.8 ng ml^{-1} (equol) and 1.4 ng ml^{-1} (genistein) with a dynamic range of 10–2500 ng ml^{-1}. Other applications of electrochemical detection include the determination of catechins and theaflavins in saliva, plasma and urine with a limit of detection of 5–10 ng ml^{-1} [30], the detection of intracellular levels of quercetin [31] and an inves-

tigation of the antioxidant activity of quercetin and its conjugates [32].

Gas chromatography coupled with mass spectrometry is a very sensitive and selective detection mode for flavonoids. As mentioned before, flavonoids are usually analysed by gas chromatography as trimethylsilyl derivatives. In the literature reviewed we found several applications of GC–MS for the analysis of flavonoids as TMS derivatives. Those include the determination of phytoestrogens (the lignans enterodiol and enterolactone, and the isoflavonoids, equol, daidzein and genistein) in serum [33]. The method used Sephadex LH-20 to isolate the phytoestrogen fraction from serum and the samples were derivatised with BSTFA. In separate work, Donovan et al. [16] also used BSTFA to derivatize catechins for determination in human plasma. The limit of detection was 0.7 nmol l^{-1} (at $S/N = 3$). The synthesis of stable deuterium-labelled analogues of flavones and flavonols [34] and phytoestrogens [35] is an important contribution because of the high degree of precision afforded by the use of such internal standards in isotope-dilution methods for the quantification of these compounds in biological fluids.

The use of high-performance liquid chromatography interfaced with atmospheric pressure mass spectrometry is perhaps the detection mode with the highest sensitivity and selectivity for analysis of flavonoids in biological fluids. Flavonoids are usually analysed using electrospray or atmospheric pressure chemical ionisation in the negative ion mode. As mentioned before, the use of an acid modifier that does not interfere with the ionisation of analytes is essential, and commonly used ones include acetic and formic acids. Applications in the literature reviewed include the use of a column-switching LC–APCI mass spectrometry method [36] for the analysis of 12 flavonoids including flavanones, dihydrochalcones (and dihydrochalcone glycosides), flavonols, flavonol glycosides and flavones. The limit of detection of the method was 0.25–2.5 ng ml^{-1} urine for aglycones and glycosides. In another study, Mauri et al. [37] used LC–ESI mass spectrometry to identify flavonoid glycosides in tomato extract and in human plasma. This study supports the controversial idea that flavonoid glycosides can be absorbed intact in humans without prior hydrolysis to their corresponding aglycones.

3. Carotenoids, retinoids, tocopherols, tocotrienols and their oxidation products

Carotenoids are a class of compounds that consist of eight isoprenoid units joined to form conjugated hydrocarbons (carotenes) and their hydroxylated derivatives (xanthophylls). The structures of some representative carotenoids are shown in Fig. 3. Carotenoids are important dietary antioxidants and some of them are precursors of vitamin A. Apart from their action as antioxidants they show a variety of biological actions that may be related to their

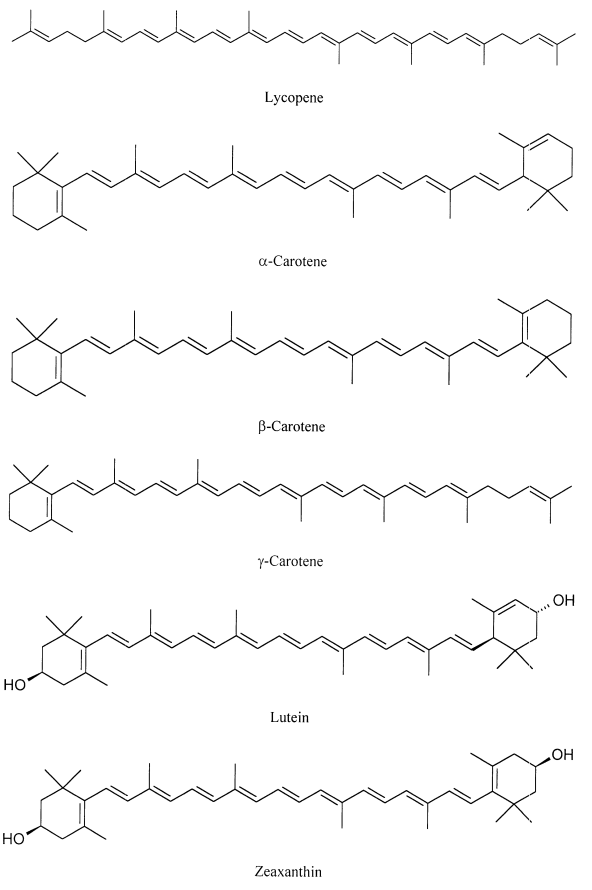
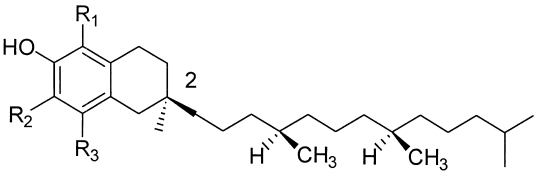


Fig. 3. Chemical structures of some biologically relevant carotenoids.

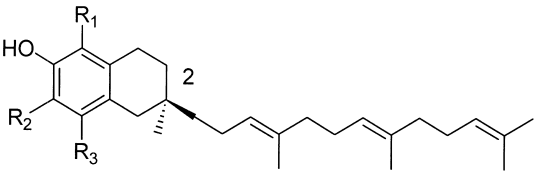
alleged anticancer action [38]. There is epidemiological evidence suggesting that consumption of carotenoid-rich fruit and vegetables is associated with reduced risk of certain tumours such as those of the lung and prostate [39,40]. However, two major trials failed to confirm epidemiological evidence, showing instead that *n*-carotene supplementation in smokers was associated with a slightly increased risk of lung cancer [41]. The role of carotenoids in cancer prevention is still open to debate, and methods for quantification of carotenoids in biological fluids are essential to resolve the question of whether or not carotenoids themselves have anticancer properties or are only markers for a diet rich in fruit and vegetables.

The retinoids are related to β-carotene in that they are comprised of retinoic acid and related compounds which may be derived from it. Retinoids are involved in the regulation of the growth and differentiation of many cell types both in fetal development and, for most cell types, throughout life. A number of nuclear receptors have been discovered for retinoids including three receptors for retinoic acid and three (retinoid X) receptors for retinoic acid metabolites. These receptors are related to the steroid/thyroid family of receptors which control DNA transcription. The naturally occurring ligands for the retinoid X receptors appear to be 9-*cis*-retinoic acid, 3,4-dehydroretinoic acid and 4-oxoretinoic acid. Very little is known about the concentration of retinoic acid metabolites in tissues and physiological fluids and this is largely due to the difficulty of measuring them in the very low levels at which they are present. Obviously there is a great interest about these compounds both in relation to fetal development and in relation to aging and the growth of cancer cells.

Vitamin E is often analysed in the same lipophilic fractions which contain carotenoids and retinoids. It is important for its ability as an anti-oxidant to protect lipids in biological membranes from oxidation. It has been proposed that it may have a role in preventing diseases such as Parkinson's disease, heart disease and cancer. Vitamin E in fact includes the α-, β-, δ-, and γ-tocopherols (Fig. 4) which are structurally related and have varying numbers of methyl groups and the tocotrienols (Fig. 4) which have a triunsaturated isoprenoid side chain. Vitamin



α–tocopherol $R_1=R_2=R_3 = CH_3$
β–tocopherol $R_1=R_2= CH_3R_3 = H$
γ–tocopherol $R_1=R_2= CH_3R_3 = H$
δ–tocopherol $R_1=R_2= H\ R_3 = H$



α–tocotrienol $R_1=R_2=R_3 = CH_3$
β–tocotrienol $R_1=R_2= CH_3\ R_3 = H$
γ–tocotrienol $R_1=R_2= CH_3\ R_3 = CH_3$
δ–tocopherol $R_1=R_2= H\ R_3 = CH_3$

Fig. 4. Tocopherols and tocotrienols.

E in the form of α-tocopherol is the principal agent responsible for inhibiting oxidative damage to the lipids within cell membranes.

Examples of method development in the analysis of carotenoids, retinoids and vitamin E for the period 1998 to early 2001 are summarized in Table 2.

3.1. Sample preparation

The instability of carotenoids to direct light, atmospheric oxygen, heat, and also extremes of pH poses a challenge in the preparations of samples of plasma, urine and tissues for the quantification of these compounds. In a recent paper by Su et al. [42] investigating the photosensitivity of carotenoids, retinol and tocopherols in human plasma, the authors found the compounds to be stable to fluorescent light for up to 72 h. However, the variation in the measurements of the analytes was greater at room temperature than when samples were kept refrigerated at −20°C and protected from light. Carotenoid

Table 2
Chromatographic methods for the determination of carotenoids, tocopherols (Ts), retinoids, tocotrienols and their oxidation products

Compound/reference	Matrix/sample preparation	Technique and chromatographic conditions	Detection/validation/comments
Retinol, α- and γ-tocopherol (T) [83]	Serum (100 μl) was deproteinated with EtOH and extracted with BuOH–EtOAc (1:1, v/v). After centrifugation the supernatant was filtered (0.45 μm filter pore size) prior to HPLC analysis	HPLC. Column: Supelcosil LC-18 (150×4.6 mm, 3 μm) with an ODS Ultrasphere 100 guard (45×4.6 mm, 5 μm). Mobile phase: (A) MeCN–BuOH (95:5 v/v) and (B) H_2O. Gradient: 0 min, 90% A, 7 min 90% A, 17 min 100% A, 22 min 90% A. Flow rate: 1.5 ml min^{-1}	Detection: method validated. Precision: 2.4–3.7% (within-day) and 3.6–4.2% (between-day). LOD: 0.35–0.45 μmol l^{-1}. Linearity, μmol l^{-1}: retinol (0.07–8), α-tocopherol (0.07–30), γ-tocopherol (0.09–25)
Lipid soluble antioxidants, retinol α- and β-carotene, α- and γ-T β-cryptoxanthin, lutein/zeaxanthin, *trans*- and *cis*-lycopene [42]	Human plasma (0.2 ml)+95% ethanol (0.2 ml containing α-T acetate 200 μg ml^{-1}, retinal Ac 570 ng ml^{-1}). Extract with hexane (1 ml containing 0.01% BHT), centrifuge, remove organic phase, evap. and dissolve in 30 μl chloroform and add 70 μl acetonitrile–MeOH (1:1) and 50 μl injected	HPLC. Column: Spherisorb ODS-2, C_{18} (250×4.6 mm, 5 μm) with a C_{18} biocompatible guard column. Mobile phase: (A) MeOH+0.05% NH_4OH, (B) MeCN+0.1% triethylamine, (C) chloroform. Gradient: 0–5 min 50% A 50–44% B 0–6% C, 5–16 min 50–55% A 44–30% B 6–15% C, 16–21 min 55–50% A 30–50% B 0% C. Flow rate: 1.4 ml min^{-1}. Retinol acetate used as I.S. for retinol, and tocopherol acetate as I.S. for α- and γ-tocopherol	UV detection (at 325 nm for retinol and retinol acetate, 292 nm for α- and γ-tocopherol and 450 nm for α- and β-carotene, β-cryptoxanthin, lutein/zeaxanthin, and lycopene). Intra-batch RSD for all analytes ≤5% except for γ-tocopherol (5.1%), and lycopene (7.3%). Inter-batch was less then 10% for all analytes. Paper also investigated photostability of analytes in plasma samples
Lycopene, β-carotene [48]	Triglyceride-rich fraction (TRF) of plasma was prepared by adding 8 ml NaCl (9 g l^{-1}) to 3–5 ml of plasma. Samples were centrifuged for 1 h at 15,000 *g*. The TRF-fraction was then extracted with *n*-heptane–ether (3:1, v/v)	HPLC. Column: Nucleosil 1005CN. Mobile phase: *n*-heptane. Flow rate: 0.7 ml min^{-1}. Ethyl-apo-8-carotenoate was used as I.S. for carotenoid determination and retinyl palmitate for retinol determination	Detection of lycopene by UV at 470 nm, β-carotene at 450 nm and retinyl palmitate at 325 nm. Intra-assay C.V. was 4.5% for lycopene and 3.9% for β-carotene
α-, β-, γ- and δ-T [52]	Human serum (100 μl) was first extracted with hexane from Pasteur pipettes filled with Extrelut (Merck) and then the hexane extract was further purified by eluting with *n*-hexane–diethylether (5:1, v/v) from Pasteur pipettes filled with silica gel. The extract was evaporated to dryness and either redissolved in mobile phase for HPLC or derivatized with MSTFA to form TMS derivatives	GC–MS. Column: RTx-5ms (30 m×0.25 mm I.D., 0.25 μm film thickness). Oven: 220°C 1 min) to 290°C (at 5°C min^{-1}), hold at 290°C for 10 min. Injection port: 275°C, splitless injection. Transfer line: 280°C, helium as carrier gas (30 cm s^{-1}). Ion source at 170°C, multiplier at 1400 V. HPLC. Column: Lichrosorb Si 60 (normal phase), (250×4.6 mm, 7 μm). Mobile phase: isooctane–isopropanol (99:0.5, v/v). Flow rate: 1.25 ml min^{-1}	MS (SIM) used for detection when GC–MS was used and fluorescence detection with HPLC (λ_{ex}=295 nm and λ_{em}=330 nm). GC–MS precision (day-to-day) is ±5% for ≥1 mg l^{-1} serum of tocopherol and ±10% for ≤1 mg l^{-1} serum of tocopherol. LOD is 40 pg using SIM. HPLC precision (day-to-day) is ±5% for ≥1 mg l^{-1} serum of tocopherol, and LOD is 50 pg
α-, β-, γ and δ-tocotrienols +α-tocopherol [84]	Human plasma (250 μl)+acetonitrile–THF (3:2 v/v, 500 μl), vortex and centrifuge 12,800 *g*, 100 μl of supernatant injected into HPLC	HPLC. Column Metaphase Crestpak C_{18} (250×4.6 mm, 5 μm), mobile phase 0.5% v/v water in methanol at 1.5 ml min^{-1}	Fluorescence detection λ_{ex} 293 nm, λ_{em} 330. Method validated. Linear ranges 40–2580 ng ml^{-1}, 34–4340 ng ml^{-1} and 16–1040 ng ml^{-1} for α-, γ- and δ-tocotrienols, respectively

Table 2. Continued

Compound/reference	Matrix/sample preparation	Technique and chromatographic conditions	Detection/validation/comments
α-, β-carotene and β-cryptoxanthin [44,85]	Cervical tissue. The tissue was ground by mechanical homogenisation with 1000 units of collagenase and 0.2 ml PBS containing 2.5 g l^{-1} ascorbic acid. The homogenate was rinsed with 2 ml EtOH and the rinsate extracted with 3×2.0 ml of hexane containing 0.2 g l^{-1} of BHT. Pooled extracts were pooled and dried under a stream of N_2. Plasma sample preparation not stated	HPLC. Plasma carotenoids: Column: Supelcosil LC-18 (250×4.6 mm, 5 μm). Mobile phase: MeCN–MeOH–CH_2Cl_2 (70:10:20, v/v) with triethylamine (0.13 ml l^{-1} acetonitrile) and ammonium acetate (0.1 g l^{-1} methanol). Cervical tissue analysis: Column: YMC C_{30} (150×4.6 mm, 5 μm) with a C_{18} guard column. Mobile phase: (A) MeOH–methyl tert.-butyl ether–NH_4Ac (95:3:2) and (B) MeOH–methyl tert.-butyl ether–NH_4Ac (20:78:2). Gradient: 1–5 min (85% A, 15% B), 5–25 min (60% A, 40% B) hold 5 min	Detection of plasma carotenoids was by UV–VIS (450 nm) and cervical tissue carotenoids detected using EAD (8 channels with from 100–520 mV in 60 mV increments). Day-to-day precision: C.V.<10%
α- and β-carotene, α-tocopherol, retinol, lycopene, lutein and β-cryptoxanthin [86]	Plasma (200 μl) or 500 μl aliquots of plasma lipoproteins were deproteinated with an equal volume of EtOH (with 0.1 g l^{-1} BHT and retinyl acetate as I.S.). Samples were extracted 2× with hexane (containing 0.1 g l^{-1} BHT) and the combined hexane layers evaporated to dryness under vacuum. Residue redissolved in ethyl ether–mobile phase A (see on the right) (1:3, v/v)	HPLC. Column: C_{30} carotenoid column (250×4.6 mm, 5 μm) with a C_{30} guard column. Mobile phase (A) MeOH (containing 1 g l^{-1} NH_4Ac), (B) methyl-tert.-butyl ether. Gradient from 100% A to 100% B over 30 min. Flow rate was 1 ml min^{-1}. Retinyl acetate was used as an I.S.	Detection was made using a UV PDA detector. Data were collected at 290, 325 and 453 nm. Inter-assay C.V. was <5% for carotenoids, retinol and α-tocopherol
Vitamin E and 7-dehydro-cholesterol (7-DHC) [87]	Human plasma (200 μl)+ethanol (200 μl), mixed, extracted with hexane (1.5 ml containing 12.5 μg ml^{-1} of BHT). Hexane evaporated and residue dissolved in methanol and 100 μl injected into HPLC	HPLC HP amino analyzer series 2. Column Supercosil LC 18 (250×5 mm, 5 μm) mobile phase methanol at 1.5 ml min^{-1}	DAD, vitamin E monitored at 292 nm and 282 nm for 7-DHC. Method validated
Zeaxanthin and lutein [47]	Serum (150 μl) was extracted with 2 ml $CHCl_3$–MeOH (2:1, v/v). The mixture was mixed, centrifuged and the chloroform layer evaporated under a stream of nitrogen. The sample was then extracted with 3 ml of hexane and centrifuged as before. Hexane and chloroform layer were combined, dried and redissolved in 150 μl of EtOH with sonication prior to HPLC analysis	HPLC. Column: C_{30} carotenoid column (150×4.6 mm, 3 μm) kept at 16°C. Mobile phase was: (A) MeOH–methyl-tert.-butyl ether–H_2O (83:15:2, v/v) with 1.5% NH_4Ac in H_2O. (B) MeOH–methyl-tert.-butyl ether–H_2O (8:90:2, v/v) with 1.5% NH_4Ac in H_2O. Flow rate: 1 ml min^{-1}	Detection using a UV PDA detector. The method claims to separate lutein, zeaxanthin, cryptoxanthin, α-carotene, 13-*cis*-β-carotene, all-*trans*-β-carotene, 9-*cis*-β-carotene, as well as four geometric isomers of lycopene (15-*cis*, 13-*cis*, 9-*cis*, and all-*trans*-lycopene). External standards were used for quantification. LOD: 0.2 pmol
Lycopene, α- and β-carotene [46]	Serum (0.5 ml) was pipetted into a centrifuge tube and 1.0 ml (2 μg ml^{-1}) of I.S. (Squalene) added. The samples were vortexed and 1 ml hexane added. After centrifugation the supernatant was removed. The extraction was repeated and extracts pooled and evaporated under a stream of N_2 and redissolved in 50 μl of EtOH	LC–MS. Column: Mightsil RP-18 (75×4.6 mm, 5 μm) kept at 30°C. Mobile phase: MeOH–MeCN (70:30, v/v). Flow rate: 1 ml min^{-1}. UV detection at 450 nm. MS conditions: needle voltage: 3 kV, multiplier at 1900 V, nebulizer gas: 400°C, and desolvator gas at 200°C	APCI (positive ion mode) MS detection with SIM ($m/z=537$ for α- and β-carotene and $m/z=411$ for squalene). C.V. for lycopene, α- and β-carotene determination were 10%, 8.4% and 5.3%, respectively. LOD was 3 ng ml^{-1} serum (at $S/N=3$).

Table 2. Continued

Compound/reference	Matrix/sample preparation	Technique and chromatographic conditions	Detection/validation/comments
α-, β-, γ- δ-Ts and α-, β-, γ- δ-tocotrienols [88]	Tissues removed from animals and immediately frozen using dry ice. For analysis tissue (1 g) pulverised at −70°C using steel mortar and pestle. Extracted with 2 ml of water and 5 ml of ethanol containing 4 μg of internal standard added, sample sonicated while cooled in ice bath, further 5 ml of ethanol added and then 10 ml of hexane. Sample vortexed and centrifuged. Upper layer removed and taken to dryness	HPLC. Column: Supelcosil LC-Diol column (250×4.6 mm, 5 μm particle size) with hexane–2-propanol (99:1) mobile phase at 1 ml min^{-1}. All vitamin E forms separated in 20 min. Elution order from diol column similar to that from silica gel	Detection FL2000 fluorescence detector λ_{ex} 296 nm, λ_{em} 330 nm. Fluorescence response for analytes linear over the range 10 to 100 ng
Retinol, α-tocopherol, and carotenoids [45]	To aliquots of plasma (200 μl) 100 μl of I.S. (5 μmol l^{-1} of retinol acetate and 40 μmol l^{-1} of tocopherol acetate) and the volume made up to 1.1 ml with distilled water. Proteins were precipitated with 70 μl of EtOH (containing 0.01% ascorbic acid) and the samples were extracted with 2.0 ml hexane. The samples were dried under a stream of N_2 and the samples dissolved in THF before being diluted with mobile phase prior to HPLC	HPLC. Column: Nucleosil ODS (250×3.2 mm, 5 μm). Mobile phase: MeOH–MeCN–THF (75:20:5) containing 0.01% ascorbic acid. Flow rate: 0.6 ml min^{-1}	Detection by UV–VIS at 325 nm for retinol and retinol acetate, 290 nm for α-tocopherol and tocopherol acetate, and 450 nm for lutein, β-cryptoxanthin, lycopene, α-carotene, and β-carotene. Recovery between 87% (β-carotene) and 96% (β-cryptoxanthin, and α-tocopherol). LOD: 0.3 μmol l^{-1} retinol, 2.5 μmol l^{-1} α-tocopherol, 0.019 μmol l^{-1} lutein and, β-cryptoxanthin, 0.028 μmol l^{-1} α-carotene, β-carotene and lycopene
Trans-β-carotene, lutein, zeaxanthin, β-cryptoxanthin, and α- and β-carotene [66]	Plasma aliquots (20 μl) were deproteinated with 100 μl EtOH and extracted with hexane (3×200 μl) containing 0.02% BHT. Extracts were dried under N_2. Cervical tissue (10 mg) was rinsed with fresh saline and homogenised in 1.8 ml of saline containing 1000 units of collagenase and 0.2 ml saline with 2.5 g l^{-1} ascorbic acid. The sample was ground by mechanical homogenisation and saponified with 5.0% ethanolic KOH, and carotenoids extracted with 3×2.0 ml of hexane containing 0.02% BHT	HPLC. Column: C_{30} polymeric stationary phase from the National Institute of Standards and Technology (250×4.6 mm, 5 μm) or a YMC C_{30} column (150×4.6 mm, 5 μm). Mobile phase: (A) MeOH–methyl-tert.-butyl ether–NH_4Ac (95:3:2, v/v) and (B) MeOH–methyl-tert.-butyl ether–NH_4Ac (25:73:2, v/v). A gradient was used: 0−5 min 87.5% A, 5–25 min linear gradient to 65% A, hold 5 min. Flow rate: not stated	An 8-channel-coulometric array electrochemical detector was used with potential settings from channels 1 to 8 of 100 to 520 mV in 60 mV increments. LOD for *trans*-β-carotene was 6.25 pg
Ubiquinol 10, ubiquinone 10, carotenoids and T [49,89]	To aliquots of neonatal plasma samples (5 or 10 μl) 50 μl of internal standard solution were added (61 pmol γ-tocotrienol, 4.2 pmol ubiquinone 7, 28 pmol ubiquinol 9, 0.1 pmol ubiquinone 9, 1.8 pmol ethyl β-apo-8′-carotenoate). Samples were extracted with 500 μl of hexane, and 400 μl of extract was redissolved in 5% 2-propanol in EtOH–MeOH (1:1, v/v) prior to HPLC	HPLC. Column: SuperPac Prep-S $RP_{c2/c18}$ (250×4.0 mm, 5 μm) with a guard column (10×4.0 mm, 5 μm). Mobile phase: 13.4 m*M* lithium perchlorate in methanol–EtOH–2-propanol (88:2:10, v/v). Flow rate: 1.2 ml min^{-1}	Electrochemical detection was used. Coulometric electrode (conditioning cell) at −0.6 V, first coulometric electrode (analytical cell) at −0.15 V, and second amperometric electrode (analytical cell) at +0.60 V. Within-day C.V. between 3 and 14%. LOD: 21–60 fmol/20 μl injection volume (at $S/N = 5$)

Table 2. Continued

Compound/reference	Matrix/sample preparation	Technique and chromatographic conditions	Detection/validation/comments
All-*trans*-retinol and all-*trans*-retinyl palmitate [63]	Serum (1 ml) containing 1.84 pmol μl^{-1} retinyl acetate as I.S. was diluted with saline and proteins precipitated with 1 ml EtOH. The solution was extracted with 3×2 ml hexane. The combined extracts were evaporated to dryness in a centrifuge/vacuum concentrator and redissolved in mobile phase.	LC–MS. Column: YMC C_{30} column (100×2.0 mm). Mobile phase: (A) MeOH–H_2O–AcOH (50:50:0.5, v/v). (B) MeOH–methyl tert.-butyl ether–AcOH (50:50:0.5, v/v). A gradient from 30–90% B in 30 min was used for separation. Flow rate: 250 $\mu l\ min^{-1}$	APCI (positive ion mode) using SIM. Nitrogen pressure 30 p.s.i., vaporizer temp: 200°C, corona current 5.0 μA, nitrogen drying gas at 250°C at a flow-rate of 7 $l\ min^{-1}$, capillary voltage: 3.8 kV, cone voltage: 60 V. LOD for all-*trans*-retinol and all-*trans*-retinyl palmitate was ca. 34 fmol μl^{-1} and 36 fmol μl^{-1}, respectively. C.V. was 1.23% and 12.5% for retinol and retinyl palmitate, respectively
α-T [90]	Human plasma (100 μl)+acetonitrile–THF (3:2 v/v, 500 μl), vortex and centrifuge 12,800 *g*, 20 μl of supernatant injected into HPLC	HPLC. Column Metaphase Crestpak C_{18} (250×4.6 mm, 5 μm), mobile phase 6% v/v THF in methanol at 1.5 $ml\ min^{-1}$	UV detection at 292 nm. Method thoroughly validated. Linear 0.42–13.5 $\mu g\ ml^{-1}$
α-T 2R, 4′R, 8′R and 2S, 4′R, 8′R isomers. α-TQ 2R, 4′R, 8′R and 2S, 4′R, 8′R isomers [60]	Rat tissue (0.5 g) or plasma (0.2 ml) mixed with 1% w/v NaCl (0.1 ml), ethanol (containing 6% w/v pyrogallol, 1 ml). Sample heated with 60% KOH (0.2 ml) 70°C for 30 min, 1% w/v NaCl (4.5 ml) added and sample shaken with 3 ml hexane–EtOAC (9:1). Centrifuge and remove 2 ml of upper layer, evap. and dissolve in 0.2 ml of hexane. Pentamethyl-6-hydroxychromone added as an internal standard.	HPLC. Column: Chiracel OD-H (250×4.5 mm) in series with Sumichiral OA4100 (250×4.5 mm). Mobile phase hexane–isopropanol (97.3:2.7 v/v) at 0.3 $ml\ min^{-1}$ at 30°C	UV detection at 268 nm. SRR- and RRR-α-T baseline separated. SRR and RRR-α-TQ ca. 25% resolved. Range 0.4–40 $\mu g\ ml^{-1}$.
Retinyl palmitate and β-carotene [91]	Serum samples (1 ml for β-carotene and 0.2 ml for retinol analysis) were mixed with 1 ml of 30% $NaCl_{(aq)}$ and 1 ml of 70% EtOH and extracted 3× with 3-ml portions of hexane. The hexane extracts were combined and evaporated under vacuum. The residue was redissolved in 200 μl MeOH–methyl tert.-butyl ether (1:1, v/v)	LC–MS. Column: YMC C_{30} column (250×4.6 mm, 3 μm) with a C_{30} guard. Mobile phase: (A) MeOH containing 1 m*M* ammonium acetate. (B) methyl tert.-butyl ether (15 to 30% B in 12 min). Flow rate: 1 $ml\ min^{-1}$	APCI (positive ion mode) MS detection with SIM. For β-carotene analysis: nebulizer (N_2) pressure: 3.1 bar, vaporizer temperature: 325°C, drying gas: 200°C at 1 $l\ min^{-1}$, capillary voltage: 2.8 kV, corona current: 4.0 μA, cone voltage: 70 V. For retinol analysis: nebulizer: 2.4 bar, vaporizer: 150°C, drying gas: 275°C at 5 $l\ min^{-1}$, capillary voltage: 2.2 kV, corona current: 2.0 μA, cone voltage: 50 V. Dynamic range: 0.4969–99.36 pmol μl^{-1}. Lower LOD: 560 fmol on column. C.V.: 4.82% for β-carotene ($n=6$)
α, β, γ, δ-T+ α-tocopherol quinone (TQ) [92]	Rat tissue extract in Tris buffer or rat plasma (0.2 ml) mixed with ethanol (2 ml cont. 0.025 w/v BHT)+0.2 ml 15% w/v ascorbic acid+0.2 ml 25% w/v pyrogallol+100 pmol of δ-tocopherol (I.S.). Heat 30 min 60°C with 1 ml% w/v KOH then add 2 ml 0.9% NaCl	HPLC. Column C_{18} neopack ODS2 (150×4.6 mm, particles 5 μm). Mobile phase 95% methanol containing 50 m*M* sodium perchlorate. Mobile phase was continuously bubbled with helium.	Dual electrode ECD assembled ''in house'' with glassy carbon electrodes and Ag/AgCl reference electrodes. Upstream electrode −500 mV and downstream +600 mV.

Table 2. Continued

Compound/reference	Matrix/sample preparation	Technique and chromatographic conditions	Detection/validation/comments
	and extract with 2 ml hexane–EtOAc (9:1) containing 0.025% w/v BHT. Centrifuge 10 min at 3000 rev./min. Remove and evap. organic layer and reconstitute in 95% methanol containing 50 m*M* sodium perchlorate		Complete reduction of TQ to hydroquinone accomplished. Method validated. LOD α-T 0.33 pmol, β-T 0.12 pmol, γ-T 0.26 pmol, δ-T 0.31 pmol. α-TQ 0.31 pmol. Oxidative stress increases TQ
α-T, TQ and epoxyquinones (TQE1 and TQE2) [59]	Silanised glassware used. Rat microsomes equivalent to 1 mg of protein mixed with 1 ml aqueous buffer then 1 ml 1 N sodium dodecyl sulphate (to denature proteins) and 1 ml of ethanol containing 50 nmol of butylated hydroxytoluene. Hexadeuterated isotopomers of the analytes (0.5 pmol). Sample extracted with hexane (2×1 ml) and centrifuged. The hexane is evaporated and acid hydrolysed to convert to acid labile oxidation products to TQ and TQE1/2. Hydrolysis products extracted in hexane and trimethylsilylated	Samples introduced into GC–MS by on column injection at 150° and separated on a 30 m×0.25 mm DB-5 ms column. Programmed: 150° (1 min) then 25° min^{-1} to 260°, then 5° min^{-1} to 280° (20 min). The on column injection method provides a method of greater sensitivity and precision than splitless injection	VG MD-800 mass spectrometer operated at 70 eV in SIM mode monitoring for the major significant ions, the spectra of the analytes and their deuterated internal standards. Calibration curves α-T, TQ TQE1 and TQE2 are linear from 25 fmol to 2000 pmol
α-T and deuterated isotopomers [58]	Sheep plasma (200 μl) mixed with 1.8 μg ml^{-1} pentamethyl 1-6-chromanol (I.S.) then ethanol (200 μl) containing 200 ng ml^{-1} BHT. Sample extracted with hexane (3×1 ml). Hexane evaporated and residue treated with BSTFA+ 10% TMCS (200 μl). Reagent evaporated and sample dissolved in 1 ml of hexane	GC Varian 3400 fitted with DB-5 column (8 m ×0.25 mm×0.25 μm). Programmable injector: 600 (0.2 min) then 250° min^{-1} to 290°, injector cooled between runs with CO_2. GC oven 60° (2 min) then 50° min^{-1} to 290° (1.9 min). Helium carrier gas 5 p.s.i. constant pressure. Retention time 4.6 min for I.S. and 7.2 min for α-T	Varian Saturn ion trap, MS–MS method optimized using reservoir of α-T. Parent ions for TMS derivatives of α-T and I.S. isolated in trap and then fragmented producing fragment ions which were monitored in quantification. LOD for α-T 89.6 fg o.c. Method could also be used for metabolic studies using D3 and D6 isotopomers of α-T as tracers
All-*trans*-retinol (R), 13-*cis*-retinoic acid (13 RA), all-*trans*-retinoic acid (RA) and 9-*cis*-retinoic acid (9 RA) [50]	Mouse embryos at 9.5 days were homogenised in ammonium acetate (0.25 *M*, pH 4.0). An aliquot of homogenate (200 μl) was transferred, internal standard (10 μl, 13-*cis*-acitretin, 0.254 ng ml^{-1}) and the acetonitrile (300 μl) was added. Vortex mix, centrifuge and 255 μl of supernatant were transferred and 85 μl water added	HPLC. Sample loaded onto extraction column (10×2.1 mm dry packed with Bondapak dimethyl ODS 37–53 μm), sample loaded in MP2+water (1:3 v/v) using Waters pump (Pump 1). The column back flushed via a switching valve (Pump 1) to a Suplex pKb 100 column (250×4.6 mm) and eluted (Pump 2) with MP2=acetonitrile–methanol–2% ammonium acetate–glacial acetic acid (79:2:16:3 v/v). For low limits of detection it was important that MP1 was prepared from the batch of MP2 used for elution	Coulometric detection using ESA detector. Guard cell set at +750 mV placed between pump 2 and column, also pulse dampener. Analytical cell with two cells: screening cell at +450 mV and analytical cell at +750 mV. Limits of detection o.c: R 12 pg, RA 7.7 pg, 9 RA 10 pg, 13 RA 12 pg. Only RA and R detected in embryos, mean values 75.8 and 279 pg/embryo, respectively.
α-T [93]	Adipose tissue (50 mg) homogenized with ethanol–water (1:1, 10 μl per mg of tissue), 50 μl of vitamin K (0.6 mg ml^{-1} in ethanol) was added to 200 μl of tissue homogenate. Samples extracted with hexane (2×1 ml) using probe sonication, then centrifuged. Supernatant evaporated and residue dissolved in $CHCl_3$–methanol (1:1)	HPLC. Column: C_{18} Nucleosil 120 (150×4.6 mm, 5 μm particle size) at 40°C. Mobile phase methanol–water (96.5:3.5) at 2 ml min^{-1}	Waters 474 fluorimeter. λ_{ex} 295 nm, λ_{em} 350 nm for vitamin E, λ_{ex} 330 nm, λ_{em} 400 nm for vitamin K. Assay linear in the range 0.4 μg–3.2 μg/sample

Table 2. Continued

Compound/reference	Matrix/sample preparation	Technique and chromatographic conditions	Detection/validation/comments
α-T and retinol [94]	Serum from neonates (0.1 ml)+ethanol (0.1 ml containing 106 μmol l^{-1} of tocopherol acetate), add hexane (0.1 ml), vortex and centrifuge. Evap. upper layer (75 μl) and dissolve residue in 100 μl of mobile phase	HPLC. Column: Nova Pak C_{18} (3.9×150 mm). Mobile phase methanol–water (95:5) at 1.5 ml min^{-1}	Waters 420-AC fluorimeter for retinoids λ_{ex} 325 nm, λ_{em} 465 nm. UV detection of α-T 280 nm. Linear ranges: retinol 0.17–4.2 μmol l^{-1}, αT 11.6–232 μmol l^{-1}
α-, β-, γ- and δ-tocopherols [52]	Human serum (100 μl) mixed with methanol (200 μl) and passed through Extrelute eluted with 5 ml of hexane, evaporated, redissolved in hexane, passed through silica gel, eluted with hexane–diethylether. Solvent evaporated, residue treated with MSTFA for GC–MS analysis or dissolved in HPLC mobile phase	GC–MS of TMS derivatives on Thermoquest GCQ. RTx-5 column (30 m×0.25 mm×0.25 μm film). Prog. 220°C (1 min) 5°C min^{-1} to 290°C (10 min). Splitless injection, helium carrier at 30 cm s^{-1} constant flow. HPLC: HP1090, Lichrosorb Si60 (250×4.6 mm×7 μm particles), isooctane–isopropanol (99.5:0.5 v/v) at 1.25 ml min^{-1}. Complete separation of all tocopherols	GC–MS: EI, source 170°C, scan 0.5 s. SIM of molecular ions and several fragment ions. HPLC. Fluorescence: λ_{ex} 295 nm, λ_{em} 330 nm. GC–MS: validated, precision 5–10%. LOD 40 pg o.c. HPLC: validated, precision 5–10%. LOD 50 pg o.c.
α-tocopherol and oxidative metabolites [53]	Human urine (0.5 ml) acidified to pH 2.5 and loaded onto C4 SPE column. Washed with water pH 2.5, eluted with methanol 2 ml. Methanol evaporated, sample dissolved in NaAc buffer pH 4.7 and incubated with glucuronidase–sulphatase (18 h) then extracted with second C4 cartridge. Eluent evap. and treated with BSTFA	GC–MS analysis of TMS derivatives HP5970. DB-1 column (30 m×0.25 mm×0.25 μm film) 120°C (2 min) 20°C min^{-1} to 200°C then 2°C min^{-1} to 240°C then 50°C to 300°C (5 min) several metabolites identified in full scan mode	HP 5970 MSD in EI mode at 70 eV. Trolox included as an internal standard to validate recovery of metabolites
Retinol and retinyl esters [51]	1.5 ml of retinyl propionate (I.S., 100 ng ml^{-1} in ethanol) were added to 0.2 ml of plasma and transferred to the freezer for 30 min then centrifuged at 1000 *g*. An aliquot (1.2 ml) was loaded onto a C_{18} extraction cartridge while mixing with aqueous ammonium acetate–ethanol–acetic acid (90:4:2), the extraction column was washed and backflushed to the analytical column via a switch valve	HPLC. Column: LichroCART cartridges in series (125×4 mm and 250×4 mm) packed with Superspher RP-18. Eluted with CH_3CN–methanol–ethanol–propanol (1:1:1:1) containing 100 μl l^{-1} of *N*-ethyldiisopropylamine (A) mixed with 1% aqueous ammonium acetate (80:20) gradient to 100% A	UV detection at 325 nm. LOQ 2.5 ng ml^{-1} in 0.2 ml of plasma for retinol and retinol palmitate. The automated process allowed analysis of >2000 samples
9-*cis*-retinoic acid [95]	Plasma (1 ml) mixed with 50 μl of I.S. (0.5 mg ml^{-1} in 1:1 methanol–acetonitrile) then 1 ml of 1 *M* phosphate buffer pH 6.0. Sample extracted with 6 ml of methyl t-butyl ether, tube shaken 10 min then frozen in dry ice acetone. Residue dissolved in 200 μl methanol then 100 μl of 5 m*M* ammonium acetate added	HPLC. Column: Microsorb Short One C_{18} (100×4.6 mm, 3 μm), column temp. 36°. Mobile phases A 5 m*M* ammonium acetate–HOAc pH 2.7, B 1% HOAc in methanol. A:B (30:70, 6 min) to A:B (20:80) at 7 min then held until 21.5 min, A:B (30:70) at 22 min until 32 min. Flow rate 1 ml min^{-1}, mobile phase sparged with helium	UV detection at 348 nm. Method fully validated, LOQ 2–5 ng ml^{-1}
R, RA, 13 RA [57]	System tested using standards at 180 μg ml^{-1} but is potentially sensitive method for bioanalysis	HPLC. Large volume injections of between 20 and 100 μl loaded onto column using system with two injection valves, the 200 μl loop in the first being filled with non-eluting mobile phase (acetonitrile–0.5% w/v ammonium acetate–water, 45:5:50). Column fused-silica 320 μm I.D.×25 cm slurry packed with Suplex pKb-100 (5 μm) in CO_2 and eluted with acetonitrile–0.5% w/v ammonium acetate–acetic acid 94.9:5:0.075 at a flow-rate of 5 μl min^{-1}. Column temperature was maintained at 50°C using a GC oven	UV detection using adapted Thermoseparations UV 2000 filled with fused-silica detection cell (20 cm×75 μm) directly mounted to the outlet of the column. When operating at temperature >50°C a fused silica restrictor was connected to the end of the column. Limit of detection for the last eluting peak RA 500 pg o.c.

analysis is usually performed under reduced light, and if storage is necessary, samples must be frozen and kept protected from light and oxygen. To protect samples from oxygen, sample vials can be flushed with argon or nitrogen and the vials should be filled in such a way as to minimize the amount of air in the container [43]. Usually an antioxidant is added to the sample to prevent oxidative degradation of carotenoids. The most common antioxidant used in the reviewed literature is butylated hydroxytoluene (BHT), although ascorbic acid (0.25%) was used in combination with BHT by Gamboa-Pinto et al. [44]. BHT is usually used at concentrations ranging from 0.01 to 0.1%. Talwar et al. [45] used ascorbic acid as an antioxidant agent in the analysis of α-tocopherol and carotenoids in plasma. The authors found that the addition of 0.01% of ascorbic acid was essential to prevent degradation of carotenoids during sample preparation, especially of lycopene and β-carotene.

When carotenoids are being measured in plasma, the first step in their extraction is the precipitation of plasma proteins. This is usually done by addition of ethanol or another apolar organic solvent such as acetonitrile and tetrahydrofuran. Carotenoids are usually extracted by liquid–liquid extraction, and the most frequently used solvent is hexane (usually containing 0.01% BHT or another antioxidant). Some authors use hexane for directly extracting carotenoids from plasma, without previous precipitation of proteins [46]. The extraction with hexane can be repeated or even combined with an extraction step utilizing another solvent or solvent system. For example, Johnson et al. [47] used a mixture of chloroform and methanol (2:1, v/v) followed by hexane extraction and then pooled the organic layers to extract zeaxanthin and lutein from plasma. The samples are then dried and reconstituted in an appropriate solvent prior to analysis. As mentioned before, the drying step should be carried out under vacuum or under a stream of nitrogen or another inert gas to prevent degradation of the carotenoids. When internal standards are used, they are usually added before extraction with hexane. In the literature reviewed the most common internal standards used were the ester ethyl-β-apo-8′-carotenoate [48,49] and squalene [46]. These compounds are commercially available.

Analysis of retinoids is carried out using similar techniques to those used for the analysis of carotenoids. Care is taken to avoid oxidation and protein precipitation is carried out using organic solvent. Automated extraction has been used for the analysis of retinoids following their extraction from tissues using an on-line extraction column and column switching [50,51].

Vitamin E may be analysed in conjunction with carotenoids and retinoids but in some cases it is the principal analyte of interest. The most common procedure for extracting vitamin E and related compounds from a volume of plasma is through addition of a similar volume of ethanol containing an anti-oxidant such as BHT or pyrogallol; tissues are extracted with ethanol containing an antioxidant and this is followed by basic hydrolysis to remove interference by other lipids. The sample is then extracted with a similar volume of hexane. Alternatively plasma extracts may be mixed with an organic solvent such as acetonitrile, protein removed by centrifugation and the supernatant injected directly into a HPLC. Solid phase extraction has been used to extract vitamin E from plasma and urine [52,53].

3.2. Chromatographic conditions

HPLC is the most frequently used technique for the analysis of carotenoids, retinoids and vitamin E (Table 2). In the literature reviewed, C_{18} still is the preferred stationary phase. However, with the recent introduction of triacontyl (C_{30}) polymeric stationary phases [54] and their commercial availability the use of these columns will certainly increase in the future. Usually referred to as ''carotenoid columns'', C_{30} columns show improved separation efficiency for isomeric carotenoids. The use of C_{30} stationary phases for the analysis of food was recently reviewed [55]. The separation of carotenoid isomers by capillary electrochromatography with C_{30}-packed capillaries was elegantly demonstrated by Sander et al. [56]. Using a 98 μm I.D.×25 cm C_{30}-packed capillary and a C_{30} 25 cm×4.6 mm HPLC column both operating at the same mobile phase linear velocity and composition, the authors calculated that for a separation of α-carotene isomers ~46,000 theoretical plates were generated using the packed capillary while the HPLC column generated only 9100 plates. The difference in efficiency was even

greater for the separation of *n*-carotene isomers with the capillary generating an impressive 280,000 theoretical plates.

Carotenoids are virtually insoluble in water and thus mobile phase composition in carotenoid analysis is usually non-aqueous, or water is incorporated as a modifier rather than as a solvent. Thus Hagiwara et al. [46] used isocratic elution with a mixture of methanol and acetonitrile (70:30, v/v) for the analysis of α-, and β-carotene and lycopene with a C_{18} reversed-phase column. More frequently analysis is carried out using gradient elution. Another solvent frequently used as mobile phase for carotenoid analysis is 17-methyl tert-butyl ether [44,47], which is usually used together with methanol or acetonitrile. Ammonium acetate and triethylamine are usually added to provide some buffering since carotenoids are sensitive to acidic conditions [42,44]. Capillary liquid chromatography has been used for the analysis of retinoids [57].

Chromatographic analysis of vitamin E and related compounds is carried out using both gas chromatography and liquid chromatography. In the case of gas chromatography, vitamin E and related compounds are converted to trimethylsilyl ethers prior to analysis [52,53,58,59] and detection is carried out using mass spectrometry in the selected ion mode. Deuterated internal standards of the analytes for use as internal standards in GC–MS analysis are relatively easily prepared [59]. In most cases HPLC utilises C_{18} columns with pure methanol or methanol containing a small amount of aqueous phase or another organic solvent as the mobile phase. In the review period separations have also been carried out on silica gel [52] and on a diol column [59]. Enantiomers of α-tocophorol and α-tocopherol quinone have been separated on chiral stationary phases [60].

3.3. Detection

Carotenoids are usually monitored by UV–Vis detection at 450 nm. The use of photodiode array detectors (PDA) for carotenoid profiling and quantifi-

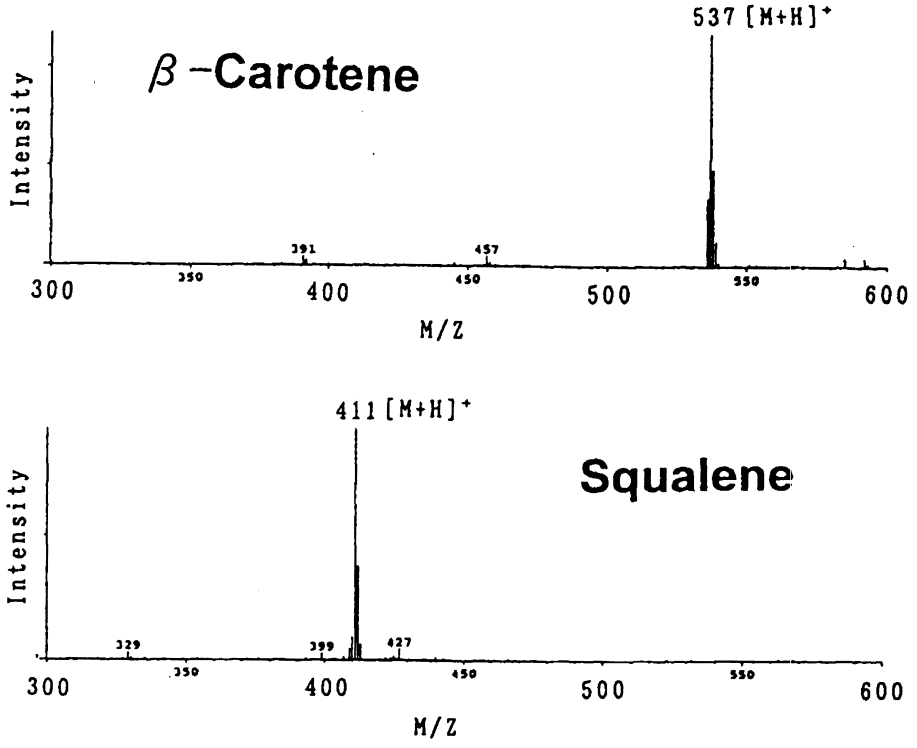


Fig. 5. APCI mass spectra of β-carotene (top) and squalene (bottom). Reproduced from Ref. [46] with kind permission of Elsevier Science.

cation from several natural sources including bacteria, pollen and algae was reported by Leenheer and Nelis [61]. Since carotenoids have several naturally occurring isomers, the use of a photodiode array detector is advantageous since the online acquisition of the UV spectra can facilitate peak assignment, and confirm peak purity. However, geometric isomers have similar absorption spectra and in the absence of authentic standards the assignment of peaks may be difficult. Moreover, stereoisomers cannot be distinguished based on their absorption spectra. To address this problem, Dachtler et al. [62] used HPLC–NMR for the separation and identification of zeaxanthin stereoisomers. Using HPLC–APCI MS and HPLC–NMR the authors describe a method for the determination of zeaxanthin stereoisomers directly from the sample (spinach or retina) with a mild extraction technique being used to minimise artefact formation and degradation of the carotenoids.

The use of LC–MS for the analysis of carotenoids is increasing. This technique offers the advantage of being very sensitive and selective. Also, LC–MS potentially simplifies sample preparation, although care must always be taken to prevent ion-suppression effects from the biological matrix components. Carotenoid analysis in biological samples by LC–MS is usually performed using atmospheric pressure ionisation techniques. Van Breemen et al. [63] investigated the use of ESI or APCI for the analysis of all-*trans*-retinol and all-*trans*-retinyl palmitate in plasma. The authors used hexane extraction for sample preparation and APCI for quantification in SIM mode. Linearity was verified with APCI but not with electrospray ionisation. The limit of detection of the APCI LC–MS method was 34 fmol μl^{-1} and 36 fmol μl^{-1} for all-*trans*-retinol and all-*trans*-retinyl palmitate, respectively. APCI was also used by Lacker et al. [64] in conjunction with C_{30} reversed-phase columns for the determination of several carotenoids, including *cis–trans* isomers of β-carotene. Peak assignment was facilitated by the use of a PDA detector. The limit of detection for β-carotene was 1 pmol. A representative positive mode APCI mass spectrum of β-carotene and squalene are shown in Fig. 5, where it can be seen how little fragmentation this ionisation mode produces. In another study, Careri et al. [65] used two C_{18} reversed-phase columns in series to determine β-carotene, β-cryptoxanthin, lutein, zeaxanthin, canthaxanthin and astaxanthin by LC–MS using ionspray and turbo-ionspray interfaces. Higher sensitivity was

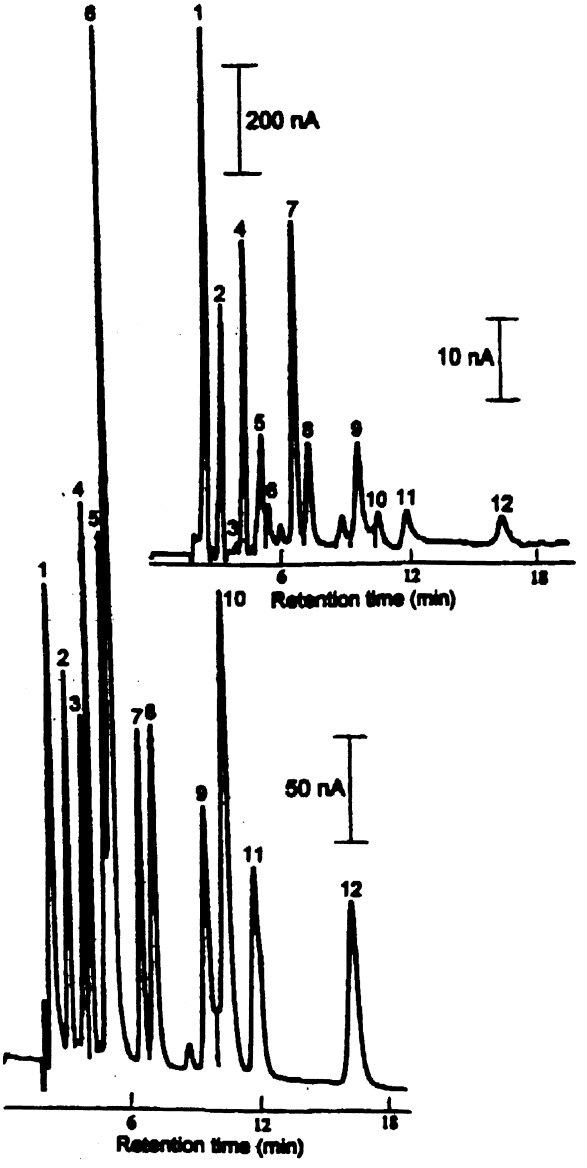


Fig. 6. HPLC chromatogram from a standard mixture of carotenoids (bottom) and from a neonatal plasma sample (top). Peak assignment: (1) 2,6-di-tert-butyl-p-cresol (2), γ-tocotrienol (3), γ-tocopherol (4), α-tocopherol (5), ethyl-β-apo-8′-carotenoate (6), β-cryptoxanthin (7), ubiquinone-7 (8), ubiquinol-9 (9), ubiquinol-10 (10), β-carotene (11), ubiquinone-9 and (12) ubiquinone-10. Electrochemical detection used. For other experimental conditions see Table 2. Adapted from Finckh et al. [49].

achieved using positive ion mode and a high drying gas temperature (500°C). The estimated limits of detection were ca. 0.1–1 ng on column.

Another option to detect carotenoids in low concentrations is the use of electrochemical detection. This detection mode can be used to achieve low limits of detection with good selectivity. Examples include the detection of ubiquinol 10, ubiquinone 1-, carotenoids and tocopherol in very small (5 or 10 μl) plasma samples [49] with LOD of 21–60 fmol/20 μl sample (at $S/N = 5$). A chromatogram of a plasma sample using this method is shown in Fig. 6. In another application, Ferruzzi et al. [66] used a coulometric array detector for the determination of *trans*-β-carotene, lutein, zeaxanthin, β-cryptoxanthin, and α- and β-carotenes. The authors used a sample preparation method requiring only 20 μl of plasma. The LOD for *trans*-β-carotene was 6.25 pg. The sensitivity for detecting retinoic acid and related compounds has recently been greatly enhanced by the development of a highly sensitive method based upon electrochemical detection [50] (Fig. 7). Coulometric detection provides the lowest limits of detection for retinoids but requires setting a relatively high oxidation potential which means that readily oxidisable compounds have to be carefully scrubbed from the mobile phase prior to entry to the detector.

4. Ascorbic acid, dehydroascorbic acid and related compounds

Ascorbic acid (AA) is the most important water soluble antioxidant and has been proposed that it is important in the prevention of diseases as diverse as cancer, atherosclerosis and Parkinson's disease. Methods for the determination of AA are relatively routine, however, they still present problems if care is not taken to minimise oxidation during sample processing and analysis. Determination of the oxidation product of AA, dehydroascorbic acid (DHAA) is also important in gaining some insight into oxidative stress within an organism and there are a number of methods for its measurement. The methods available for determining DHAA in biological matrices have been recently reviewed [67]. Future advances in the measurement of AA and DHAA will be aimed at measuring small amounts of these materials in specific tissues in real time (Table 3).

4.1. Sample preparation

AA is highly water soluble and is thus not extracted into an organic solvent. Sample processing involves removing protein present in the sample from solution followed in most cases by analysis

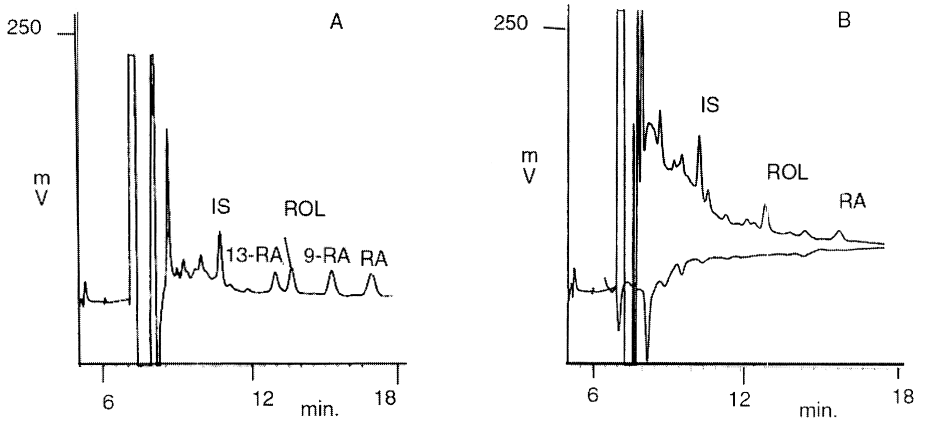


Fig. 7. (A) Separation of retinoid standards 13-*cis*-retinoic acid (13-RA) 330 pg o.c., (retinol (ROL) 438 pg o.c., 9-*cis*-retinoic acid (9-RA) 330 pg o.c. and all-*trans*-RA (RA) 329 pg o.c. on a pKb column using acetonitrile–methanol–2% ammonium acetate–acetic acid (79:2:16:3). (B) Trace from albumin blank in comparison with an extract from 1.64 mouse embryos, ROL was 279 pg/embryo and RA was 75.8 pg/embryo. Reproduced from Ref. [50] with kind permission of Elsevier Science.

Table 3
Separation methods for the determination of ascorbic acid and related compounds in biological fluids

Compound/reference	Matrix/sample preparation	Technique and chromatographic conditions	Detection/validation/comments
L-ascorbic acid, dehydroascorbic acid, ascorbic-2-mono and polyphosphate, ascorbate monosulphate [96]	Fish tissues homogenized in ice-cold 0.2 M NaAc pH 4.8. Extracts divided in two and one half treated with ascorbate oxidase the other stabilised with 0.2% w/v dithioerythritol. Enzyme-treated sample deproteinated with 5% $HClO_4$ and centrifuged at 12,000 *g*	HPLC. Column: Vydac C_{18} (4.6×250 mm, 5 μm). Mobile phase: 0.04 *M* NaAc, 0.5 m*M* tetrabutylammonium phosphate adjusted to pH 3.76 with 85% H_3PO_4, 24 ml of methanol added and solution adjusted to 1000 ml with water, flow-rate 0.6 ml min^{-1} at 23°C	Detection DAD set to monitor 250 nm. Method validated. LOD for ascorbic acid 0.2 μg ml^{-1}
AA [97]	Fresh plasma (100 μl) mixed with 10% v/v perchloric acid (100 μl) containing 1% w/v metaphosphoric acid (mPA) in a brown centrifuge tube. Urine (100 μl) mixed with water (900 μl) and then 500 μl mixed with 500 μl of 10% v/v perchloric acid containing 1% w/v mPA in a brown centrifuge tube. Samples centrifuged 12,000 *g*, 5 min, 4°C and filtered through a 0.2 μm filter. Samples stored in liquid nitrogen until analysis	HPLC: HP 1050. Column: Prodigy ODS3 (300×4.6 mm, 5 μm particles), 30 μl injection. Mobile phase 20 m*M* ammonium dihydrogen phosphate with 0.015% mPA adjusted to pH 3.5 at 0.75 ml min^{-1}	DAD detection at 245 nm with a reference wavelength at 550 nm. LOD for plasma and urine 0.09 μg ml^{-1}. Linearity 1–100 μg ml^{-1}. Samples were stable for 60 min on ice in darkness for at least 60 min. Concentrations in volunteers 7–17.6 μg ml^{-1} (urine) and 0.1–8.1 μg ml^{-1} (plasma). The column was carefully selected
Ascorbic acid dehydroascorbic acid (DHAA), uric acid isoascorbic acid [96]	Method development studying standard solutions of the analytes. The DHA is treated with dithiothreitol to reduce it to ascorbic acid. Standards stabilized with EDTA. Dihydroxybenzylamine used as internal standard	HPLC: Shimadzu LC-9A. Column Hamilton PRP-1 polymeric reverse phase (250×4.1 mm). Mobile phase phosphate buffer 0.05 *M* pH 2.5 containing 5 m*M* cetyltrimethyl ammonium bromide	Eluent monitored by UV and ECD using a Gibson EC detector set at +0.65 V
AA and DHAA [71]	Plasma (0.5 ml) mixed with 0.5 ml of 10% MPA and sample centrifuged (14,000 *g*, 10 min) one volume of supernatant added to two volumes of 0.4 *M* acetate buffer pH 3.9. Sample (0.5 ml) transferred to Vectaspin micro and centrifuged	HPLC. Column: Jupiter C_{18} (250×4.6 mm 5 μm particles with 300 Å pore size), mobile phase 2.3 m*M* dodecyltrimethyl ammonium chloride and 2.5 m*M* EDTA in 66 m*M* phosphate/20 m*M* acetate buffer adjusted to pH 4.5 at 1.2 ml min^{-1}. Post column derivatisation with 28 m*M* *o*-phenyldiamine in 12 m*M* sodium citrate and 55 m*M* EDTA at pH 3.7	Detection for AA ESA Coulochem ECD with guard cell at +200 mV and a dual analytical cell operating at −200 mV and +150 mV and DHAA detected with Jasco FP fluorescence detector, λ_{ex} 350 nm, λ_{em} 430 nm. AA and DHAA analysed in plasma in the ranges 0.1–10 μg and 0.05–5 μg, respectively
AA, DHAA [98]	Plasma, serum or urine samples (200 μl) mixed with 90% methanol–water–1 m*M* EDTA (800 μl). Samples cooled in ice to precipitate protein and centrifuged. For DHAA analysis the samples are treated with 10 m*M* 2,3-dimercapto-1-propanol for 10 min, reagent is removed by extraction into ether prior to analysis	HPLC. Column: e.g. Luna C_{18} (250×4.6 mm, 5 μm). Mobile phase methanol (25% for plasma and 30% for urine samples) with 0.05 *M* Na phosphate, 0.05 *M* Na acetate, 189 μ*M* dodecyltrimethylammonium bromide, 36.6 μ*M* tetraoctylammonium bromide adjusted to pH 4.8 at 1 ml min^{-1}	Detection with 5200 Series ESA coulometric detector set at +0.25 V. LOD 0.5 pmol with routine detection of concentrations as low as 5 nM. System, apart from columns, passivated by flushing with 6 N nitric acid
AA [68,69]	Ascorbic acid solution (0.25 μm) was stabilized with mobile phase containing 20 m*M* monosodium glutamate (MSG) adjusted to pH 2.1 with phosphoric acid. Solution remained stable in the presence of Cu^{2+} and Fe^{3+}. AA 400 ng ml^{-1} was injected prior to analysis to obtain optimized peak height. A similar study was also carried out using disodium 5′-guanosine monophosphate as a stabiliser	HPLC. Column Inertsil OD-3 (150×4.6 mm, 5 μm). Mobile phase 20 mM MSG adjusted to pH 2.1 with phosphoric acid at 0.8 ml min^{-1}. 5 μl of sample injected	Hitachi ED 623 ECD set at 400 mV. Method validated, linear response over the range 0.1–50 ng o.c.

Table 3. Continued

Compound/reference	Matrix/sample preparation	Technique and chromatographic conditions	Detection/validation/comments
AA, DHAA [99]	Tissue samples homogenised in 0.3% metaphosphoric acid containing EDTA (0.1 m*M*) and thiourea (1 m*M*), sample tube filled with inert gas then centrifuged and supernatant analysed. For DHAA portion of the supernatant is treated with mercaptoethanol	Column: C_{18} Waters cartridge in radial compression module. Mobile phase 0.2 *M* phosphate buffer pH 3.0 at 1 ml min^{-1}	Detection with 5100 A ESA coulometric detector
AA, DHAA [70]	Plasma mixed with 10% MPA+2 m*M* EDTA and centrifuged, tissues extracted with 5% MPA+1 m*M* EDTA. Samples frozen immediately with dry ice and stored at −70°. Samples thawed and 100 μl mixed with 400 μl of 1 m*M* EDTA for AA measurement, 10 μl injected into HPLC. DHAA determined by mixing 100 μl of sample with 50 μl of trizma buffer containing tris(2-carboxyethyl) phosphine.HCl (TCEP 2 m*M*), after 5 min pH adjusted to pH 4.7 with 350 μl of McIlvain buffer and sample (10 μl) analysed without further processing	HP1100 pump. Column Luna C_{18} (150×4.6 mm, 3 μm). Mobile phase phosphate–acetate buffer at pH 5.4 containing 1 m*M* EDTA, 50 mg dodecyltrimethyl ammonium chloride/20 mg tetraoctyl ammonium bromide l^{-1} at a flow-rate of 0.6 ml min^{-1} at 30°	Detection with ESA Coulochem II detector with cells operated at −200 mV and +300 mV. Calibration curve for AA linear in the range 5 to 100 μ*M*. TCEP gives complete reduction in 5 min even at low pH and it also acts as a very effective stabilising reagent after reduction has occurred
AA, lactic acid and pyruvate [72,73]	Microdialysis probe inserted into the brain of anaesthetised gerbil and then blood flow cut off for 10 min. Reperfusion allowed to occur for 2 h. Dialysis probes perfused with Ringers solution at 2 μl min^{-1}.	Dialysates collected every 3 min in a 5-μl loop and directly injected into HPLC. Polypore column (100×4.6 mm, 10 μm) mobile phase 4 m*M* sulphonic acid sparged with helium, flow-rate 0.5 ml min^{-1}	UV detection with BAS UV-116A. AA, lactic acid and pyruvic acid determined. AA decreased at beginning of reperfusion and increased to 260% of basal bevel at 2 h
AA [74]	Plasma (10 μl) from paediatric subjects mixed with 2.5 ng $^{13}C_6$-ascorbic acid and 1 ng of $^{13}C_4$-threonic acid and 1 μl of 25% v/v trichloroacetic acid. Samples dried by vacuum centrifugation and reacted with *N*-t-butyldimethylsilyltrifluoroacetamide (25 μl) in acetonitrile (50 μl) for 30 min at 60°C. 2 μl of sample injected into GC–MS	GC–MS: HP5971A. Column: Supelco SPB1 (15 m×0.25 mm) 80–300°C at 30°C min^{-1}	Analytes detected in SIM mode. Method validated. Calibration was carried out by standard addition. Levels of AA in patient plasma were 11.9±5 μg ml^{-1}

using HPLC. Protein precipitation reagents include: perchloric acid, metaphosphoric acid and methanol–water. It is also important to stabilize the ascorbic acid against oxidation and reagents which have been used for this during the review period include: dithioerythritol, monosodium glutamate [68], 5′-guanosine monophosphate [69] and EDTA. If measurement of DHAA is required the most frequently used approach is to carry out reduction. Reducing agents used include: dithioerythritol, 2,3-mercaptopropan-1-ol and mercaptoethanol. A recently introduced, very effective, reducing agent is tris-(2-carboxyethyl)phosphine hydrochloride [70]. DHAA has also been measured directly using post-column derivatisation [71] and AA has been measured directly without sample processing utilizing a microdialysis probe [72,73], Fig. 8.

4.2. Chromatographic methods

Most methods have involved separation by HPLC on C_{18} columns either with or without the use of an ion pairing reagent. Polymeric columns are also used [72,73]. Ion pairing reagents include: tetrabutylammonium phosphate, dodecyltrimethylammonium chloride, dodecyltrimethylammonium bromide and tetraoctylammonium bromide and cetyltrimethylammonium bromide. Stabilisers are also incorporated

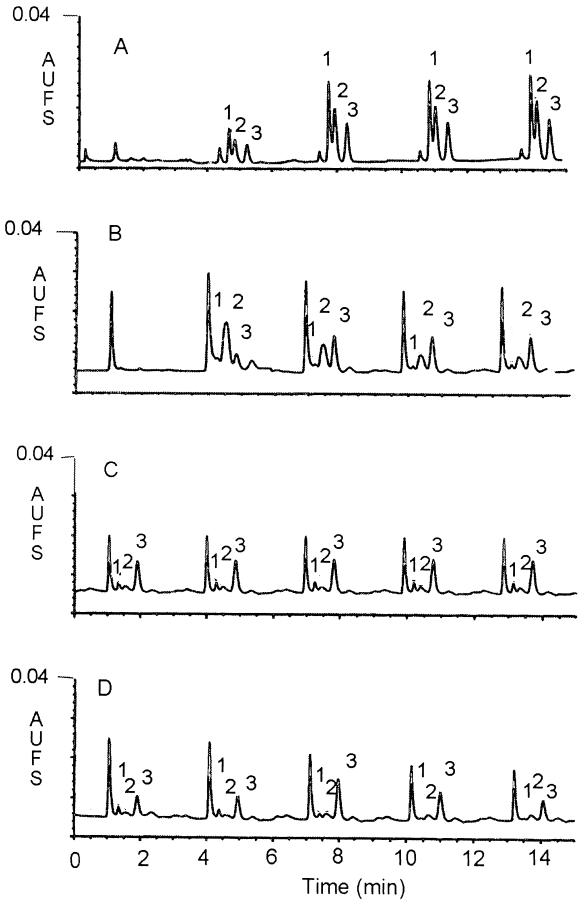


Fig. 8. Chromatograms of dialysate obtained at 3-min sampling intervals. Pyruvate (1), ascorbic acid (2), lactic acid (3). (A) Standard mixture. (B) After implantation of dialysis probe. (C) Basal levels prior to ligation. (D) 10-min ligation followed by reperfusion of gerbil brain. Reproduced from Ref. [72] with kind permission of Elsevier Science.

into the mobile phase and include: metaphosphoric acid, EDTA, monosodium glutamate [68] and 5′-guanosine monophosphate [69].

4.3. Detection

Detection methods used included: DAD or UV, coulometric, electrochemical and fluorescence after post-column derivatisation of DHAA. Apart from HPLC methods a highly sensitive method was developed for the determination of AA in small volumes of plasma (10 μl) from pediatric patients utilizing gas chromatography mass spectrometry of t-butyldimethylsilyl derivatives [74]. Fig. 8 shows the change in ascorbic, lactic and pyruvic acid concentrations following insertion of a microdialysis probe into gerbil brain and following this with cuffing of the blood supply to the brain for 10 min and reperfusion for 2 h. Ascorbic acid reached a maximum of 260% of basal levels 60 min after the start of reperfusion.

5. Nomenclature

AA	ascorbic acid
APCI	atmospheric pressure chemical ionisation
BHT	butylated hydroxytoluene
BSA	*N*,*O*-(bis)trimethylsilyl acetamide
BSTFA	*N*,*O*-bis(trimethylsilyl) trifluoroacetamide
CZE	capillary zone electrophoresis
DHAA	dihydroascorbic acid
EAD	electrode array detection
ECD	electrochemical detector
ESI	electrospray ionisation
GC–MS	gas chromatography mass spectrometry
I.S.	internal standard
LOD	limit of detection
LOQ	limit of quantification
MEKC	micellar electrokinetic capillary chromatography
MSTFA	*N*-methyl-*N*-trimethylsilyltrifluoroacetamide
OC	on-column
ODS	octadecylsilane
PDA	photodiode array
RP	reversed-phase
S/*N*	signal-to-noise ratio
SIM	selected ion monitoring
SPE	solid-phase extraction
TBDMS	tertiarybutyldimethylsilyl
TMS	trimethylsilyl

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