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REVIEW

CHROMATOGRAPHY OF FAT-SOLUBLE VITAMINS IN CLINICAL CHEMISTRY

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LIST OF ABBREVIATIONS

BHT	Butylated hydroxytoluene
$(CH_3)_4NB_3H_8$	Tetramethylammonium octahydridotriborate
CI	Chemical ionization
DBP	Vitamin D-binding protein
ECD	Electron-capture detection
ED	Electrochemical detection
EDTA	Ethylenediaminetetraacetic acid

EI	Electron impact
FFAP	Free fatty acid phase
FHID	Fast heavy ion-induced desorption
FID	Flame ionization detection
GC	Gas chromatography
GC–MS	Gas chromatography–mass spectrometry
HAPS	Hydroxyalkoxypropyl-Sephadex
HFBA	Heptafluorobutyric anhydride
HPLC	High-performance liquid chromatography
I.S.	Internal standard
ITS	Isotachysterol
LC	Liquid chromatography
LC–MS	Liquid chromatography–mass spectrometry
MID	Multiple-ion detection
MS	Mass spectrometry
$NaBH_4$	Sodium borohydride
NARP	Non-aqueous reversed-phase
25-OHD	25-Hydroxyvitamin D
1,25-$(OH)_2$D	1α,25-Dihydroxyvitamin D
RBP	Retinol-binding protein
SDS	Sodium dodecyl sulphate
TBDMS	3-*tert.*-Butyldimethylsilyl
THF	Tetrahydrofuran
TLC	Thin-layer chromatography
TMS	Trimethylsilyl
α-TQ	α-Tocopherylquinone
UV	Ultraviolet
VLDL	Very-low-density lipoprotein

1. INTRODUCTION

The importance of fat-soluble vitamins in fundamental biochemistry, nutrition and medicine is generally recognized. Assays for these compounds in biological materials traditionally relied on colorimetry or fluorimetry, often in conjunction with thin-layer chromatography (TLC) or open-column chromatography. As a result, the methods were generally time-consuming, subject to interferences and suffered from poor sensitivity. The advent of modern chromatography truly revolutionized the analytical methodology for fat-soluble vitamins. Gas chromatography (GC) has, however, found limited application, particularly to the thermolabile isoprenoid vitamins A and K, and has been rapidly superseded by liquid chromatography (LC) on microparticulate supports. This versatile technique permits separations at room temperature, without the need for derivatization, and affords superior resolving power, sensitivity and speed.

This paper is concerned with modern LC and, to a lesser extent, GC methods for the determination of fat-soluble vitamins in serum and tissues. Although essentially it deals with recent (after 1980) literature only, most of the principles

underlying the assays have been outlined in a recent book [1] and still remain valid.

2. VITAMIN A

2.1. Background

Vitamin A [retinol or 9,13-dimethyl-7-(1,1,5-trimethyl-6-cyclohexene-5-yl)-7,9,11,13-nonatetraene-15-ol] (Fig. 1) is essential for cellular differentiation, normal vision, growth and reproduction.

Excellent reviews on the biological activity of vitamin A have been written by Moore [2,3] and Olson [4]. In food, vitamin A is present mainly as the retinyl ester. In the intestine, the dietary esters are hydrolysed and the resulting retinol is absorbed in the mucosa cells. In the intestinal mucosal cells absorbed retinol is re-esterified (mainly with palmitic acid) and associated with lymph chylomicrons. These esters enter the bloodstream [5] and the chylomicrons are then rapidly converted to chylomicron remnants by the action of lipoprotein lipase. The hydrolysed retinyl esters are re-esterified, again mainly with palmitic acid, and taken up in the liver by the hepatocytes [6]. Free retinol, mobilized from these stores after hydrolysis, is then transported in the plasma by a specific transport protein (retinol-binding protein, RBP) and with pre-albumin [7,8].

Vitamin A is metabolized in two major ways, by oxidation of the C-4 position of the cyclohexene to 4-hydroxy-, 4-keto-, and to 5,6-epoxyretinoic acid [9,10] or by oxidation at the C-15 position to retinal and irreversibly to retinoic acid. Retinoic acid itself is further metabolized by conjugation with glucuronic acid or taurine [11,12], in some instances after oxidative cleavage of the side-chain [13]. These polar metabolites are excreted in the urine or bile. A significant portion of retinoyl β-glucuronide is then recycled to the liver by the enterohepatic circulation [14,15]. In addition to the well known biological activity of retinol, a large number of retinoic acid analogues, retinoids, are currently under investigation for their antineoplastic activity and for the treatment of psoriasis and acnea. Excellent reviews on this subject have been written by Bollag [16,17].

The transport, storage and utilization of vitamin A in humans is affected by many gastrointestinal disorders, such as diarrhoea and sprue. Also, liver diseases and chronic intake of alcohol impair the liver storage of vitamin A and finally can result in a decreased vitamin A level in serum. Further, measurement of vitamin A together with the different retinyl ester forms can give clear information for the diagnosis of a fat malabsorption syndrome [4].

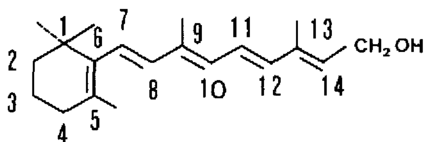


Fig. 1. Structure of retinol.

2.2. Isolation, extraction and clean-up

Again, we shall focus only on the isolation of retinyl esters and retinol from biological matrices of human origin.

To avoid isomerization and oxidation of the vitamin A compounds, all manipulations, such as the collection, extraction and storage of the samples, must be carried out in subdued light and in the absence of drastic reagents. Reference compounds, stock solutions and samples should be stored in the dark at low temperature [18,19] and preferably under a nitrogen atmosphere. With these precautions, together with the use of an appropriate internal standard (I.S.), reliable results can be obtained in a quantitative measurement of retinol and/or retinyl esters. When the level of total retinol is sought regardless of whether it is present in its free or esterified form, a saponification step is included. Saponification consists in digestion of the sample in alcoholic or aqueous potassium hydroxide followed by extraction of the lipids with an organic solvent (e.g., *n*-hexane).

For the detection and identification of various retinoids in human epidermis, Vahlquist [20] performed alkaline hydrolysis at 80°C for 30 min. To prevent degradation (oxidation or isomerization), ascorbic acid, ethylenediaminetetraacetic acid (EDTA) and butylated hydroxytoluene (BHT) are added to the sample. Furr et al. [21] allowed the hydrolysis to proceed overnight at room temperature in a 10% methanolic solution of sodium hydroxide. Under these milder conditions they could omit the addition of antioxidants such as BHT, ascorbic acid or pyrogallol. The last compound was used in a 1% concentration during the digestion procedure (20 min at 60°C) as described by Ross [22]. Even under milder conditions and with the addition of antioxidants, heat and alkali can cause extensive degradation of the retinoids; consequently, direct extraction procedures have gained interest in recent years.

If we are interested in the various ester forms of retinol together with retinol itself, then a direct extraction procedure without any digestion step is necessary.

Denaturation of the transport proteins prevents irreversible sorption of proteins on the stationary phase in a further chromatographic step and liberates retinol from its specific binding protein. Common solvents for the denaturation of serum proteins are methanol [23], ethanol [24–31], and acetonitrile [32,33], and they are used in an amount equal to or double that of the serum. Finally, the extraction is performed with one of the common solvents for fat-soluble compounds (e.g., *n*-hexane, light petroleum or cyclohexane). An alternative for the denaturation of serum proteins is direct addition of an organic extraction solvent mixture, e.g., butanol–acetonitrile [34] or butanol–ethyl acetate [35]. For the extraction of retinol and/or retinyl esters from other biological samples (e.g., human liver, milk or corpus luteum), a homogenization step has to be included in the analysis. The water is removed from the tissue sample either by lyophilization [36] or by grinding with anhydrous sodium sulphate [21,22] prior to extraction with dichloromethane [21], methanol [36] or hexane [22]. For samples with a high lipid content, preliminary purification of the crude extract may be necessary in order to separate retinol and retinyl esters as a class from most other lipids, especially triglycerides. Open-column chromatography on aluminium ox-

ide has been described by Ross [37] for this purpose. For the analysis of vitamin A in softer tissues, such as human cheek epithelial cells, no homogenization is needed and a simple Bligh–Dyer extraction [38] is satisfactory [39].

In the last few years a large number of papers describing the simultaneous determination of vitamins A and E in biological samples of human origin have appeared. This is meaningful in view of the recently suggested biological interaction between the two vitamins. As both compounds are lipophilic molecules, extraction procedures do not differ very much. In almost all papers both vitamin A and E are extracted with *n*-hexane after denaturation of the proteins with ethanol [40–48]. In a few papers denaturation of the proteins is also performed with the extraction solvent [49,50].

Before going to the chromatographic section of this chapter, we should consider the choice of the internal standard. In analyses of both exogenous and endogenous compounds most errors occur during extraction, subsequent evaporation and derivatization or during the chromatographic step. Suitable internal standards should minimize these errors by compensation, provided that they are added at the earliest possible stage. A basic requirement therefore is a close structural similarity with the compound to be assayed. Too often, however, internal standards do not bear any relationship to the compound of interest, and this practice should be strongly discouraged. Therefore, omission of internal standardization, even with a simple sample manipulation [25,30,32,41,44,49] is unacceptable, as there is no permanent control over each individual sample analysed in a series of unknowns.

Owing to the lack of structural similarity to vitamin A, α-naphthol is unacceptable as an internal standard [23,39], whereas retinyl acetate, propionate and heptanoate for vitamin A analysis and retinyl acetate and tocol or tocopheryl acetate are suitable internal standards for vitamin A and E determinations.

2.3. Gas chromatography

The application of GC to retinoid analysis dates back to the early 1960s and we encourage the reader to consult some reviews for specific experimental data for these older procedures [1,51,52].

Owing to the instability of conjugated unsaturated compounds, such as retinol, GC has only limited value in retinoid analysis. Diatomaceous earth supports should be acid-washed, treated with a base and then treated with a silanizing material to block all polar surface groups that could interact with the sample. Typical supports therefore include Gas-Chrom Q and Chromosorb W AW-DMCS (80–120 mesh). The liquid phases include SE-30, SE-52, OV-17 and QF-1, all used at concentrations of 1–3%. Finally, treatment of the column with an antioxidant, such as β-carotene or hydroquinone, is also helpful in reducing the destruction of the more labile compounds. In 1983 gas chromatography–mass spectrometry (GC–MS) was applied to the evaluation of total body reserves of vitamin A in humans [53]. After administration of dideuterated vitamin A, the resulting mixture of normal and dideuterated forms was isolated from plasma and measured by GC–MS by following the molecular ions of m/z 268 and 270. Both anhydroretinol and

dideuterated anhydroretinol were formed spontaneously in good yield on the GC column (3% OV-101 or OV-210 on 100–120 mesh Gas-Chrom Q). During the passage through the GC column, even at 233 °C, a significant amount of isomerization occurred, as indicated by five minor peaks eluting slightly before the all-*trans* peak. Under chemical ionization (CI) conditions with isobutane as reagent gas the $[M+H]^+$ peaks at m/z 269 and 271 for anhydroretinol and dideuterated anhydroretinol, respectively, predominated, and also the isobutane addition peak at m/z 325 and 327 ($[M+57]^+$) appeared. However, considerable fragmentation still occurred and electron impact (EI) proved to be more sensitive than CI under the conditions used. Although GC, especially when coupled with MS, is an extremely useful technique, in the last few years most research groups in the vitamin A field have focused on milder chromatographic separation procedures, such as high-performance liquid chromatography (HPLC).

2.4. Liquid chromatography

HPLC permits rapid and non-destructive quantitative analyses at lower temperatures. Consequently, HPLC has become an extremely valuable method for the separation, identification and quantitation of different vitamin A compounds. In this section we shall evaluate more recent applications of HPLC to biomedical and clinical studies on vitamin A. For readers interested in older applications we refer to other reviews [1,52].

2.4.1. Chromatographic systems

2.4.1.1. Adsorption chromatography

In this technique a polar column packing material, such as silica or to a lesser extent alumina (commercially available as LiChrosorb, LiChrospher, Spherisorb, Nucleosil, Zorbax, etc.) is eluted with a non-polar eluent (e.g., *n*-hexane or light petroleum). The addition of a small percentage of water or alcohol to the hexane moderates the highly active sites and increases the surface homogeneity of the column packing.

Adsorption chromatography is known to produce a better resolution of the different isomers of retinol [54] and retinyl esters [55]. These separations, however, are only necessary for studies of the isomeric forms in the eye. Only a few research groups have used adsorption chromatography for the analysis of retinol. Using an isopropanol-moderated system the occurrence of retinol instead of retinal in the corpus luteum could be established [56]. In vitamin A analysis adsorption chromatography allows the direct injection of the extraction solvent (also mostly consisting of *n*-hexane) and there is no need for an evaporation and resuspension step [23,30,31,41]. A typical chromatogram from such a type of analysis is given in Fig. 2.

Gradient elution, which is unusual in adsorption chromatography, allowed the simultaneous determination of retinol and the major circulating retinyl esters (eluting in a single peak). It is difficult, however, to believe that a re-equilibration time of 3 min is sufficient for the adsorption system described [31].

Only one simultaneous determination of vitamins A and E has been performed

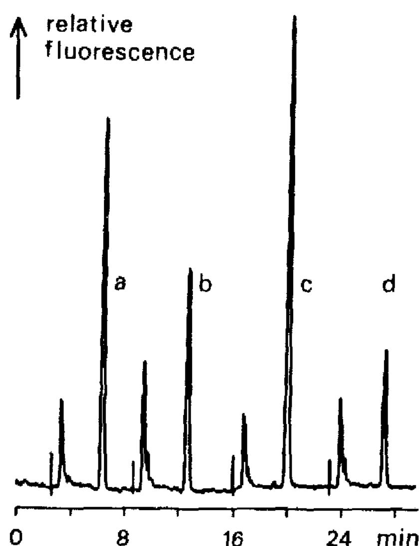


Fig. 2. Typical HPLC elution profiles of a standard solution (a, 50 nmol/l) and of *n*-hexane extracts of plasma samples (b, c and d, 1.19, 2.55 and 0.74 μmol/l vitamin A in plasma, respectively). Polygosil 60-5, 5 μm (8 cm $\times$ 0.46 cm I.D.) column, eluted isocratically with *n*-hexane–dichloromethane–2-propanol (90:10:1, v/v/v) at a flow-rate of 1.2 ml/min. Fluorescence detection at the excitation/emission wavelength pair 333/470 nm. From ref. 30, with permission.

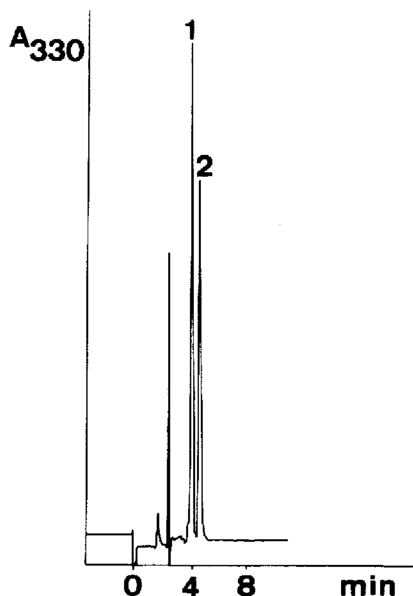
on a silica system, again providing compatibility with the extraction solvent used. However, even this simplification of the procedure cannot allow internal standardization to be omitted, as also admitted by the author [44].

2.4.1.2. Bonded-phase chromatography

2.4.1.2.1. Normal phase. Normal-phase columns, such as diol- or cyano-bonded phases, offer several advantages over silica adsorption phases. The columns are easier to clean and irreversible adsorption of polar compounds is greatly reduced. Only a few papers have described the application of normal-phase chromatography on a diol [25] or cyano [32] phase for the quantitative measurement of vitamin A in human serum. Again the extraction solvent can be injected directly on to the HPLC system. However, in both papers internal standardization was omitted.

The beneficial effect of 2-alkanols (e.g., 2-octanol or 2-nonanol) on the separation of retinol isomers has also been described [36,57]. Eluting a cyano column, combined in series with a reversed-phase column, with two different eluent systems, four isomers of retinol can be nearly baseline separated.

2.4.1.2.2. Reversed phase. It is well known that reversed-phase packing materials are not very sensitive to small changes in the water content of the eluent. Also, columns can be rapidly re-equilibrated after gradient elution without drastic changes in retention time. Reversed-phase HPLC is also advantageous in metabolism studies as more polar compounds (metabolites) will elute before the parent compound. Pure methanol can be used to elute retinol from reversed-phase packing materials, e.g., μBondapak [28] and RP-18 [24]. However, organic solvents such as methanol and acetonitrile have also been used in admix-



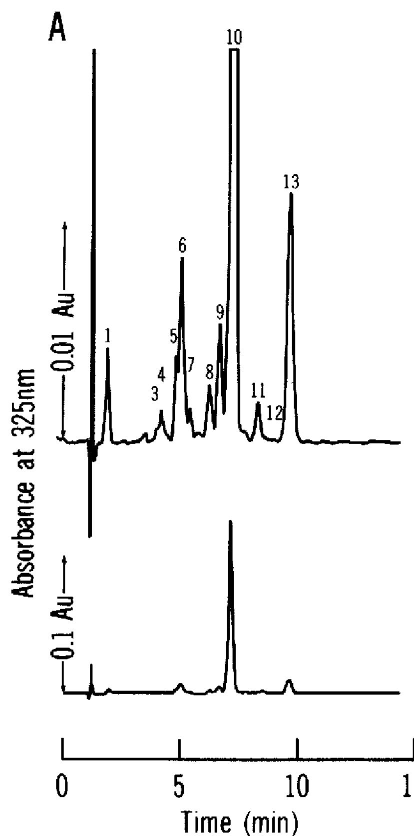


Fig. 3. Chromatogram of a serum extract under NARP conditions. Column, Zorbax-ODS, 7 μm (25 cm × 0.46 cm I.D.); eluent, acetonitrile–dichloromethane–methanol (70:15:15, v/v/v); flow-rate, 1 ml/min; detection, 330 nm. Peaks: 1 = retinol; 2 = I.S. (retinyl propionate). From ref. 33, with permission.

Fig. 4. Isocratic NARP HPLC trace of a rat liver extract. Column, Resolve C_{18}, 5 μm (15 cm × 0.39 cm I.D.); eluent, acetonitrile–dichloromethane (80:20, v/v); flow-rate, 1.5 ml/min; detection, 325 nm. Peaks: 1 = retinol; 3 = retinyl linolenate; 4 = retinyl laurate; 5 and 12 = non-retinoid interferences; 6 = retinyl linoleate; 7 = retinyl myristate + palmitoleate; 8 = retinyl pentadecanoate; 9 = retinyl oleate; 10 = retinyl palmitate; 11 = retinyl heptadecanoate; 13 = retinyl stearate. From ref. 63, with permission.

tures with a small percentage of water to affect the rate of elution of retinol or the internal standard used [19,26,29]. Further, ammonium acetate is sometimes added to the eluent [35] as a masking agent for the residual silanol groups on chromatographic packing materials [58]. Very recently, Curley et al. [59] also described the use of ammonium acetate. Further, they advised the use of methanol–water mixtures with end-capped packings (Ultrasphere-ODS) and acetonitrile–water with non-end-capped materials (Ultrasil-ODS) for the separation of retinol from retinal and retinoic acid.

By eluting highly retentive reversed-phase packings (Zorbax-ODS) with non-

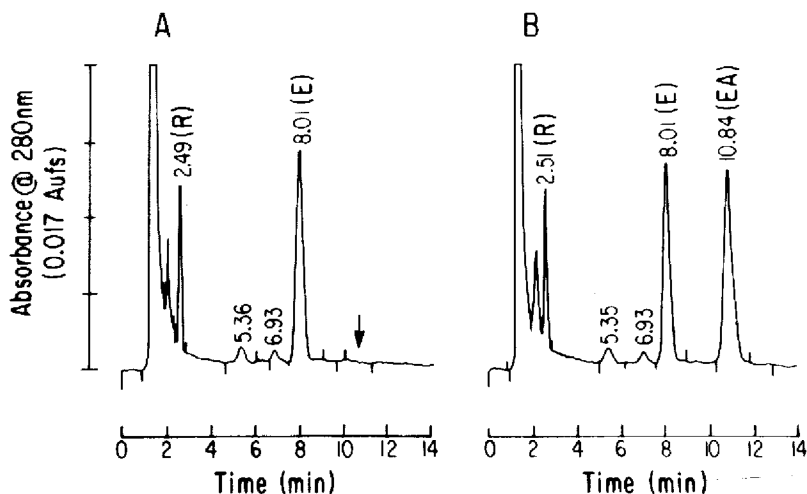


Fig. 5. Simultaneous determination of retinol (R) and vitamin E (E) with tocopheryl acetate (EA) as an I.S. Column, μBondapak RP-18, 10 μm (30 cm × 0.39 cm I.D.); eluent, methanol-water (95:5, v/v); flow-rate, 2.5 ml/min; detection, 280 nm. A is a serum extract without I.S. added; B, I.S. added. From ref. 50, with permission.

aqueous mobile phases, Parris [60] obtained very effective separations of water-insoluble, non-polar compounds. It is obvious that improved solubility of the sample in the totally organic eluent prevents precipitation on the column or incomplete elution of the sample. Owing to a higher possible sample load, an improvement in the detection limit can also be observed. In 1983 Nelis et al. [33] applied these non-aqueous reversed-phase (NARP) conditions to the quantitative measurement of retinol in human serum. The elution of the Zorbax-ODS column was performed isocratically with acetonitrile–dichloromethane–methanol, and retinyl propionate was used as an internal standard (Fig. 3).

For the separation of the different retinyl ester forms, the use of methanol–water [61] or acetonitrile–water [37] results in very long chromatographic runs. Gradient systems, still including water in the mobile phase, can overcome this problem of long retention [21]. Another multi-step, three-solvent gradient based on methanol, water and chloroform even allowed the separation of fourteen retinoids including various retinyl esters in one single run of less than 50 min [62]. However, isocratic NARP conditions with acetonitrile–dichloromethane as eluent even provide a more rapid and satisfactory separation of the most prevalent retinyl esters in biological matrices [63], as demonstrated in Fig. 4. The incorporation of silver ions in the mobile phase [64] also allows the different ester forms to be separated. However, using this procedure the cleaning of all glassware that contained this mobile phase is very time-consuming.

For the simultaneous quantitation of vitamins A and E the eluents used consist basically of pure methanol [46] or 4–5% water in methanol [40,42,47,50] (Fig. 5).

With these aqueous mobile phases, the samples have to be dissolved and in-

jected in a stronger solvent (e.g., pure methanol or ethanol). However, this can cause precipitation of lipids on top of the column, resulting in an increased backpressure and loss of efficiency. Hildebrandt et al. [49] extracted serum samples with isopropanol, and an unusual eluent (isopropanol plus an aqueous solution of heptanesulphonic acid) was used to separate vitamins A and E. However, this group also overlooked internal standardization [49]. Finally, an isocratic NARP procedure allowed the separation and quantitation of retinol, vitamin E and various carotenoids in less than 20 min, making this method applicable to larger epidemiological studies [43].

2.4.2. Detection

Owing to the high values of the molar absorptivity and the λ_{max} of the retinol molecule, ultraviolet (UV) monitoring has been the most widely used method of detection of retinoids.

Usually UV detection for retinol analysis is performed at fairly high wavelengths (310–340 nm), resulting in a detection limit of 10–20 ng/ml using 100 μl [26,31] to 500 μl [22,35,41] or even up to 1 ml [34] of serum sample. For the detection and quantitation of retinyl esters, UV detection is used in almost all studies [21,37,61,63–65].

A second method of detection depends on the ability of retinoids to fluoresce. By selecting an excitation wavelength of 325 nm and an emission wavelength of 470 nm, very sensitive detection of both retinol and its esters can be achieved. By employing fluorescence detection the specific measurement of retinol or retinyl esters is approximately one order of magnitude better than assays with UV detection, often resulting in analyses of extremely small sample volumes, ranging from 50 μl [28,32] to even 1 μl [30]. Typical chromatograms have been presented in Fig. 2.

As to the simultaneous determination of vitamins A and E, UV absorbance detection is necessarily a compromise. At 330 nm (λ_{max} of retinol) tocopherol is virtually non-absorbing, whereas at the λ_{max} of tocopherol there is a fair amount of retinol absorbance left. Accordingly, 290 nm was selected as the detection wavelength by Driskell et al. [40], whereas others used 280 nm [42,50], which affords lower sensitivity and specificity. Other research groups changed the detection wavelength during the chromatographic run to detect both compounds (and the internal standard) at their λ_{max} [43,47,49]. Very recently the simultaneous use of UV and fluorescence detection was reported by Todd and Brown [66]. In their procedure, retinyl acetate is the I.S. for retinol and tocopheryl acetate compensates for vitamin E losses. The same principle of double internal standardization was applied by Borland and Shenkin [67], although they used UV detection only. Fluorescence detection with automatic change of the excitation and emission wavelengths has been applied only once for the measurement of vitamins A and E, by Rhys Williams [44]. In this normal-phase eluent both tocopherol and retinol show a five- to six-fold increase in fluorescence intensity over that in a reversed-phase eluent. The latter procedure would really be excellent if tocol were used as an internal standard.

Recently, electrochemical detection (ED) of both vitamins A and E has also

been studied [48], as shown in Fig. 6. Under reversed-phase conditions (94% methanol in 0.05 *M* sodium perchlorate) both tocopherol and tocol show hydrodynamic voltammograms with a plateau current of +0.4 and +0.55 V, respectively. Retinol initiates its electrochemical response only at +0.55 V and does not reach a steady state even at +0.9 V [48,68,69]. Very recently, McCrehan and Schönberger [69] applied ED to the simultaneous determination of retinol, α-tocopherol and β-carotene on a Vydac column eluted with water–methanol–*n*-butanol. Although ED of vitamin A does not result in the same gain in sensitivity as for vitamin E and β-carotene, ED will certainly provide a useful alternative to UV and fluorescence detection in future research on vitamins A and E.

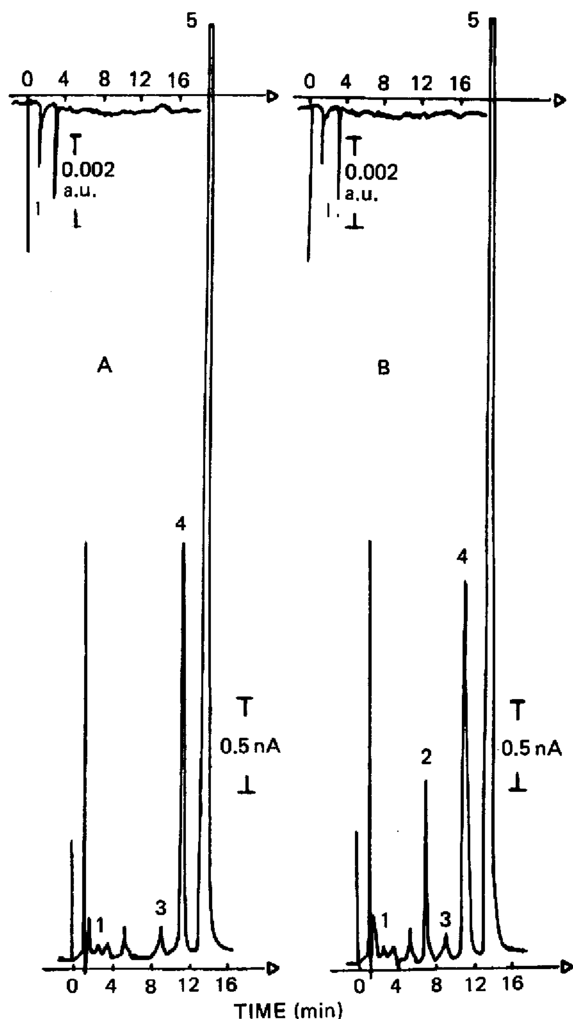


Fig. 6. Chromatograms of extracts from (A) healthy human plasma and (B) human plasma spiked with tocol as internal standard. Top chromatograms are UV absorptions at 313 nm. Bottom chromatograms are electrochemical responses at +0.6 V versus Ag/AgCl. Peaks: 1 = retinol; 2 = tocol; 3 = δ-tocopherol; 4 = β- and γ-tocopherol; 5 = α-tocopherol. From ref. 48, with permission.

3. β-CAROTENE

3.1. Background

β-Carotene is prominent among the carotenoids, a class of polyene pigments biosynthesized in plants via the mevalonate pathway [70]. Animals and humans assimilate these compounds through dietary consumption [71,72]. Following absorption from the food, carotenoids appear unaltered in the blood, although some derivatives are partly metabolized to retinal (vitamin A) [71,72]. The latter, including β-carotene, α-carotene, β-cryptoxanthin and γ-carotene, are designated with the collective term "provitamin A" [71,72].

The structural formulae of the major carotenoids occurring in human plasma are depicted in Fig. 7. β-Carotene has maximum provitamin A activity because it is converted, by the enzyme β-carotene 15,15′-dioxygenase, to two molecules of retinal [71,72]. Other provitamin A carotenoids display roughly 50% of the biopotency of β-carotene [71,72]. Lutein, zeaxanthin and lycopene lack provitamin A activity, although in lower animals lutein can act as a precursor of 3-dehydroretinol, vitamin A_2 [73].

Whether the assessment of the vitamin A nutritional status should include a determination of β-carotene in plasma is controversial [74]. Unlike retinol, β-carotene cannot be mobilized on demand from a specific storage site in the body, e.g., the liver or depot fat [75]. Consequently, its plasma levels partly or wholly reflect eating habits [76]. Some disorders, e.g., anorexia nervosa, are associated

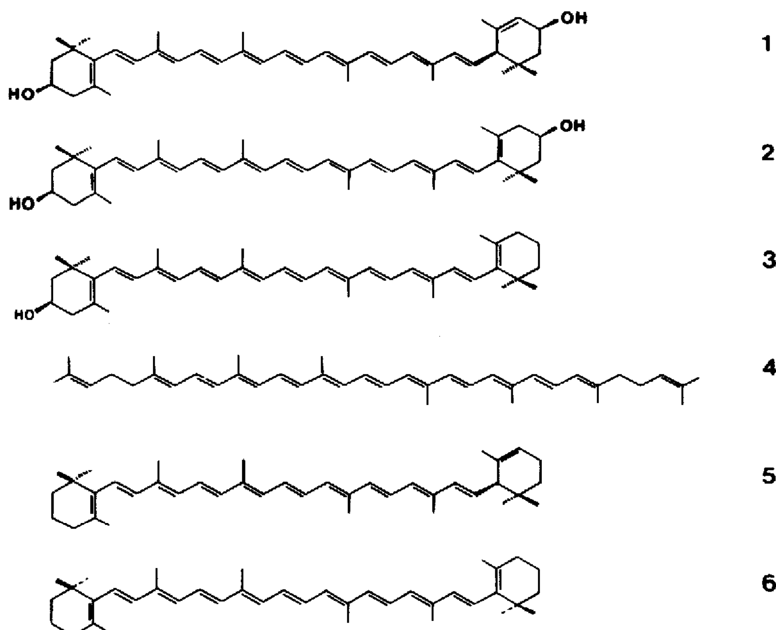


Fig. 7. Structures of the principal carotenoids in human plasma/serum. (1) lutein; (2) zeaxanthin; (3) β-cryptoxanthin; (4) lycopene; (5) α-carotene; (6) β-carotene.

with hypercarotenaemia [77]. This state can also be caused by massive ingestion of β-carotene for therapeutic reasons, i.e, the treatment of photosensitivity [78]. Lipid malabsorption results in β-carotene deficiency, a key diagnostic parameter in this pathology [79]. Like other isoprenoid vitamins, β-carotene and possibly other carotenoids are thought to exert a chemopreventive action on carcinogenesis [76,80]. In view of this purported relationship, the simultaneous determination of carotenoids, retinol and tocopherol in plasma is meaningful.

3.2. Isolation, extraction and clean-up

Modern carotenoid assays in plasma are mostly based on LC. Hence, the extent of sample pre-treatment will be primarily determined by the specificity of this technique. There are no reports of the determination of carotenoids in human tissues, despite their known affinity for lipid-rich sites in the body [75]. A standard analytical scheme consists of deproteinization of plasma or serum (0.1–0.5 ml) with ethanol, followed by lipid extraction with hexane or light petroleum [27,41,43,69,81–88]. Saponification does not appear to be necessary but is sometimes performed [85]. Some investigators have omitted the deproteinization step [89,90] or prefer to add antioxidants [87]. Occasionally, hexane has been replaced with chloroform as an extraction solvent [91]. The organic layer is isolated, evaporated to dryness under nitrogen and the residue is dissolved in a suitable solvent prior to injection on to the LC column. During the sample preparation precautions should be taken to protect the vulnerable carotenoids from light, air, heat, acids and oxidants [92]. A few workers have attempted to simplify the extraction method by eliminating the evaporation step. Thus, Peng et al. [93] and Nierenberg [94] deproteinized the plasma with perchloric acid and extracted the carotenoids with tetrahydrofuran (THF)–ethyl acetate. After centrifugation, an aliquot of the supernatant could be directly injected on to the column. Brief exposure of the sample to the harsh perchloric acid allegedly did not lead to carotenoid deterioration. However, stabilization of the THF to keep it peroxide-free was found to be essential. Evidently, because no concentration step is involved, the sensitivity may become critical, the limiting factors being the sample size (usually 0.5 ml) and the volume of the extraction solvent. This limitation also applies to methods in which an extract in hexane is injected on to a normal-phase column, without an evaporation step [41].

Despite the relative uniformity of the methods in the literature, a note of caution should be made in this respect. Some workers have expressed concern about poor recoveries of β-carotene from plasma using the ethanol–hexane approach [95]. Specifically, part of the β-carotene might coprecipitate with the proteins, remain tightly bound and resist further extraction attempts. Anomalously low recoveries of retinyl esters from serum using this extraction method have also been reported [96]. Another factor possibly underlying incomplete recovery is the reconstitution of the residue obtained after evaporation. Solvents stronger than ethanol, e.g., diethyl ether, are recommended for this purpose [87]. Incomplete solubilization of lipid extracts in methanol has previously been shown to result in low recoveries of retinyl esters [96]. A useful procedure is to dissolve

the residue in the strongest component of the chromatographic solvent (e.g., dichloromethane) and subsequently add methanol and/or acetonitrile. This in turn argues in favour of NARP or normal-phase systems with strong mobile phases, containing chloroform or dichloromethane, the preferred solvents for carotenoids (see Section 3.4.1).

The selection of a suitable internal standard for β-carotene represents a major obstacle. Dimethyl-β-carotene, as proposed by Driskell et al. [81], apparently meets the requirements of an ideal structural analogue. It possesses similar physicochemical characteristics, including spectral properties, and in reversed-phase chromatography elutes after β-carotene, in a blank position of the chromatogram. Unfortunately, the compound has to be synthesized and is so unstable that it can hardly be stored without deterioration, not even at −70°C [97].

Other carotenoids suggested as internal standards include β-apo-8′-carotenal [98], β-apo-8′-carotenoic acid ethyl ester [83] and echinenone [84]. These oxygenated derivatives, however, are much more polar than the unsubstituted β-carotene and hence elute well before the latter in reversed-phase systems. They would be more appropriate as potential internal standards for the determination of certain xanthophylls [99]. The β-carotene analogue which, at present, most closely approaches the ideal situation is decapreno-β-carotene [69,97]. This synthetic derivative has two isoprene units more than β-carotene. A disadvantage is the substantial bathochromic shift in its absorption maximum (λ_{max} 502 nm) with respect to β-carotene (λ_{max} 450 nm), which necessitates the addition of an excess of I.S. to equal the response of β-carotene (at 450 nm). The same drawback would apply to torulene as an I.S. (λ_{max} 480 nm), especially when using a fixed-wavelength detector at the non-optimal wavelength of 436 nm [87]. Other β-carotene analogues with longer or shorter polyene chains, e.g., "C_{37}-β-carotene" [100] or nonapreno-β-carotene (C_{45}) [101], would be more appropriate choices in terms of their spectral properties, but none of these compounds is readily available. This is also the case for esters of isozeaxanthin [102].

3.3. Gas chromatography

The status of GC as an analytical tool for carotenoids was thoroughly reviewed by Taylor and Ikawa [103] in 1980. Since then, there has been no follow-up on this topic. To increase their volatility and reduce their thermolability, carotenoids have been chromatographed as perhydro derivatives. Although there is no known biomedical application of this approach, it remains potentially valuable as a basis for GC–MS identification work [103].

3.4. Liquid chromatography

LC has superseded the classical non-specific spectrophotometric assays of "total carotene", which do not distinguish between provitamin A and non-provitamin A carotenoids. The latter type of assays grossly overestimate the β-carotene content of plasma [88].

3.4.1. Chromatographic systems

3.4.1.1. Adsorption chromatography

In principle, adsorption chromatography is unattractive for quantitative work on carotenoids for a number of reasons. Underivatized silica has been suspected to cause on-column degradation of carotenoids [104], displays little or no retentivity for β-carotene and fails to separate the positional isomers α- and β-carotene [105]. However, there is one report of its use in connection with the determination of β-carotene in human serum [41]. β-Carotene could be separated from lycopene but not from α-carotene. The major application of silica lies in the resolution of geometrical (*cis/trans*) isomers of carotenoids [105], which is of little concern with regard to biological samples of human origin, however. This separation can also be achieved on certain polymeric reversed phases [69], but the actual content of *cis-β*-carotene in serum appears to be negligible [69]. Alumina is less agressive than silica, is capable of separating positional isomers [106] and has a higher affinity for β-carotene. However, very stringent precautions, i.e., careful control of the water content of the mobile phase and the temperature, are necessary in order to obtain reproducible results [107].

3.4.1.2. Bonded-phase chromatography

3.4.1.2.1. Normal phase. Polar bonded phases, e.g., amino and cyano, are superior to silica and alumina in terms of stability and reproducibility, but are less efficient for the separation of carotenoid *cis/trans* isomers. However, they suffer from a similar drawback as silica in that β-carotene has little retention on these materials. Conversely, lutein and zeaxanthin are strongly retained. Therefore, a concurrent determination of xanthophylls and carotenes in serum extracts usually involves gradient elution [89,90].

3.4.1.2.2. Reversed phase. Most LC systems for the determination of β-carotene in human plasma or serum are based on NARP chromatography [27,43,81–84,86–88,91]. Typical eluents consist of acetonitrile as a base solvent and chloroform [27,43,82,91], dichloromethane [81,84,88] or THF [83,87] as a modifier to control the eluotropic strength. Selectivity in NARP chromatography is often profoundly affected by the addition of methanol [99,108]. Some investigators prefer to incorporate a small amount of aqueous ammonium acetate in an essentially organic eluent, presumably with the aim of improving the peak shape [93,94]. On most C_{18} packing materials the xanthophylls show little retention under the strong elution conditions used for β-carotene. Accordingly, these polar derivatives are disregarded in certain carotenoid assays [81,82,88,91,93,94].

To cover the whole spectrum of polar and non-polar carotenoids of interest, gradient elution may be required [85]. However, some non-end-capped bonded phases, notably Zorbax-ODS, display a "low" selectivity for carotenoids in that they interact more strongly with xanthophylls than with unsubstituted carotenes [108]. As a result isocratic separations become feasible, even of compounds with a very substantial polarity difference such as retinol and β-carotene [108]. In the original NARP system serum extracts containing lutein, zeaxanthin, β-cryptoxanthin, lycopene, α-carotene and β-carotene were resolved in approximately 30 min [108]. Faster isocratic NARP separations (13–15 min) of the major plasma carotenoids were achieved using higher flow-rates [84], shorter columns (15 cm)

[87] or 3-μm particles [88]. A representative carotenoid profile of plasma is shown in Fig. 8.

The eluents in these NARP procedures are mixtures of acetonitrile, methanol and dichloromethane. However, β-carotene, unlike the xanthophylls, is reportedly poorly recovered from new, unconditioned Zorbax-ODS columns using this type of eluent [109]. Replacement of the original eluent with acetonitrile–ethyl acetate–decanol appears to overcome this undesirable effect [109]. Conversely, poorly retentive C_{18} materials, notably some polymeric phases (Vydac 201TP), have to be eluted with semi-aqueous eluents, which may also lead to losses of β-carotene through adsorption on the column frit [69].

Methods for the simultaneous determination of carotenoids and isoprenoid vitamins receive proper attention in the sections on vitamins A and E. Isocratic separations [43,86] have tended to replace the earlier gradient procedures [27], although on the polymeric Vydac 201TP phase gradient elution remains desirable [69]. Thus lycopene, α-carotene, β-carotene and various vitamin A and E derivatives have been separated on a 3-μm column in less than 15 min [86] (Fig. 9).

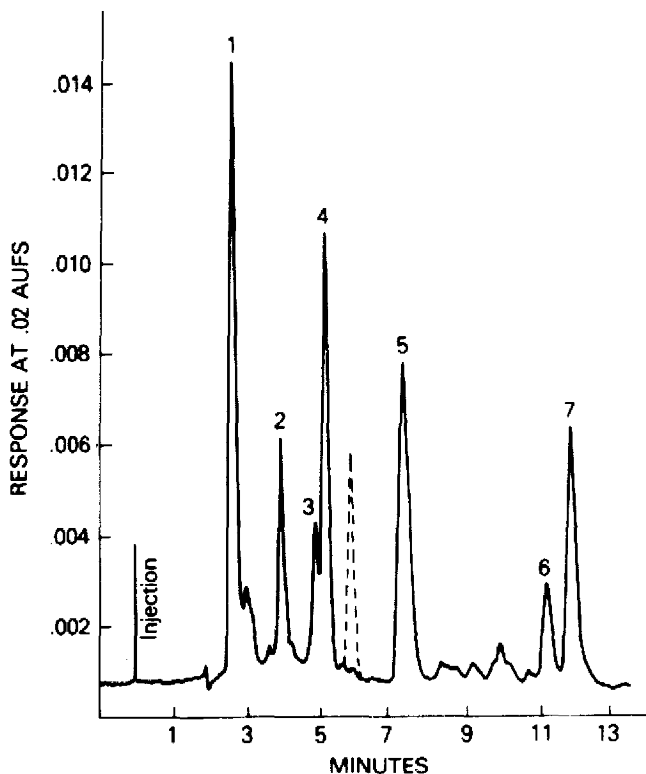


Fig. 8. Carotenoid profile of normal plasma (solid line). Column, 5-μm Supelcosil LC-18 (25 cm × 0.46 cm I.D.); eluent, acetonitrile–dichloromethane–methanol (70:20:10, v/v/v); flow-rate, 1.7 ml/min; detection, 436 nm. Peaks: 1 = lutein + zeaxanthin; 2 = unknown; 3 = "pre-cryptoxanthin"; 4 = β-cryptoxanthin; 5 = lycopene; 6 = α-carotene; 7 = β-carotene. The dotted line indicates the elution position of echinenone (I.S.). From ref. 84, with permission.

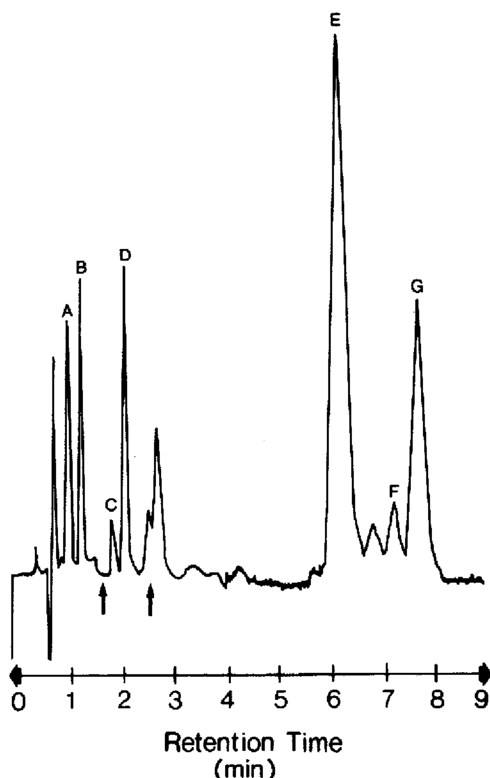


Fig. 9. Chromatogram of fat-soluble vitamins extracted from plasma. Column, 3 μm Hypersil ODS (6 cm × 0.46 cm I.D.); eluent, methanol; flow-rate, 0.9 ml/min. Peaks: A = retinol; B = retinyl acetate; C = γ-tocopherol; D = α-tocopherol; E = lycopene; F = α-carotene; G = β-carotene. Peaks A and B were measured at 305 nm, C and D at 290 nm and E–G at 460 nm. The arrows indicate the wavelength changes. From ref. 86, with permission.

3.4.2. Detection

Carotenoids absorb light in a favourable region of the spectrum (440–500 nm), with molar absorptivities well above 100 000 l mol^{-1} cm^{-1}. Consequently, spectrophotometric detection affords a high selectivity and sufficient sensitivity to cover the range of physiological carotenoid levels in plasma. In practice, the detection of carotenoids in LC has become virtually restricted to this single technique, especially as these compounds exhibit no native fluorescence [110]. However, carotenoids are electrochemically active [111]. This property was recently exploited in a method for the simultaneous determination of retinol, tocopherols and β-carotene, which represents the first example of ED of β-carotene [69]. A detection limit of 2.1 μg l^{-1} for this compound was reportedly achieved [69].

In absorption detection, variable- and fixed-wavelength detectors have been used, the latter operating at the non-optimal wavelength of 436 nm [27,41,84,85,87,88,93,94]. A multi-channel photodiode array detector adds an extra dimension in that it can monitor and memorize "on-line" the highly

characteristic absorption spectra of carotenoids, thus often permitting their tentative identification [112]. This versatile instrument also permits wavelength switching during the chromatographic run to include compounds other than carotenoids, i.e., retinoids and tocopherols [43,86]. This is further discussed in the appropriate sections on vitamins A and E.

4. VITAMIN D

4.1. Background

Humans derive their vitamin D (vitamin D_3 and D_2) from two sources. Vitamin D_3 is biosynthesized in the skin on irradiation of provitamin D_3 with UV light, whereas vitamin D_2 is absorbed from the diet. Vitamin D is converted in the liver to 25-hydroxyvitamin D (25-OHD), which itself is further metabolized in the kidney to 1α, 25-dihydroxyvitamin D [1,25-$(OH)_2D$] and other dihydroxylated compounds. Although 25-OHD is the major circulating metabolite, 1,25-$(OH)_2D$ is known to be biochemically the active form in the regulation of calcium and phosphorus metabolism and bone resorption.

The levels of vitamin D and 25-OHD are an indicator of the vitamin D status in man. Decreased vitamin D and 25-OHD concentrations can be caused by deficient food intake, malabsorption, lack of exposure to UV light combined with a bad diet, liver cirrhosis or chronic or acute hepatitis (resulting in decreased 25-hydroxylation). Excessive vitamin D intake may lead to hypervitaminosis. Decreased levels of 1,25-$(OH)_2D$ are associated with hypoparathyroidism, pseudohypoparathyroidism, Fanconi syndrome, vitamin D-dependent rickets type I, neonatal hypocalcaemia, osteomalacia, osteoporosis and renal osteodystrophy. Increased 1,25-$(OH)_2D$ levels may suggest hyperparathyroidism, sometimes calcium nephrolithiasis, vitamin D-dependent rickets type II or sarcoidosis [113,114]. Many other vitamin D metabolites are of unknown importance. The occurrence of several metabolites complicates the measurement of the vitamin D compounds in serum because of their potential interference in the assay. Further, the quantitation of vitamin D and metabolites in plasma remains difficult owing to their low concentrations and the instability of the molecule. Before the determination of vitamin D or one of its metabolites, an extensive sample clean-up has to be carried out to eliminate the interference from other vitamin D compounds and

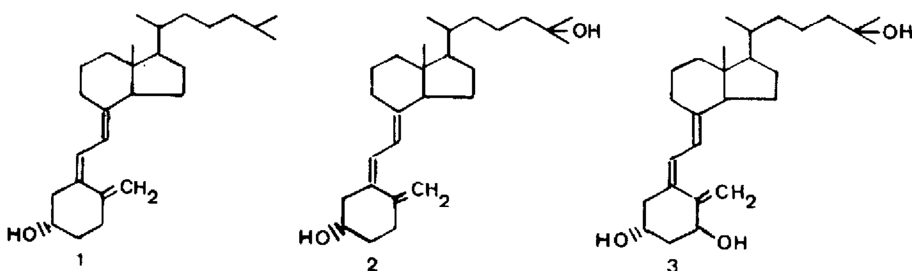


Fig. 10. Structures of (1) vitamin D_3, (2) 25-OHD_3 and (3) 1,25-$(OH)_2D_3$.

unidentified serum constituents. To this end a variety of open columns are used, with or without a preliminary extraction. Sometimes one or even two HPLC steps are necessary for the purification before the final measurement. There is a current tendency towards simplifying the time-consuming clean-up steps by using new purification procedures and developing more specific assays.

Work carried out in this field up to 1981 has been reviewed in an excellent paper by Jones et al. [115]. Because the literature on this topic is extensive, the scope of this paper has been limited to vitamin D, 25-OHD and 1,25-$(OH)_2D$. Structural formulae of these metabolites are shown in Fig. 10.

4.2. Isolation, extraction and clean-up

4.2.1. Saponification

Saponification can be useful for eliminating the bulk of neutral lipids in biological samples. It is used in the analysis of milk [116], where the fat is saponified by treatment with ethanolic potassium hydroxide. Subsequently the unsaponified fraction is extracted with light petroleum and washed with distilled water to eliminate soap and residual potassium hydroxide. A similar saponification procedure was adopted by Masuda et al. [117], who isolated the vitamin D–vitamin D-binding protein (DBP) bonded fraction from serum by affinity chromatography. If possible, saponification should be avoided because of the instability of vitamin D metabolites and because of the lack of convenience [115].

4.2.2. Extraction

Extraction with an organic solvent has the advantage that vitamin D and most of its metabolites are extracted as fat-soluble seco-steroids, whereas hydrophilic compounds are mostly eliminated.

Solvent systems for extraction fall into two basic categories, depending on the desired selectivity (i.e., total or selective lipid extraction) [115]. Total lipid extraction may be necessary when a simultaneous analysis of several vitamin D metabolites with a wide range of polarities is required. The Bligh–Dyer extraction procedure [118] is used in many laboratories [119–128]. Several investigators have replaced chloroform with dichloromethane in order to reduce the evaporation time. In addition, the latter extracts contained less lipid material [129–142]. This extraction method was also used for the isolation of vitamin D and its metabolites from human milk [143–145], followed by precipitation of the neutral lipids with absolute methanol and the polar lipids with diethyl ether. Also with ethanol a total lipid extraction is obtained. It is a rapid method for the precipitation of the proteins and the simultaneous extraction of vitamin D metabolites [120,146,147]. Hollis and Frank [140] used ethanol to extract a serum sample that had been diluted with an equal volume of saturated ammonium sulphate solution.

To obtain a selective lipid extract, more polar solvents that simultaneously precipitate the proteins are used. Mixtures of a non-polar solvent (e.g., *n*-hexane or cyclohexane) and ethyl acetate [147,148], methanol [149], isopropanol [150] or *n*-butanol [151] have been recommended for the extraction of vitamin D and

its hydroxylated metabolites. Other workers prefer to denature the sample first with methanol [152] or acetonitrile [153] or to treat it with alkali [147] before extraction with *n*-hexane. Fohlman et al. [154] selectively isolated vitamin D metabolites by affinity chromatography before their extraction with *n*-hexane. Mixtures of benzene or toluene [155,156] with isopropanol [157] or methanol [158] have also been applied to the extraction of vitamin D and its metabolites. Treatment of the sample with ammonium sulphate [158] or ammonium carbonate prior to the extraction [159,160] has been described for the isolation of the hydroxylated metabolites.

Diethyl ether alone [155,161–168] or combined with methanol [120] and/or dichloromethane [169] is a very common extraction solvent for vitamin D. It can even extract 1,25-$(OH)_2D$ from acetone [170] or acetone–dichloroethane-treated plasma samples [171]. However, peroxides present in the diethyl ether can react with the *cis*-triene part of vitamin D, and hence result in a loss of the compounds.

Mini-column systems such as Clin-Elut [172–175] and Extrelut [176–179] can eliminate almost completely triglycerides and cholesterol from diluted samples. Acidic lipid constituents can be removed by a final wash of the organic extract with an alkaline buffer. Sometimes BHT at a concentration of 0.1 mg/ml serves as an antioxidant during extraction and chromatography. The application of mini-columns is still hampered by batch-to-batch variability, resulting in incomplete and irreproducible recoveries. Also, interferents originating from the plastic tubes or the packing itself still remain a serious drawback for the use of these columns, even in routine clinical methods.

Open-column chromatography on silicic acid [150,161,162,170,174], Sephadex LH-20 [119,121–126,128–130,133,135,163,164,166,168–171], Lipidex 5000 (a less polar hydroxyalkoxy derivative of Sephadex LH-20) [129,140,142,146,169], Celite [170], Sep-Pak silica or C_{18} and Bond-Elut silica, C_{18} or NH_2 [128,141,169,180–198] can also be used to separate vitamin D from 25-OHD, 1,25-$(OH)_2D$ and sometimes other vitamin D metabolites. However, the retention times and recoveries can differ significantly from batch to batch. Further, different studies demonstrated that a gel chromatographic clean-up on Sephadex LH-20 or Lipidex 5000 is never sufficiently selective to allow a final measurement of 25-OHD_3 in human serum by HPLC with UV detection [128,146]. On the other hand, commercially available cartridges such as Sep-Pak C_{18} lead to cleaner HPLC profiles [184]. Using these materials some investigators could even eliminate the HPLC purification step in their analysis of 1,25-$(OH)_2D$ in human serum by a radioligand assay [140,181,196].

Finally, we draw the attention to the issue of internal standardization. Radioactively (tritium) labelled vitamin D (metabolites) are added to each sample before starting the extraction. Addition of the I.S. to one or two vials in a whole batch of samples is a bad approach as recoveries in individual samples are not compensated for.

4.3. Gas chromatography

GC analysis of vitamin D compounds is less popular than HPLC analysis for various reasons. First, at elevated temperatures vitamin D and its hydroxylated

metabolites undergo thermal rearrangement involving B-ring closure with formation of two isomers, i.e., the pyro and the isopyro forms. Second, clean-up of serum samples is more complex for final GC analysis than for HPLC analysis, and finally GC of vitamin D is always hampered by the presence of large amounts of cholesterol in the samples. The removal of cholesterol is therefore essential for obtaining clean chromatograms.

Derivatization to the isotachysterol (ITS) isomers prevents thermal cyclization due to the rearrangement of the A-ring. However this derivatization is not applicable to the 1α-OH function.

For the analysis of 25-OHD_3 by GC–MS with multiple-ion detection (MID), Björkhem and Holmberg [159] formed 3-*tert.*-butyldimethylsilyl-25-trimethylsilyl (TBDMS-TMS) derivatives. Other groups applied the same procedure for the analysis of 1,25-$(OH)_2D_3$ [128]. For the formation of unesterified ITS forms of the vitamin, Seamark et al. [131] used hydrochloric acid in dichloroethane, chloroform or dichloromethane at 0°C. Although these isomers already chromatographed as single peaks, the formation of TMS ethers was useful in preventing adsorption and destruction and resulted in improved linearity [146,188].

The simultaneous formation of ITS isomers and derivatization of the hydroxylic functions (except the 25-OH group) can be achieved by using heptafluorobutyric anhydride (HFBA) [199].

Losses through destruction and adsorption during the GC analysis of trace amounts of metabolites in human serum can be prevented by adding 200 ng of carrier vitamin D.

4.3.1. Chromatographic systems

GC systems reported for vitamin D analysis consist of silanized glass columns filled mainly with 1.5% SE-30, 2% OV-1 on Chromosorb or Celite 545 and 1% free fatty acid phase (FFAP) on Gas-Chrom Q.

4.3.2. Detection

4.3.2.1. Flame ionization and electron-capture detection

Flame ionization detection (FID) has been described for the analysis of vitamin D [132,199] but is not sufficiently sensitive for the measurement of the vitamin in human plasma samples. Electron-capture detection (ECD) is sensitive enough, as demonstrated on standards [199], but lacks specificity to allow the quantitation of vitamin D in human plasma [115].

4.3.2.2. Mass spectrometric detection

Coupling of a mass spectrometer to a GC apparatus gives a sensitivity comparable to that of ECD. However, the superior specificity of the GC–MS combination is well known. GC–MS with MID was used by De Leenheer and Cruyl [199] for the analysis of plasma vitamin D_3. They focused on the ion of m/z 580, the molecular ion of $isotachysterol_3$ heptafluorobutyrate, and on that of m/z 594, the molecular ion of the internal standard $dihydrotachysterol_2$ heptafluorobutyrate.

Mass fragmentography of serum vitamin D and hydroxylated metabolites was carried out [132,146] on ITS-TMS ether derivatives of vitamin D and metabo-

lites using a multiple-ion detector. The molecular ions have m/z 456.7 (vitamin D_3), 468.7 (vitamin D_2) and 544.7 (25-OHD_3).

MID was also applied by Björkhem and Holmberg [159] and later by other investigators [168,197]. They monitored the ions of m/z 586 and 589 of TBDMS-TMS derivatives of 25-OHD_3 and its deuterated form and m/z 452 and 455 of TMS ether derivatives of 1,25-$(OH)_2D$ and its deuterated form.

These methods were further used to evaluate different methods for the determination of 25-OHD with UV or radioligand assay [128,197].

Fohlman et al. [154] used fast heavy ion-induced desorption (FHID) mass spectrometry on different vitamin D compounds. The samples have to be deposited on a thin film to permit this approach.

4.4. Liquid chromatography

4.4.1. Chromatographic systems

Modern LC on microparticulate materials has superseded GC for the resolution of vitamin D derivatives. Individual hydroxylated metabolites are most efficiently separated by normal-phase chromatography, whereas the use of reversed-phase procedures has become virtually restricted to the differentiation of the vitamin D_2 and D_3 forms. Depending on the plasma/serum levels of the compound(s) of interest, on-line absorption detection or an off-line radioligand assay is indicated for quantitation. Specifically, UV detection is sufficiently sensitive to permit the determination of vitamin D and 25-OHD, although in general a multi-dimensional approach (coupling of normal- and reversed-phase systems) is required in order to eliminate interferences. However, this technique completely fails to detect physiological amounts of 1,25-$(OH)_2D$. Nevertheless, HPLC remains an essential part of the purification prior to most of the radioligand assays of this compound. The latter are subject to considerable cross-reaction from related metabolites.

4.4.1.1. Adsorption chromatography

On silica (Zorbax-Sil, Spherisorb, μPorasil, LiChrosorb, Nucleosil 50-7), the separation of vitamin D derivatives takes place according to the number and position of the hydroxyl groups in the molecule. Binary mobile phases usually consist of hexane as a base solvent and isopropanol as a polar modifier [121, 123, 133, 137, 139, 143, 148, 150, 152, 155, 156, 161, 164, 169, 170, 172, 180–182, 184, 187, 192, 195, 197, 198, 200, 201]. A small volume of methanol may be added to minimize peak tailing [164,183,186,200]. Alternatively, mixtures of dichloromethane and an alcohol can be used [122,135,169]. Ternary solvent systems containing hexane and variable percentages of a chlorinated hydrocarbon and an alcohol often display a unique selectivity with regard to the D_2 and D_3 forms [200], which normally co-elute in normal-phase chromatography. Disadvantages of these eluents include poor solvent miscibility and sample solubility.

A representative separation of vitamin D metabolites on silica is shown in Fig. 11. In a typical procedure, crude lipid extracts are injected, the compound of interest, e.g., 1,25-$(OH)_2D$, is collected and, following evaporation of the solvent, subjected to the radioligand assay. The elution position of the derivative is marked

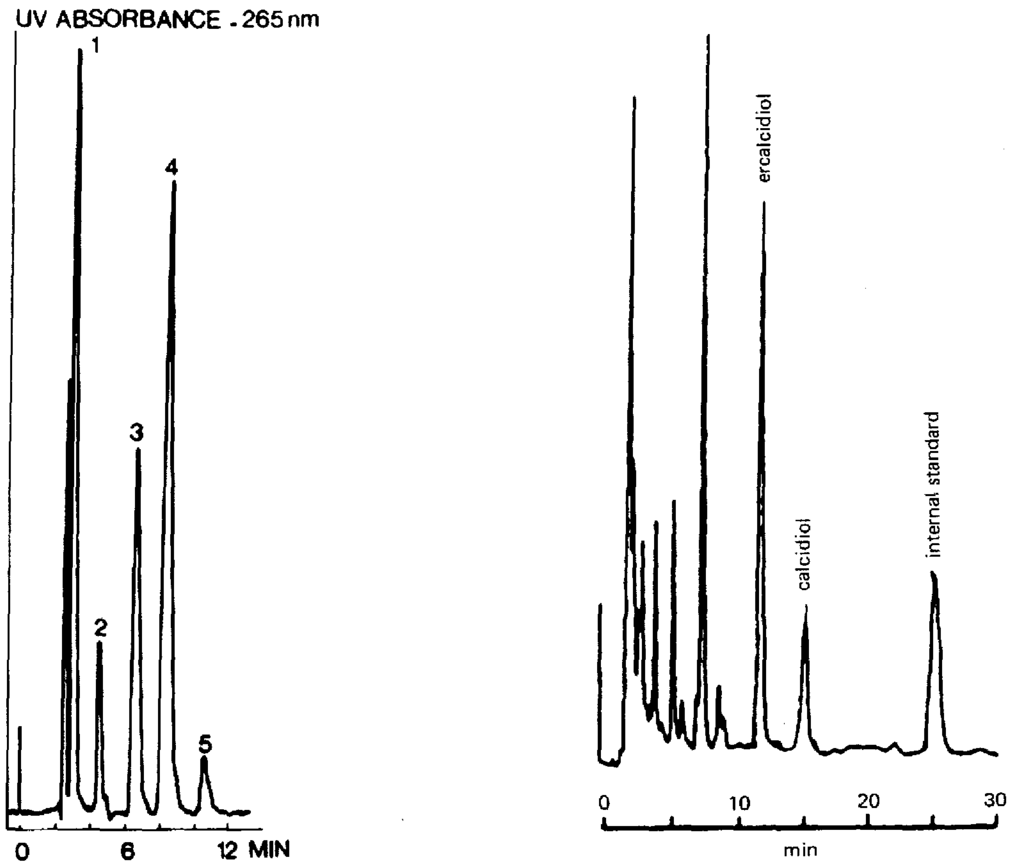


Fig. 11. Chromatogram from standards of (1) vitamin D_3, (2) 25-OHD$_3$, (3) 24,25-(OH)$_2$D$_3$, (4) 25,26-(OH)$_2$D$_3$ and (5) 1,25-(OH)$_2$D$_3$ eluted on a silica column developed with hexane–isopropanol–methanol (87:10:3, v/v/v) at a flow-rate of 1.5 ml/min.

Fig. 12. Chromatogram from a purified serum extract showing elution positions of ercalcidiol (25-OHD$_2$), calcidiol (25-OHD$_3$) and *trans*-calcidiol (internal standard). HPLC was carried out on a Zobax-Sil column developed with hexane–isopropanol (98:2, v/v) at a flow-rate of 2.0 ml/min. Detection was by UV absorbance at 254 nm. The serum sample came from a patient treated with vitamin D_2. From ref. 198, with permission.

by injecting a reference standard in conjunction with UV detection, or its radiolabelled equivalent. Care should be taken not to contaminate the 1,25-(OH)$_2$D fractions with other vitamin D metabolites, especially the closely eluting 25,26-(OH)$_2$D, which exhibits a high degree of cross-reactivity.

Normal-phase systems are used by many investigators for the quantitative determination (with UV detection) of total 25-OHD [117,153,183,192,197] or the separate determination of 25-OHD$_2$ and 25-OHD$_3$ [123,133,137,181,182,198], as shown in Fig. 12. To obtain a clean chromatogram, this HPLC system is often preceded by another normal-phase system or by reversed-phase chromatography.

4.4.1.2. Bonded-phase chromatography

4.4.1.2.1. Normal phase. Nitro polar bonded phases (e.g., Nucleosil 10 NO_2) [151] may be a substitute for silica as column material. This approach in conjunction with a step gradient deserves special mention. In this procedure, elution of the column with pure hexane removes non-polar lipids while retaining the vitamin D metabolites. These in turn are eluted using hexane–isopropanol–water mixtures and quantitatively determined in a radioligand assay.

4.4.1.2.2. Reversed phase. Several reasons underly the limited popularity of reversed-phase chromatography in the vitamin D area. First, the resolving power of reversed-phase chromatography for the dihydroxy metabolites is inferior to that of normal-phase chromatography. Further, combination with a radioligand assay requires the evaporation of semi-aqueous solvent mixtures, which is inconvenient. Finally, the presence of dissolved oxygen in the water may cause oxidation of the labile vitamin D molecule [115].

As mentioned above, the technique remains valuable for the resolution of vitamins D_2 and D_3, as part of their separate determination in plasma or serum. Both C_{18} (Zorbax-ODS, Ultrasphere-ODS, Hypersil-ODS) [129,136,138,150, 161,169,183] and C_8 (Ultrasphere-octyl) [152] columns have found application, with water–methanol or water–acetonitrile as mobile phases. However, the presence of an excess of interfering UV-absorbing compounds in biological extracts prevents the use of a single reversed-phase system for the quantitative determination of vitamins D_2 and D_3. In practice, the actual separation is always preceded by a normal-phase clean-up system [129,136,138,150,161,169,183]. Fig. 13 represents a chromatogram of a serum extract obtained with this multi-dimensional approach.

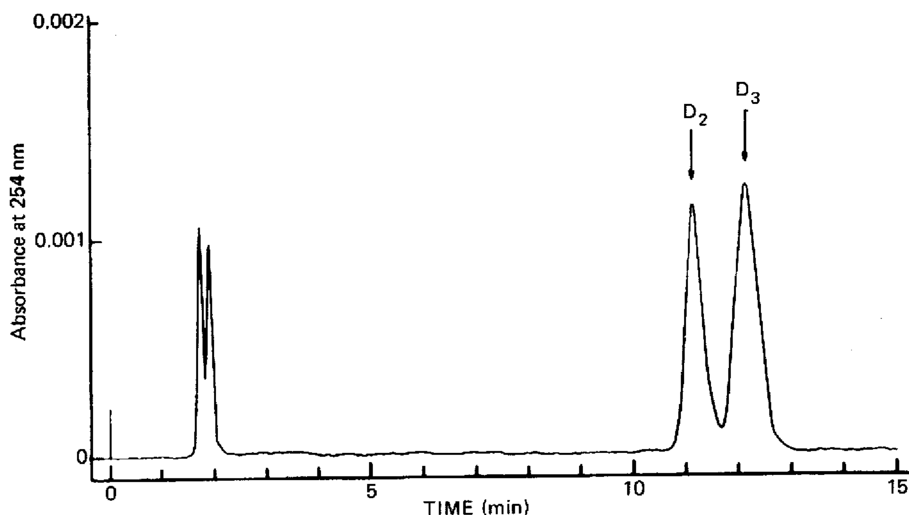


Fig. 13. Separation of vitamins D_2 and D_3 (standards) on a reversed-phase Zorbax-ODS column eluted with methanol-water (98:2, v/v) at a flow-rate of 1.5 ml/min. From ref. 129, with permission.

4.4.2. Detection

Until recently, on-line detection of vitamin D and its metabolites in LC has relied on their UV-absorbing properties (λ_{max}=265 nm). Although the corresponding molar absorptivities are low (ϵ=17 500 l mol^{-1} cm^{-1} at 265 nm or 15 000 l mol^{-1} cm^{-1} at 254 nm for vitamin D and 18 000 l mol^{-1} cm^{-1} at 265 nm or 16 900 l mol^{-1} cm^{-1} at 254 nm for 25-OHD), both fixed- (254 nm) and variable-wavelength detectors provide sufficient sensitivity to detect selected derivatives (see above) in plasma or serum. In contrast, the optimal wavelength of detection does not ensure the selectivity required to distinguish vitamin D and 25-OHD from an excess of UV-absorbing co-extractants, hence the need for a multi-dimensional approach, as discussed earlier, to remove these interferences.

Both the sensitivity and the specificity of the assays can be significantly improved by converting vitamin D and 25-OHD to ITS derivatives. This derivatization results in a bathochromic shift of the absorption maximum and an overall gain in sensitivity by a factor of approximately 2. Several assays employ this derivatization for detection at either 290 nm [146,201] or 301 nm [185,188].

Vitamin D and its metabolites do not exhibit native fluorescence but are electrochemically active. The latter property has recently been exploited in the development of a serum assay for 25-OHD_3, based on reversed-phase chromatography and amperometric detection [153]. The detection limit was estimated to be 1 ng of 25-OHD_3 (absolute amount injected).

5. VITAMIN E

5.1. Background

Vitamin E is a collective term for tocopherols and tocotrienols, a series of potent antioxidants chemically derived from 6-chromanol [202]. The structural difference between the two groups of compounds lies in the degree of saturation of an isoprenoid side-chain [202]. Tocotrienols and tocopherols are abundant in seeds, grains and vegetable oils [203], but only tocopherols are absorbed to an appreciable extent by humans [203]. Individual tocopherols, i.e., α-, β-, γ- and δ-tocopherol, are distinguished by the number and the position of methyl substituents on the chromanol nucleus (Fig. 14).

The vitamin E nutritional status in man is primarily determined by α-tocopherol, biologically the most active homologue [203] and the predominant form in plasma (88%) [203,204]; β- and γ-tocopherol contribute only 2 and 10%, respectively, to the vitamin E plasma levels [204], and the δ-form is detected only after massive ingestion of, e.g., soybean fat emulsions [205]. Transport of tocopherols in plasma occurs by β-lipoproteins [206].

Although the exact biochemical function of vitamin E is controversial [203], α-tocopherol derives a significant part of its biological activity from its antioxidant properties [203,207,208]. As a free radical scavenger it protects membrane lipids from peroxidation [207,208]. Free radical mediated peroxidative processes have been linked to ageing, the onset of heart disease and cancer and the toxicity of xenobiotics [209]. Vitamin E deficiency states are associated with a number

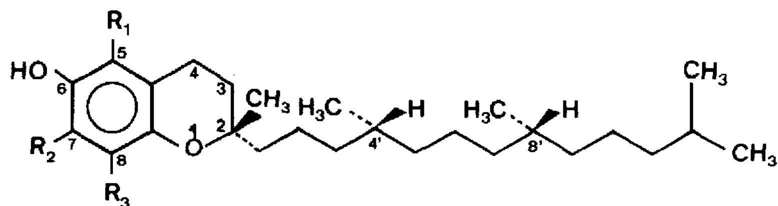


Fig. 14. Structures of tocopherols. The stereochemical notation only pertains to the natural α-, β- and γ-tocopherols (2*R*,4′*R*,8′*R* configuration).

Compound	R_1	R_2	R_3
α-Tocopherol	CH_3	CH_3	CH_3
β-Tocopherol	CH_3	H	CH_3
γ-Tocopherol	H	CH_3	CH_3
δ-Tocopherol	H	H	CH_3
5,7-Dimethyltocol	CH_3	CH_3	H
Tocol	H	H	H

of pathological conditions, including bronchopulmonary dysplasia, retrolental fibroplasia, haemolytic anaemia in premature infants, malabsorption syndromes caused by bowel and pancreatic diseases and various neuromuscular disorders [210–212]. Hence close monitoring of tocopherol levels is desirable in certain groups of patients.

The appropriateness of α-tocopherol plasma levels as an indicator of vitamin E nutritional status is, however, increasingly under debate [213]. Rather than the plasma concentration the tocopherol/lipid ratio could be a more reliable index [213]. Alternatively, the tocopherol levels in biomembranes, e.g., erythrocytes and platelets, would even better reflect the vitamin E nutritional status, as they are independent of plasma lipid [213]. Likewise, a direct determination in tissues is equally relevant. For example, abetalipoproteinaemia has been monitored by measuring α-tocopherol in adipose tissue biopsies [214]. Although many assays focus on α-tocopherol only, the determination of the γ/α-tocopherol index in plasma may be a valuable index for assessing compliance in trials involving supplementation with α-tocopherol [215].

The simultaneous monitoring of plasma vitamin A and E levels is meaningful in view of the purported interaction between the two vitamins [216]. In addition, studies on the possible chemopreventive action of isoprenoid compounds on carcinogenesis [217] warrant the determination of retinol, α-tocopherol and β-carotene and other carotenoids.

There is current interest in the biological fate of α-tocopherylquinone (α-TQ), a potential metabolite of α-tocopherol [203]. Although its authentic presence in vivo is still uncertain, this compound may be considered to be an indicator of oxidative stress on the cellular level [218]. In vitro exposure of erythrocytes to peroxide readily generates the quinone from α-tocopherol [218]. Both the quinone and α-tocopherol are sometimes determined concurrently with ubiquinones and ubiquinols, another class of redox-active compounds [219]. However, unlike the assays of α-tocopherol, retinol and β-carotene, the determination of quinones

is not yet part of routine clinical chemistry practice but remains restricted, for the time being, to fundamental biochemical studies.

5.2. Isolation, extraction and clean-up

In principle, any lipid extraction procedure should be useful for the isolation of tocopherols from a biological matrix. Tissues and faeces are homogenized in an aqueous or semi-aqueous medium prior to extraction [214,221,227] or in the extracting solvent itself [228–230]. Erythrocytes and platelets are processed as suspensions. During sample preparation, precautions should be taken to protect the labile tocopherols against oxidative destruction, which is readily catalysed by heat, light, alkaline pH conditions and metal ions [203,220]. As a rule, GC assays of tocopherols require more extensive clean-up schemes than their LC counterparts, including saponification, removal of cholesterol and derivatization [220]. In many GC systems cholesterol coelutes with α-tocopherol. Unless a highly specific detector is used (see Section 5.3.2), this interference has to be exhaustively removed using such approaches as digitonin precipitation, TLC and open-column chromatography [220]. A complementary TLC step may also serve the function of separating β- and γ-tocopherols [220]. Except when containing special binary stationary phases, packed GC columns indeed fail to distinguish between the two positional isomers [220].

The introduction of modern LC on microparticulate materials in connection with absorbance, fluorescence or electrochemical detection has tremendously simplified sample pre-treatment. This area has recently been reviewed [231]. A typical analysis scheme consists in a protein precipitation step using ethanol or methanol, lipid extraction with hexane or heptane, evaporation of the organic layer to dryness, reconstitution of the residue with a solvent compatible with the chromatography and injection of an aliquot on to the LC column [27, 40, 42, 43, 46–48, 69, 86, 204, 205, 215, 221–223, 225, 232–241].

In some instances, chloroform is preferable as an extraction solvent, e.g., as part of a Bligh–Dyer extraction [39,242,243]. Saponification is generally omitted but may remain useful for the analysis of tissues [214,244,245], erythrocytes [213,245] and, according to a few workers, plasma [213,244,245]. There is indeed evidence indicating that the amount of co-existing fat considerably affects the recovery of tocopherols from tissues [245].

The addition of antioxidants, although a common practice [46,219,221,226,232,235], has little effect on the recovery of tocopherols from human plasma [50,234], unlike that from rat plasma [234]. In contrast, the use of pyrogallol or ascorbic acid is essential, in connection with the analysis of red blood cells, to prevent dramatic losses of tocopherols due to interaction with co-extracted, iron-containing pigments [213,224,238,243,245]. This risk is allegedly minimized in the method of Burton et al. [224], which utilizes pre-treatment of the matrix with sodium dodecyl sulphate (SDS) prior to extraction with ethanol–heptane. The use of SDS makes membrane lipids more amenable to extraction and results in colourless, cleaner extracts, even from erythrocytes. Burton's et al. [224] improved method, originally devised for plasma, erythrocytes and liver

homogenates, has been adopted by other workers, specifically for the analysis of plasma [219,226] and muscular tissue [225]. In recent years there has been a trend towards simplifying the sample pre-treatment in order to increase the sample throughput in routine analysis. Various workers have attempted to eliminate the evaporation step by using lipid-extracting solvents which are directly compatible with the chromatographic system. If hexane is retained as an extraction solvent, normal-phase chromatography is indicated [41,44,246], although there is one report of the use of a reversed-phase column, operated in a normal-phase mode [244]. Extracts in butanol–ethyl acetate (1:1, v/v) have been directly injected onto reversed-phase columns without adversely affecting the performance [50,227].

Alternatively, a monophasic extraction may be performed using acetone [228], isopropanol [49], ethanol [247] or methanol [242]. These water-miscible solvents precipitate the proteins and, at the same time, extract the tocopherols in the supernatant. However, it should be realized that methods involving no concentration (evaporation) step put more stringent demands on the detector sensitivity. Consequently, the use of fluorescence [242] or electrochemical detectors [247] is indicated in order to keep the sample size as low as possible. Thus the sample volume has been reduced to 10–50 μl, which is particularly interesting in paediatric clinical chemistry. Lehmann and Martin [242] extracted 50 μl of serum with 3 ml of methanol and injected 75 μl of the supernatant, the equivalent of as little as 1.2 μl of serum.

Apart from extra detector sensitivity, a monophasic extraction also requires enhanced selectivity. The complexity of a "total" extract of plasma in acetone can be judged from Fig. 15 (top). The large extraneous peak was absent from extracts obtained with hexane (Fig. 15, bottom). Repeated injection of such extracts is likely to impair the column lifetime. Care should also be taken not to inject extracts in solvents which are stronger than the mobile phase. The poor peak shape and/or low efficiency resulting from the injection of extracts in acetone [228] (Fig. 15) or ethanol [247] may be rationalized on this basis.

The merits of an I.S. used to compensate for analytical variability are generally acknowledged. However, many vitamin E assays do not involve internal standardization [41, 44, 49, 214, 219, 221, 223, 226, 233, 239, 244, 247]. One of the reasons for this neglect is the non-availability of an "ideal" structural analogue of α-tocopherol. So far tocol seems to be the most appropriate choice [39, 43, 48, 69, 205, 213, 215, 235, 243, 245, 248], although its structure differs from that of α-tocopherol by three methyl groups. Because of this substantial difference in lipophilicity, both compounds elute too remote from each other in some chromatographic systems [248]. In addition, tocol is believed to be unstable during saponification and is poorly extracted in the presence of an excess of lipids [245]. The synthetic 5,7-dimethyltocol would be a superior alternative [222] but, unfortunately, this compound is very difficult to acquire.

A readily available vitamin E derivative is α-tocopheryl acetate, which is frequently used for internal standardization [42,43,228,230,232,238]. However, the use of this compound is restricted to reversed-phase chromatography with UV detection, because it is neither fluorescent nor electrochemically active and elutes

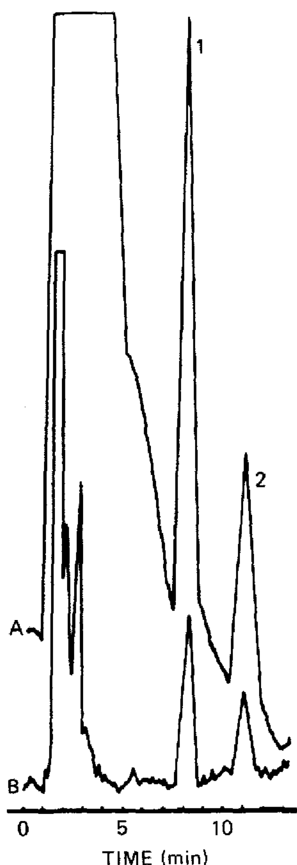


Fig. 15. Chromatograms of rat plasma obtained with a monophasic extraction approach (acetone) (top) and a biphasic extraction procedure (ethanol-hexane) (bottom). Column, 10-μm LiChrosorb RP-18 (25 cm × 0.46 cm I.D.); eluent, methanol-water (98:2, v/v); flow-rate, 2 ml/min; detection, 292 nm. Peaks: 1 = α-tocopherol; 2 = α-tocopheryl acetate. From ref. 228, with permission.

virtually unretained in normal-phase systems. δ-Tocopherol has been suggested as an I.S., mainly for assays based on LC with ED [237,240], but, as mentioned above, this compound may occur in plasma under certain conditions. For the simultaneous determination of vitamins A (retinol) and E, most investigators currently use the commercially available retinyl acetate as an internal standard [27,40,42,46,47,86,234], sometimes in combination with α-tocopheryl acetate [42] or, as an alternative to either of those, tocol [69].

5.3. Gas chromatography

5.3.1. Chromatographic systems

In recent years LC has totally superseded GC as a routine technique for the determination of tocopherols in biological materials. Since 1980 more than 50 papers have reported biomedical applications of LC in the vitamin E area, whereas only a few papers have dealt with GC methods. The merits and shortcomings of

GC for vitamin E analysis have been extensively reviewed previously [220]. Major efforts were directed in the past towards separating positional isomers and eliminating the interference of cholesterol (see Section 5.2). The former has been accomplished using packed columns with special binary liquid phases or, more conveniently, using capillary GC.

Derivatization of tocopherols to TMS ethers, acyl esters or rarely quinones generally improves their GC properties. Recent (1980) biomedical GC methods describe the determination of α-tocopherol in plasma [249], red blood cells [250,251], liver [252] and lung tissue [253] (see Section 5.3.2).

5.3.2. Detection

GC vitamin E assays using packed columns and FID or ECD are gradually becoming obsolete. The persisting value of GC for vitamin E determination rather lies in its excellent compatibility with MS. Up to now there have been no reports on the LC–MS separation of tocopherols. GC–MS continues to yield unsurpassed sensitivity and selectivity. Thus, Thomas et al. [253] exploited these properties to determine picogram amounts of α-tocopherol in exceptionally small samples (5–10 μg) of lung tissue. Masses 502 and 515, being the molecular ions of the TMS ethers of α-tocopherol and the I.S. d_{13}-deuterated α-tocopherol, respectively, were monitored. The extraction procedure was extremely simple in that it was performed with the silylating agent itself, added directly to the dried tissue sample. Despite the absence of a clean-up procedure, the large excess of TMS-cholesterol, coeluting with TMS-α-tocopherol, did not interfere, owing to the high specificity of the MS detector.

5.4. Liquid chromatography

5.4.1. Chromatographic systems

Normal- and reversed-phase systems have their own specific applications in vitamin E chromatography. Homologues are well separated on reversed-phase columns, whereas the resolution of the positional isomers β- and γ-tocopherol requires a normal-phase procedure. Traditionally, silica was used for this purpose, but in recent years more stable polar bonded phases have come to replace it as a support. For a review of the literature before 1980 we refer to the above-quoted paper [220].

5.4.1.1. Adsorption chromatography

Pioneering work on the resolution of β- and γ-tocopherol by adsorption chromatography on silica was conducted in the late 1970s. Standard eluents reported for this particular separation were hexane–diisopropyl ether or, alternatively, hexane–isopropanol or hexane–ethanol [220]. Similar eluent combinations continue to be used in more recent procedures [44,215,221,239]. However, because of the limited relevance of β-tocopherol and its negligible content in plasma, there is rarely a real need for its separation from γ-tocopherol. Therefore, most LC methods for vitamin E use reversed-phase chromatography, in which β- and γ-tocopherol coelute as one peak, yet distinctly resolved from α-tocopherol. However, there may be a place for adsorption chromatography in vitamin E analysis.

Regardless of its separating capacity for positional isomers, some investigators prefer normal-phase systems because of their compatibility with the injection of biological extracts in hexane [44]. Increasingly, however, bonded phases are tending to replace silica in this respect [246].

5.4.1.2. Bonded-phase chromatography

5.4.1.2.1. Normal phase. The four natural tocopherols can be conveniently separated on amino bonded phases [205,213,222,245] using the same mobile phases as previously used on silica. One assay of vitamins A and E in serum was based on a cyano column and hexane–isopropanol as the eluent [246]. As mentioned above (see Section 5.4.1.1), the main rationale for this system was its compatibility with the injection of serum extracts in hexane. For the same reason, a Partisil PXS reversed-phase column was eluted with a similar solvent mixture [244], most peculiar for a reversed-phase material. Obviously this column can be used in a normal-phase mode because it is non-end-capped and therefore contains accessible polar sites. However, the use of hexane is likely to modify the bonded phase, thus making the column unsuitable for subsequent "real" reversed-phase work.

5.4.1.2.2. Reversed phase. More than 90% of all current vitamin E assays in plasma/serum [27, 40, 42, 43, 46–50, 86, 215, 219, 223, 226, 228, 232, 234, 236–238, 240, 242], erythrocytes [238,243,245], platelets [242], faeces [227] and tissues [39, 214, 223, 228, 230, 241, 244, 245] are based on reversed-phase chromatography. The resolution of retinol, tocol, δ-tocopherol, $\beta+\gamma$-tocopherol, α-tocopherol and α-tocopheryl acetate, in this order of elution, appears to be straightforward on any C_{18} or C_8 reversed-phase column. Elution is commonly carried out with methanol–water mixtures. The simultaneous chromatography of retinol, α-tocopherol and carotenoids usually requires stronger, non-aqueous mobile phases, e.g., methanol [86] or mixtures of methanol–acetonitrile with chloroform or dichloromethane [27,43]. Gradient elution, often starting from semi-aqueous conditions, was found to be necessary in some instances [27], particularly in connection with poorly retentive polymeric phases [69]. Recent improvements in the reversed-phase separation of tocopherols are concerned with low-dispersion chromatography using 3-μm particles [86,241] and/or shorter columns [48,86,240,241,247]. These faster and more sensitive systems (see Fig. 9) supersede the older procedures, which were frequently based on the popular 30 cm$\times$0.39 cm I.D. 10-μm μBondapak column [40, 42, 50, 214, 227, 232, 234, 242].

5.4.2. Detection

In keeping with tradition [220], absorption and fluorescence detection continue to form the backbone of LC assays of vitamin E. However, ED is now steadily advancing. Absorption is monitored at 292 nm, the λ_{max} of α-tocopherol [40,228,234,238], or, alternatively, if a fixed-wavelength detector is used, at 280 nm [42,50,222,230,232,235]. Despite operating at a non-optimal wavelength, the latter generally affords comparable sensitivity because of better signal-to-noise ratios and increased stability. Minimal detectable amounts of α-tocopherol in absorption detection are roughly of the order of 30–60 ng. Better figures could

still result from low-dispersion chromatography (see above). A representative chromatogram of a plasma extract with detection at 292 nm is shown in Fig. 16.

When increased sensitivity and/or selectivity are required, fluorescence detection is a straightforward choice. Tocopherols exhibit native fluorescence (λ_{exc} 205 and 295 nm, λ_{em} 330 nm). Working at the lower wavelength of excitation, a concept originally proposed by Hatam and Kayden [254], entails improved sensitivity, obviously at the expense of selectivity. This approach has found a limited follow-up [221,244] in that most LC determinations (with fluorescence detection) of vitamin E in plasma [41, 44, 205, 213, 215, 239, 242, 245], erythrocytes [243], platelets [242] and tissues [39,228, 244] utilize the long wavelength of excitation (ca. 295 nm). The method of Lehmann and Martin [242] stands out as a model example of the superior performance of fluorescence detection.

As discussed above (see Section 5.2), the exceptional sensitivity (detection limit 0.1 ng of α-tocopherol) and selectivity of this technique permit a mono-

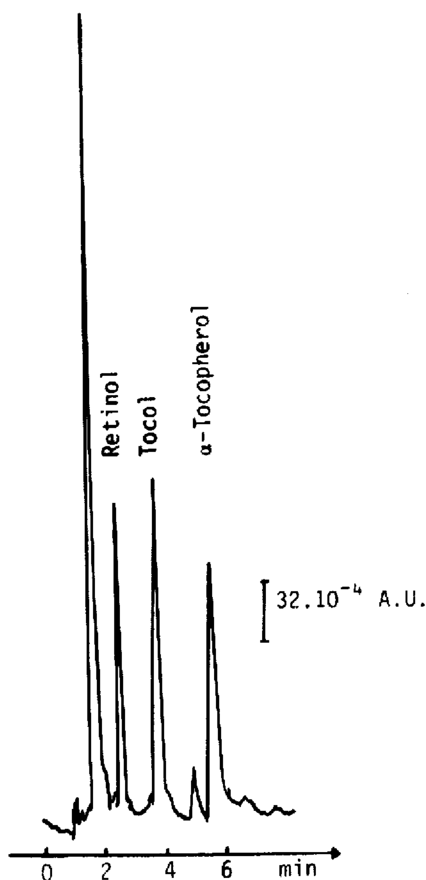


Fig. 16. Chromatogram of a plasma extract from a normal subject. Column, 10-μm RP-18 (RSL) (25 cm × 0.46 cm I.D.); eluent, methanol; flow-rate, 2 ml/min; detection, 292 nm. The small peak before α-tocopherol is probably due to $\beta+\gamma$-tocopherol (from V.O. De Bevere and A.P. De Leenheer, unpublished work).

phasic extraction, without clean-up, of as little as 50 μl of plasma. Fig. 17 illustrates the patterns of extracts of plasma and platelets obtained with this technique. The chromatogram of human plasma (left) compares favourably with one obtained on (rat) plasma using another monophasic extraction method (acetone) but with UV detection at 292 nm (Fig. 15, top). From this figure, the value of a double-phase extraction (Fig. 15, bottom) in connection with a poorly selective detection technique can be appreciated.

Because of the different spectral properties of vitamins A and E, their simultaneous monitoring poses additional problems. At 325 nm, the λ_{max} of retinol, α-tocopherol is non-absorbing, whereas retinol does not fluoresce under conditions set for α-tocopherol. However, retinol does absorb light at the λ_{max} of α-tocopherol. In order to obtain maximum sensitivity for both compounds, the detector wavelength must be switched during the run from 325 to 292 nm. This can be done either manually [49,246] or, more conveniently, using a programmable in-

Fig. 17. Chromatogram of extracts of plasma (left) and platelets (right). Column, 10-μm μBondapak C_{18} (30 cm $\times$ 0.39 cm I.D.); eluent, methanol-water (98:2, v/v); flow-rate, 1.4 ml/min; detection, fluorescence (Perkin-Elmer 650-10S), λ_{exc} 292 nm, λ_{em} 325 nm. Peaks: 1 = γ-tocopherol; 2 = α-tocopherol. From ref. 242, with permission.

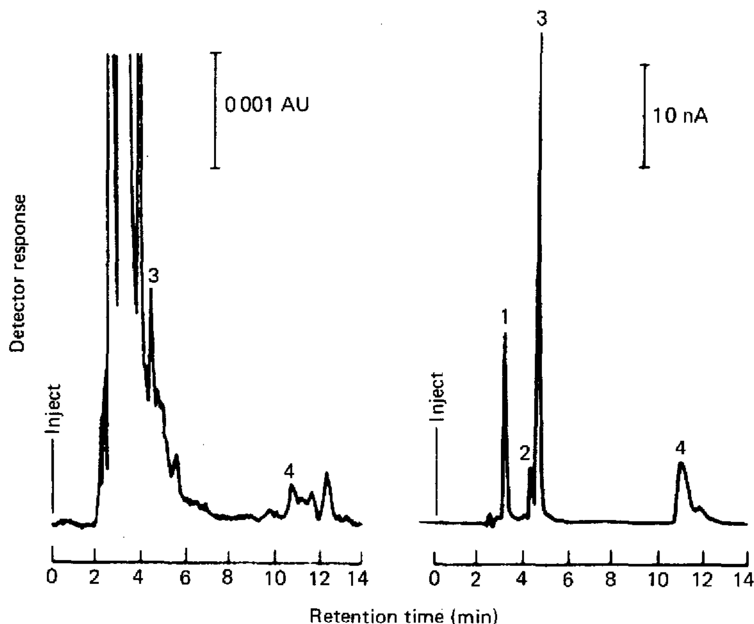


Fig. 18. HPLC profile of human blood obtained with (left) UV and (right) electrochemical detection. Column, 5-μm Ultrasphere ODS (25 cm × 0.46 cm I.D.); eluent, methanol–ethanol (1:9, v/v) containing 20 m*M* lithium perchlorate; detection, ED (BAS LC 4B), +0.50 V. Peaks: 1 = BHT; 2 = $\beta+\gamma$-tocopherol; 3 = α-tocopherol; 4 = unknown. From ref. 226, with permission.

strument [69], preferably a multi-channel photodiode array detector [43,86]. A third wavelength of 450 nm can be included to detect β-carotene [27,43,86].

Alternatively, the absorbance can be monitored at a single wavelength, i.e., 280–300 nm [40,42,50,232,234,246] (with some loss of sensitivity for retinol) or using a dual-channel detector at two separate wavelengths [27,47]. Russell et al. [46] linked a UV and a fluorescence detector in series. Time switching between both detectors afforded optimal sensitivity for retinol (absorbance detection at 325 nm) and α-tocopherol (fluorescence detection). The use of a programmable fluorescence detector permits the simultaneous monitoring of both retinol and α-tocopherol at their respective optimal wavelengths of excitation and emission [44].

ED of vitamin E was pioneered in 1979 by Ikenoya et al. [255]. They described a reversed-phase system with sodium perchlorate as a supporting electrolyte in a non-aqueous mobile phase. Using amperometric detection, picogram amounts of tocopherols could be determined in plasma. Recently, a detection limit of 1 fmol (0.4 pg) was reportedly achieved [241]. This unprecedented sensitivity permits the handling of very small samples (down to 10 μl of plasma or even less) [240,247] and the use of simple sample pre-treatment schemes, e.g., monophasic extractions [247].

Most systems are based on similar chromatographic conditions to those described by Ikenoya et al. [255], using sodium perchlorate [48,229,237,241,245], lithium perchlorate [219,226] or tetraethylammonium perchlorate [247] as elec-

trolyte. Others preferred sodium acetate [240], ammonium acetate [69] or tetraethylammonium acetate [256] and found the pH of the eluent to be either critical [240] or unimportant [69] for optimal sensitivity. Both amperometric [48, 69, 219, 226, 229, 237, 240, 241, 245] and coulometric [225,247,256] detectors have been used, operated in the oxidation mode to detect tocopherols in extracts of plasma/serum [48, 69, 219, 226, 237, 240, 245, 247], erythrocytes [245] and tissues [219, 225, 226, 229, 241, 245, 256], including biopsies [225]. The major strength of ED lies in its capability to detect α-TQ and other quinones also [219,225,226,229,241,256]. The levels of α-TQ in biological material are generally too low to allow UV detection. Because the compound does not fluoresce, ED is the only useful alternative.

For a concurrent determination of tocopherols and quinones, a dual electrode set-up is required [225,241,256]. The first electrode, at a negative potential, reduces the quinones whereas the second electrode, carrying a positive voltage, oxidizes the corresponding reduction products in addition to the native tocopherols. A recent method involving the simultaneous ED of retinol, tocopherols and β-carotene [69] has been discussed in Section 3. In addition to unsurpassed sensitivity, ED also yields extra selectivity. This is exemplified in Fig. 18, which represents HPLC profiles of whole blood obtained with UV detection and ED, respectively. In the UV trace, the α-tocopherol peak is barely visible. From the above examples it is clear that ED is becoming a powerful analytical tool in vitamin E research. The stability, low price and convenience of modern apparatus even make ED attractive as a routine technique in clinical chemistry.

6. VITAMIN K

6.1. Background

Vitamin K was discovered by Henrik Dam in an attempt to demonstrate the possible essential requirement of cholesterol in the diet of the chick. He described haemorrhagic symptoms in chicks fed with an ether-extracted diet [257]. The antihaemorrhagic fat-soluble compound was called vitamin K_1 or phylloquinone and was identified as 2-methyl-3-phytyl-1,4-naphthoquinone. Later, other forms of the vitamin were also demonstrated, all of them containing an unsaturated side-chain. This group of multiprenylmenaquinones was called vitamin K_2. The parent compound of this series, 2-methyl-1,4-naphthoquinone or menadione, also showed biological activity and is now widely used as a supplement in animal feeds [258]. The chemical structures of the different vitamins K are shown in Fig. 19.

After the characterization and synthesis of vitamin K, its mechanism of action was studied. It was shown that the synthesis of plasma prothrombin (factor II) and factors VII, IX and X was substantially depressed in vitamin K deficiency. Further studies demonstrated that vitamin K did not directly influence the de novo synthesis of these blood-clotting factors [259], but that it was necessary for the conversion of glutamyl residues to γ-carboxyglutamyl residues in these vitamin K-dependent blood-clotting factors II, VII, IX and X [260] and also in other discovered proteins (proteins C and S) [261,262]. The adjacent carboxyl groups

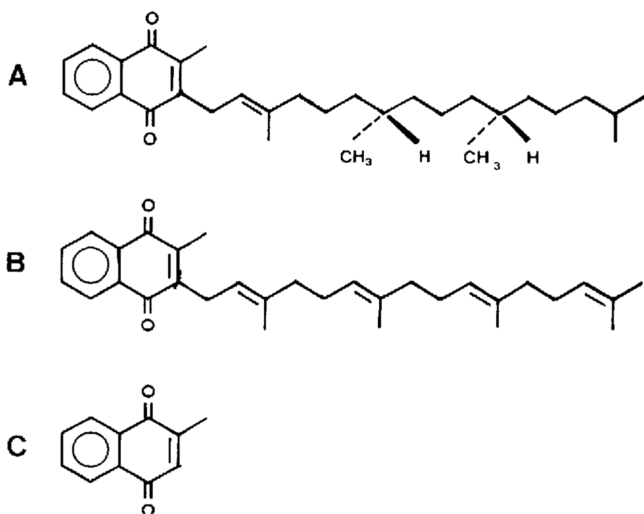


Fig. 19. Structures of (A) phylloquinone (vitamin K_1), (B) menaquinone (vitamin K_2) and (C) menadione (vitamin K_3). The particular form of menaquinone shown has four prenyl units, hence menaquinone-4.

of these γ-carboxyglutamyl residues provide the vitamin K-dependent proteins with characteristic calcium- and phospholipid-binding properties that are essential for their function.

The molecular role of vitamin K in the above carboxylation reaction has not yet been totally elucidated, however. Biochemical and metabolic studies on vitamin K require both highly sensitive and specific techniques. The presence of interfering lipids, the instability of the molecule and the extremely low physiological level render the quantitative determination a real analytical challenge and may be the cause of the still existing controversy over the exact endogenous level of vitamin K_1. The aim of this section is to evaluate some applications of chromatographic techniques such as GC and especially HPLC in clinical studies of vitamin K_1 in humans.

6.2. Isolation, extraction and clean-up

Almost all investigators, independently of each other, arrived at similar experimental designs for the assay of vitamin K_1 in biological samples. First, one should avoid strong light and alkali and, as endogenous levels are in the picogram range, care should be taken to prevent contamination. In human blood vitamin K is transported by lipoproteins, especially by the very-low-density lipoprotein (VLDL) fraction [263]. When analysing this matrix, denaturation of the lipoproteins is necessary before vitamin K can be effectively extracted. Denaturation is mostly performed by adding double the amount of ethanol to the serum [264–271], while others have used methanol [272] or isopropanol [273] for this purpose. This denaturation significantly improves the recovery, and Langenberg [274] found the optimal concentations of methanol, ethanol and isopropanol to

be 80, 70 and 60%, respectively. The highest recovery was obtained with isopropanol; however, over 10% of isopropanol went over to the *n*-hexane layer used for extraction, thus increasing the evaporation time and coextracting many interfering polar compounds. In our hands the use of double the amount of ethanol resulted in the highest recovery. After denaturation, extraction of the vitamin is performed by either a monophasic extraction using methanol and chloroform [272] following Bligh and Dyer [275] or by a biphasic extraction with *n*-hexane in nearly all other instances [264–271,273]. Although the recovery with these methods is high, the selectivity is rather poor and many interfering lipophilic compounds are coextracted. This necessitates in almost all procedures two stages of HPLC in which the retention mechanisms are different and complementary.

To extract other liquid tissues, such as human milk, the method of Folch et al. [276] with chloroform–methanol (2:1, v/v) has been successfully applied [277]. Again, excessive amounts of polar lipids, especially phospholipids, are coextracted. In addition to the HPLC stages a preliminary purification of the crude extract was required in order to remove these interferents. This can be achieved by either conventional column chromatography on silica gel [277] or on commercial silica [269] or reversed-phase [272] cartridges. On a silica column the non-polar fraction and vitamin K are rapidly eluted whereas triglycerides, steroids and phospholipids remain on the silica. Alternative purifications have also been tried on hydroxyalkoxypropyl-Sephadex (HAPS) [278] and by enzymatic hydrolysis with lipase [279] for the analysis of human milk. All these purifications markedly reduce the amount to be fractionated by HPLC and prolong the column lifetime.

Before describing the final chromatographic steps, we should draw attention to the use of an I.S. A typical problem with a system with multiple chromatographic steps is the choice of an appropriate I.S. General requirements such as structural analogy, absence from the sample, similar stability and equal extraction recovery to the compound of interest must be fulfilled. However, while the I.S. should coelute with the compound of interest during all chromatographic purification steps, it must be separated in the final chromatography. Disregarding the addition of an I.S. [269,272] or choosing tocopheryl acetate [280] is unacceptable for this type of analysis. Structural analogues such as vitamin $K_{1(15)}$ [268], vitamin $K_{1(25)}$ [270,271], vitamin $K_{2(30)}$ [273], [^{3}H]vitamin $K_{1(20)}$ [265], dihydrovitamin $K_{1(20)}$ [281] and vitamin K_1 epoxide [266] are supposed to be excellent choices.

6.3. Gas chromatography

Most papers deal with the separation and quantitation of standard mixtures of vitamin K_1 and menaquinones, and are beyond the scope of this review. Only a few attempts to measure vitamin $K_{1(20)}$ in human biological matrices by GC have been reported. Two of the most obvious reasons for this relative unpopularity of GC are the long retention times and the on-column degradation of vitamin K-related compounds. Bechtold and Jähnchen [282] developed a GC method on a packed column without derivatization for the quantitation of vitamin K_1, vitamin

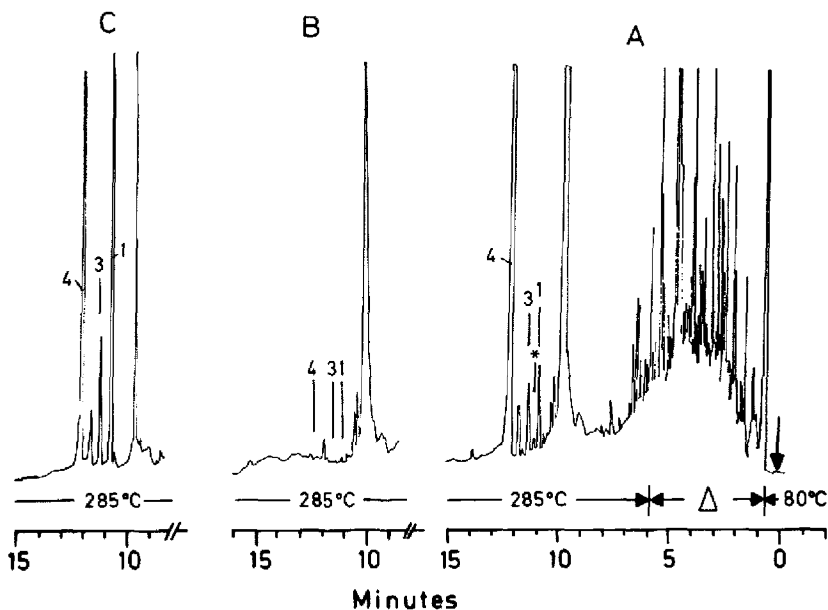


Fig. 20. Chromatograms of vitamin $K_{1(20)}$ (peak 3), $K_{1(20)}$ 2,3 epoxide (peak 1) and $K_{2(20)}$ (I.S.) from plasma extracts. (A) Extract of 0.4 ml of human plasma to which vitamin $K_{1(20)}$ (15 ng/ml) and *trans*-vitamin $K_{1(20)}$ 2,3-epoxide (15 ng/ml) and vitamin $K_{2(20)}$ (250 ng/ml) were added. (B) Blank extract of 0.4 ml of human plasma. (C) Extract of 0.4 ml of plasma from a patient under treatment with phenprocoumon, receiving 10 mg of vitamin $K_{1(20)}$ IV. From ref. 284, with permission.

K_1 epoxide and menaquinone-4 in plasma samples. They used a 3% OV-17 column operated at 302°C with ECD and did not observe any degradation. The high stationary phase load (above 3%) and omission of derivatization both seem to prevent degradation of the compounds [283]. In a more recent publication, Bechtold et al. [284] described the application of a fused-silica capillary column (25 m×0.32 mm I.D.) with a chemically bonded stationary phase (CP-Sil 5) operated at 285°C followed by ECD. They did not obtain a lower detection limit for vitamin K_1 in plasma than in the packed column analysis, but substantial chromatographic advantages were described, such as baseline separation of vitamin K_1 and the isomers of vitamin K_1 epoxide, longer column lifetime and better reproducibility (Fig. 20). Nevertheless, they were unable to quantitate endogenous vitamin K_1 levels in human plasma by this method.

6.4. Liquid chromatography

LC eliminates the risk of degradation of a compound by heat or light. The highly lipophilic and neutral character of the K vitamins renders them compatible with a wide range of organic solvents used as mobile phases in HPLC. Further, a high molar absorptivity allows sensitive photometric detection. The main advantages, however, of HPLC over other chromatographic techniques (e.g., TLC) lie in the combination of sensitivity and selectivity, resulting in a high degree of reproducibility and accuracy of the measurement. Excellent reviews on this sub-

ject have appeared [1,285]. In this section we shall make a critical evaluation of the published methodologies from the viewpoints of both its clinical applicability and its potential in future biomedical research.

6.4.1. *Chromatographic systems*

Although chromatography on open-column systems or on silica cartridges removes many less polar and more polar lipids from vitamin K_1, many compounds (including cholesteryl esters) still remain. Therefore, a procedure involving two sequential stages of HPLC is necessary in order to separate these interfering lipids from the vitamin. Irrespective of the final detection mode, almost all procedures for the quantitation of endogenous vitamin K_1 levels described in the literature used adsorption chromatography as the primary purification stage. The fraction containing the vitamin K-related compounds (and the I.S.) is then collected and further analysed on the reversed-phase system. It is highly recommended to use two separate LC systems, one for the adsorption and another for the reversed-phase mode. If only one system is available, extreme care should be taken not to contaminate the silica column with traces of methanol, as this would drastically change the retention behaviour of the compounds.

6.4.1.1. *Adsorption chromatography*

The interactions between a lipophilic molecule, such as vitamin K, and the polar silanol groups on the silica surface are very weak. Therefore, eluents of very low eluotropic strength should be used. The basic solvent is always a saturated hydrocarbon (*n*-hexane or isooctane [280]) moderated by the addition of a small percentage of a solvent of greater eluotropic strength such as 0.2% acetonitrile [267,286,287], 1% ethyl acetate [280] or 1.5 or 3% diisopropyl ether [265,270]. In 1982, Lefevere et al. [265] introduced on-line removal of the bulk lipids on a guard-column. Lipids eluting before or after vitamin K were directed to waste and during the silica chromatography of vitamin K itself, the guard column was back-flushed. This, however, requires long equilibration times and makes it less suitable for the routine analysis of large numbers of samples.

All silica columns used in the first HPLC stage have semi-preparative dimensions, allowing a greater sample load. From the silica column, a fraction containing both vitamin K_1 and the I.S. is collected. To avoid interfering peaks in the final chromatogram, the smallest possible volume should be collected. One should take care, however, not to lose one of the compounds of interest. Owing to the instability of retention times, especially in adsorption chromatography, blind collection by reference to retention times of pure standards should be carefully checked.

A better way is to relate the elution position of vitamin K_1 to the retention behaviour of UV-absorbing contaminants present in every sample. One can also add an external marker (e.g., chlorovitamin K_1) as an indicator for the collection of the vitamin K-related compounds [265]. Again, it should be emphasized that the I.S. and the compound of interest must elute very close to each other in the first HPLC system, allowing the collection of a small volume. As a consequence, analyses of this type are generally limited to a single vitamin K-related compound at a time. A single research group [272] is running the final analytical step on an

adsorption column after a Sep-Pak C_{18} purification. However, the chromatogram shown in their paper clearly demonstrates the need for further purification, even for the analysis of serum samples with a high vitamin K_1 content.

6.4.1.2. Bonded-phase chromatography

6.4.1.2.1. Normal phase. Chromatography of K vitamins on polar bonded phases (CN, NH_2 or diol) has been applied only once to biological samples of human origin. It gave similar results to those obtained by adsorption chromatography [277] (see Fig. 21). On standards it was seen that lower concentrations of the moderator were needed for the K vitamins while the elution order was the same as that for the parent underivatized silicas. Problems of reproducibility in preparing an eluent with a very low concentration of modifier (down to 0.05% for acetonitrile in *n*-hexane on a CN column) may be the cause of the unpopularity of these packing materials.

6.4.1.2.2. Reversed phase. Reversed-phase chromatography is the method of choice for the final step of vitamin K assays because it is the most effective way of separating vitamin K_1 from lipids with closely related polarities and from its own structural analogues [e.g., vitamin $K_{1(15)}$ or $K_{1(25)}$] used as an internal standard. Whenever possible, non-aqueous mobile phases (mixtures of methanol or acetonitrile and dichloromethane) should be used in combination with highly retentive columns. In these mobile phases vitamin K and the remaining coextracted lipids are freely soluble. Further, these non-aqueous eluents have a lower viscosity, resulting in lower operating pressures and extended column lifetimes.

Sometimes, incorporation of water may be necessary to dissolve the supporting electrolytes needed in ED [267] or electrochemical reactions [286]. Under these circumstances, the decrease in efficiency in a semi-aqueous medium is compensated for by the gain in selectivity obtained by ED. Haroon et al. [281] had to add water to the mobile phase of the final reversed-phase system to dissolve zinc

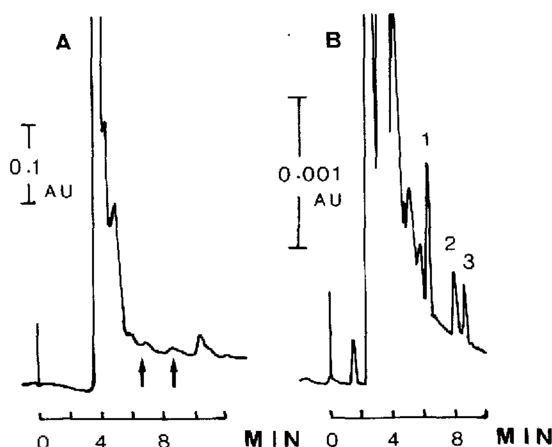


Fig. 21. (A) Semipreparative stage on Partisil 10 PAC with hexane–50% water-saturated dichloromethane (8:2, v/v) as mobile phase and UV detection at 254 nm. (B) Analytical reversed-phase stage on Zorbax-ODS with acetonitrile–dichloromethane (7:3, v/v) as mobile phase and UV detection at 270 nm. Peaks: 1 = vitamin K_1 epoxide (I.S.); 2 = chlorovitamin K_1 (marker compound); 3 = vitamin K_1. From ref. 227, with permission.

chloride as a reducing reagent. These derivatization techniques and special detection modes will be treated later. The reason why Abe et al. [264] incorporated up to 5% of water in the eluent is unclear, as their derivatization reagent, sodium borohydride gradually degrades in an aqueous medium. For the analysis of pharmacological levels of vitamin K_1, reversed-phase chromatography can be used without the prior adsorption chromatographic clean-up [271]. However, for quantitation of endogenous levels it was necessary to combine the reversed-phase with a detection mode more specific than UV absorption (e.g., electrochemical [269,273] or fluorescence detection [264]).

6.4.2. Detection

UV detection is by far the most commonly used detection mode in HPLC. Major advantages include the ease of operation, the high degree of reproducibility and its compatibility with most chromatographic systems. Limitations of UV detection are the lower sensitivity and selectivity. The naphthoquinone structure of vitamin K_1 with a high molar absorptivity ($\epsilon = 20\,000$ l mol^{-1} cm^{-1} at 248 nm) allows detection down to 500 pg. For the epoxide metabolite, however, the minimal detectable amount is three times higher.

The selectivity of UV detection at 254 or 248 nm is much less favourable, and consequently for the analysis of endogenous levels of vitamin K_1 in biological matrices complicated and extensive clean-up steps are necessary [265, 266, 268, 272, 277, 280, 286, 287]. In our opinion, UV detection is only suitable for quantitation of the high plasma levels after administration of pharmacological doses of vitamin K_1 [271,288].

The introduction of more sensitive and selective detection modes provided the opportunity for measuring vitamin K-related compounds in the low picogram range. In the late 1970s Ikenoya and co-workers [289,290] published an amperometric detection method for relatively high levels of phylloquinone. Reductive ED of endogenous levels of vitamin K_1 has been described by Ueno and Suttie [267], but practical problems such as oxygen interference and passivation of the working electrode resulting in sloping baselines and a decrease in sensitivity restricted the practical utility of reductive ED [291]. The introduction of dual-electrode ED eliminates oxygen interference, as reduction of this molecule is irreversible, and increases the sensitivity of the detection. In this procedure the compounds are first reduced at the first electrode and are then detected downstream at the second electrode (arranged in series) by re-oxidation. Increased sensitivity is also obtained by the use of porous, graphite electrodes, enabling a greater proportion of the analyte to be electrolysed and being less sensitive to passivation than thin-layer electrodes [292,293] (Fig. 22). Methods using the redox ED achieve detection limits of 50 pg/ml for vitamin K_1 using 2 ml of plasma, being at least one order of magnitude better than that for the reductive-mode ED.

In addition to UV and ED, fluorescence detection has also been applied in vitamin K analyses. However, vitamin K does not exhibit native fluorescence and only the fluorescence of reduction products (hydroquinone forms) or photodecomposition products can be monitored. Using a post-column photochemical reaction detection system, Lefevere et al. [294] were able to quantitate endogenous

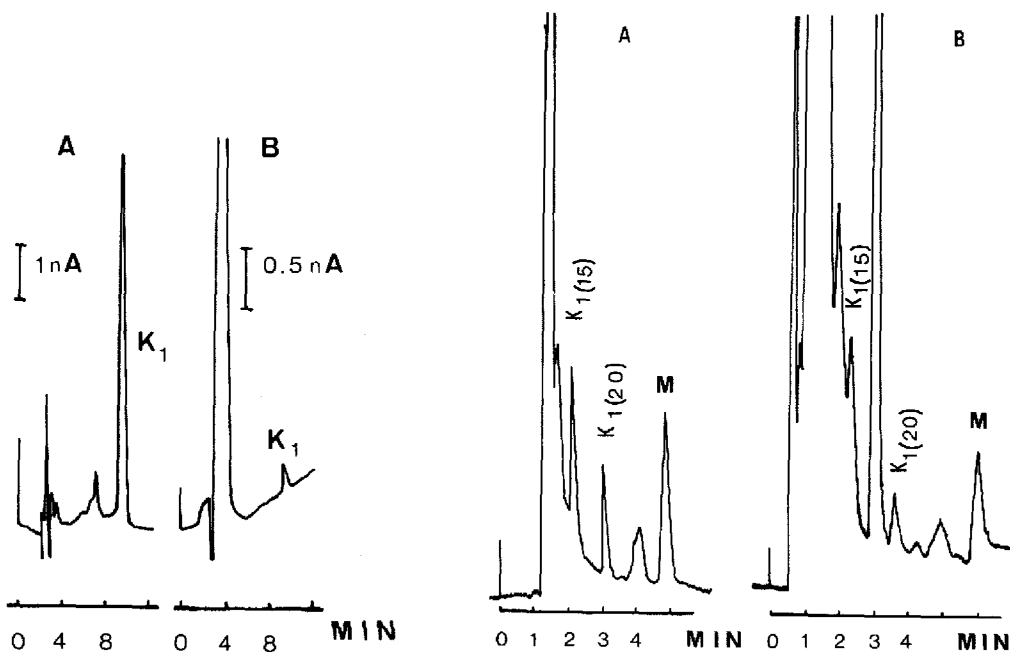


Fig. 22. Chromatograms obtained for 1-ng injections of phylloquinone using LC–ED in (a) redox mode and (b) reductive mode. From ref. 293, with permission.

Fig. 23. Comparison between (A) fluorescence and (B) UV detection for the determination of vitamin $K_{1(20)}$ in serum after multi-dimensional separation on silica and RP-18 columns. M is a marker compound, $K_{1(25)}$. From ref. 294, with permission.

vitamin K_1 levels, but not vitamin K_1 epoxide. Typical chromatograms are shown in Fig. 23. An alternative approach using a coulometric detector as a post-column reactor to reduce phylloquinone to the fluorescent hydroquinone has been successfully applied by several groups [269,273,286,294,295]. With a slight modification (incorporation of a potentiostat with higher capacity) Langenberg and Tjaden [297] were also able to quantitate vitamin K_1 epoxide in human plasma.

To minimize the amount of equipment required and to eliminate cell contamination problems, chemical reactions were also devised to reduce vitamin K_1 to its fluorescent hydroquinone form. With a post-column solid-phase reactor filled with zinc particles, Haroon et al. [281] reduced vitamin K_1 almost quantitatively (efficiency $>90\%$) without interference from oxygen (Fig. 24). Vitamin K_1 epoxide could also be measured successfully by this procedure. A wet-chemical reduction in a post-column reactor with sodium borohydride was described by Abe et al. [264]. However, they used water in the chromatographic eluent and the sodium borohydride reagent is known to decompose gradually both in ethanol and in water. To compensate for the loss in reactivity of the reagent, the use of an I.S. is highly recommended. By increasing the reaction temperature to 37°C and by performing the reaction in an air-segmented system, Lefevere et al. [294] were able to increase the sensitivity three-fold.

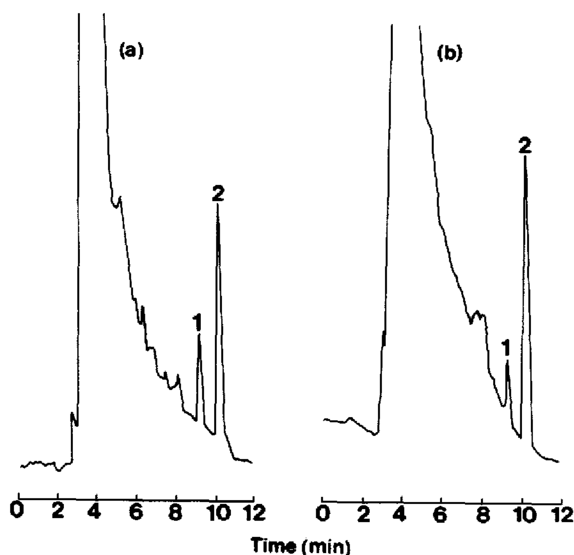


Fig. 24. Reversed-phase chromatograms for the assay of vitamin $K_{1(20)}$ in (a) pool plasma and (b) individual plasma sample. Peaks: 1 = K_1; 2 = dihydro-K_1 (I.S.). From ref. 281, with permission.

In our laboratory we developed a wet-chemical post-column reaction system with the totally new reagent, tetramethylammonium octahydridotriborate, $(CH_3)_4NB_3H_8$, which is more stable than sodium borohydride ($NaBH_4$) even in methanol. To avoid the problem of air segmentation with a debubbling system, we applied a knitted coil open-tubular reaction coil [298]. Owing to the special geometric configuration of this coil, no peak broadening occurs, even without air segmentation [299]. The reduction reaction takes place only at high temperatures (80°C), which allowed us to incorporate the reagent directly in the eluent. Both vitamin $K_{1(20)}$ and the I.S., vitamin $K_{1(25)}$, are chromatographed unreacted at room temperature, whereas both compounds are reduced in the post-column reaction. In this way we can eliminate the use of two different pumps and avoid long equilibration times [300].

Using either the two-pump or the one-pump system with $(CH_3)_4NB_3H_8$ as a reagent, we determined the reference interval for endogenous vitamin $K_{1(20)}$ in serum from healthy volunteers and from newborn babies [270]. With either electrochemical or fluorescence detection with wet-chemical reaction the reference intervals found for endogenous vitamin K_1 agreed very well [270,293] and were around 300 pg/ml in plasma. Suttie and co-workers [267,286] found a reference interval from 270 to 1950 pg/ml whereas procedures using UV detection clearly sufffered from interference, as they obtained mean levels of 2.4 or 2.6 ng/ml [265,272]. The individual levels did not show a Gaussian distribution but an apparent log-normal distribution [265,270]. In their paper, Ueno and Suttie [267] depicted a skewed distribution of serum vitamin $K_{1(20)}$ concentrations. However, they accepted a Gaussian distribution without any confirmatory test for it. In our opinion, both electrochemical and fluorimetric detection greatly increase the selectivity and the sensitivity for vitamin K-related compounds and, further, they

are less sensitive to interfering peaks. This has resulted in the use of smaller amounts of biological matrices, especially in the monitoring of vitamin $K_{1(20)}$ in newborn babies [301].

7. CONCLUSIONS

A critical survey of the recent literature reporting chromatographic assays for fat-soluble vitamins in biological materials of human origin confirms the previous trends [1] of the rapid advance of LC as a standard analytical technique. Most methods employ bonded phases, particularly reversed-phase materials. Smaller particles (3 μm) and shorter columns are being increasingly used to improve speed and sensitivity. Although absorbance and fluorescence detection can cover most applications, ED may be the ultimate answer to the demands of enhanced sensitivity and selectivity, the overall aim being the reduction of the sample size and the simplification of the sample preparation. With the present trends continuing, the value of GC has become virtually restricted to applications of GC–MS. The superior performance of this technique could be advantageously exploited for the future development of reference methods to validate the present LC determinations of fat-soluble vitamins.

8. SUMMARY

A review is presented of current gas and liquid chromatographic methods for the determination of the fat-soluble vitamins A, D, E and K and the provitamin A β-carotene in biological samples of human origin. For each vitamin, the discussion successively focuses on procedures for sample preparation, gas and liquid chromatographic systems and principles of detection. The emphasis is on liquid chromatography, which is gradually becoming a standard technique in fat-soluble vitamin assays. New trends in the liquid chromatography of these compounds include the use of smaller particles and shorter columns, to improve speed, and the advance of electrochemical detection as an alternative to absorbance and fluorescence detection. Bonded phases, both normal and reversed phase, tend to be preferred over underivatized silica as column supports. Gas chromatography remains of particular value in combination with mass spectrometry, a technique which may form the basis of reference methods. In general, despite the availability of well established analytical methods for fat-soluble vitamins, the wealth of recent literature in this area indicates that there continues to be a need for new assays with enhanced speed, specificity and sensitivity.

9. ADDENDUM: NORMAL VALUES FOR FAT-SOLUBLE VITAMINS

Tables 1–5 give endogenous and reference levels of the various vitamins in human plasma/serum.

TABLE 1

ENDOGENOUS RETINOL LEVELS IN HUMAN SERUM

Authors	Ref.	Sex*	Age (years)	Mean value (ng/ml)	S.D. (ng/ml)	n
Biesalski et al.	32			510	151	450
Vuilleumier et al.	41	M		663	123	75
		F		534	128	75
Hildebrandt et al.	49	M	0–15	459	173	40
		F	0–15	512	164	40
		M	16–35	849	195	27
		F	16–35	705	187	30
		M	36–60	973	174	29
		F	36–60	867	126	26
		M	61–90	937	169	28
		F	61–90	747	184	25
Rhys Williams	44			2.08 μmol/l	–	10
Cuesta Sanz and Santa-Cruz	47	M		630	100	22
		F		500	120	32
Speek et al.	30			0.79 μmol/l	–	429
Herbeth et al.	303	M		606	115	186
		F		456	90	173
Russell et al.	46			553	130	288

*M = male; F = female.

TABLE 2

SELECTED REFERENCE VALUES FOR β-CAROTENE IN HUMAN PLASMA/SERUM

Authors	Ref.	Sex	Mean value (μg/l)	S.D. (μg/l)	n
Driskell et al.	81		330	205	20
Vuilleumier et al.	41	M	315	155	75
		F	437	310	75
Katrangi et al.	82	M	331	191	8
		F	351	164	18
Nierenberg	94		274	103	17
Thompson et al.	85		151*	86*	34
			294**	194**	34
Milne and Botnen	86		182	84	12
Gatautis and Pearson	88	M	116	63	25
		F	154	104	25
Stacewicz-Sapuntzakis et al.	87	M	168	105	55
		F	237	147	55
Ito et al.	302	M	298	264	356
		F	616	396	567

*Non-smokers.
**Smokers.

TABLE 3

SELECTED REFERENCE VALUES FOR VITAMIN D AND METABOLITES IN HUMAN PLASMA/SERUM

Authors	Ref.	Assay*	Compound	Mean value	S.D.	*n*
Aksnes	161	A	Vitamin D_3	4.6 μg/l	3.5 μg/l	10
Seamark et al.	132	B	Vitamin D_3	8.1 μg/l	4.8 μg/l	15
Aksnes	161	A	25-OHD_3	23.0 μg/l	4.9 μg/l	10
	161	C	25-OHD_3	23.5 μg/l	5.2 μg/l	10
Delvin et al.	162	C	25-OHD_3	20.2 μg/l**	4.3 μg/l	40
Seamark et al.	132	B	25-OHD_3	17.9 μg/l	7.4 μg/l	18
	132	B	25-OHD_2	0.9 μg/l	0.2 μg/l	3
Parviainen et al.	150	C	25-OHD	19.4 μg/l	14.0 μg/l	41
Jongen et al.	151	C	25-OHD	20.5 μg/l***	6.6 μg/l	20
Parviainen et al.	123	A	25-OHD	21.7 μg/l	4.9 μg/l	16
Kohl and Schaefer	181	A	25-OHD	21.4 μg/l***	6.7 μg/l	20
	181	A	25-OHD	28.0 μg/l§	8.4 μg/l	18
Trafford et al.	146	A	25-OHD_3	23.9 μg/l§	11.0 μg/l	11
	146	A	25-OHD_2	4.4 μg/l§	1.3 μg/l	11
Keck and Krüskemper	135	C	25-OHD	12.2 μg/l§	5.9 μg/l	17
	135	C	25-OHD	3.3 μg/l***	0.9 μg/l	17
Turnbull et al.	185	A	25-OHD_3	20.1 μg/l	7.8 μg/l	24
	185	A	25-OHD_2	3.7 μg/l	1.6 μg/l	22
Imawari et al.	160	C	25-OHD	19 μg/l	5 μg/l	13
Loo and Brien	152	A	25-OHD_3	33.6 μg/l	6.2 μg/l	8
Bouillon et al.	147	D	25-OHD_3	37 μg/l	36 μg/l	55
	147	C	25-OHD_3	43 μg/l	43 μg/l	55
	147	A	25-OHD_3	34 μg/l	35 μg/l	55
Kao and Heser	192	C	25-OHD	18 μg/l	5 μg/l	34
	192	C	25-OHD_3	12.5 μg/l	4.4 μg/l	34
	192	C	25-OHD_2	5.9 μg/l	3.0 μg/l	34
Stern et al.	155	E	1,25-$(OH)_2D$	33.4 ng/l	6.4 ng/l	16
Aksnes	161	F	1,25-$(OH)_2D$	32.5 ng/l	8.5 ng/l	20
Mallon et al.	130	F	1,25-$(OH)_2D$	33.0 ng/l	7.9 ng/l	15
Peacock et al.	171	D	1,25-$(OH)_2D$	46.0 ng/l	12.2 ng/l	42
Bouillon et al.	180	D	1,25-$(OH)_2D_3$	38 ng/l	12 ng/l	54
Bishop et al.	164	F	1,25-$(OH)_2D_3$	36 ng/l	17 ng/l	35
Dabek et al.	119	F	1,25-$(OH)_2D$	44.2 ng/l	15.0 ng/l	39
Dokoh et al.	172	F	1,25-$(OH)_2D$	55.2 ng/l	13.6 ng/l	20
Jongen et al.	151	F	1,25-$(OH)_2D$	51.7 ng/l	10.8 ng/l	20
Gray et al.	121	D	1,25-$(OH)_2D_3$	57.3 ng/l	29.1 ng/l	26
Duncan et al.	167	F	1,25-$(OH)_2D$	34.7 ng/l	10.7 ng/l	20
Imawari et al.	160	F	1,25-$(OH)_2D$	37 ng/l	11 ng/l	13
Scharla et al.	178	D	1,25-$(OH)_2D_3$	55 ng/l	12 ng/l	30
Reinhardt et al.	191	F	1,25-$(OH)_2D$	37.4 ng/l	2.2 ng/l	22
Kao and Heser	192	F	1,25-$(OH)_2D$	25 ng/l	7 ng/l	34
De Leenheer and Bauwens	156	D	1,25-$(OH)_2D$	51.8 ng/l	15.2 ng/l	91
	156	F	1,25-$(OH)_2D$	53.9 ng/l	15.5 ng/l	46
Nicholson et al.	174	G	1,25-$(OH)_2D$	39.1 ng/l	10.4 ng/l	34
Hollis	196	F	1,25-$(OH)_2D$	28.2 ng/l	11.3 ng/l	29

*A = UV; B = MS; C = competitive protein-binding assay; D = radioimmunoassay; E = bioassay; F = radioreceptor assay; G = cytoreceptor assay.

**Children.

***Winter.

§Summer.

TABLE 4

SELECTED REFERENCE VALUES FOR α-TOCOPHEROL IN HUMAN PLASMA/SERUM

Authors	Ref.		Mean value (mg/l)	S.D. (mg/l)	*n*
Kato et al.	205		9.1	–	19
Leclercq and Bourgeay-Causse	232		13.2	3.2	74
Lehmann and Martin	242		8.0	2.1	10
			9.4	1.8	10
Vuilleumier et al.	41	Male:	13.1	3.2	75
		Female:	12.1	2.5	75
Willett et al.	217		12.6	0.4	210
Vandewoude et al.	237		12.1	4.1	25
Nierenberg and Lester	50		15.6	7.4	41
Mino et al.	213	Children:	6.8	0.1	261
		Adults:	10.0	0.7	26
Biesalski et al.	246	Male:	11.1	2.2	115
		Female:	11.1	2.2	63
Milne and Botnen	86		9.6	3.3	12
Cuesta Sanz and Santa-Cruz	47	Male:	11.8	2.3	22
		Female:	11.8	2.8	32

TABLE 5

ENDOGENOUS VITAMIN K_1 LEVELS IN HUMAN SERUM

Authors	Ref.	Mean value (pg/ml)	S.D. (pg/ml)	*n*
Shearer et al.	266	260	144	30
Lefevere et al.	265	3000	1700	40
		2600*	–	
Pietersma-de Bruyn and van Haard	272	2475	1440	25
Ueno and Suttie	267	1100	420	26
Langenberg	274	960	–	10
Lambert et al.	270	309	216	50
		247*	–	
Van Haard et al.	296	1700	900	72
Haroon et al.	281	560	–	22
Mummah-Schendel and Suttie	286	1300	640	95
		1100*	–	

*Median value.

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