Review

Chromatographic determination of vitamins in foods

Anna Rizzolo*

IVTPA, Istituto Sperimentale per la Valorizzazione Tecnologica deli Prodotti Agricoli, via Venezian 26, 20133 Milan (Italy)

Stefano Polesello

Dipartimento di Chimica Inorganica, Metallorganica e Analitica, Università di Milano, via Venezian 21, 20133 Milan (Italy)

ABSTRACT

Chromatographic methods for the determination of water- and fat-soluble vitamins in foods are reviewed. For each vitamin, sample preparation, detection problems and chromatographic conditions are presented and discussed. High-performance liquid chromatography (HPLC) is becoming a standard method in vitamin assay, especially for routine work. HPLC systems can be automated using in-line solid-phase extraction and column switchings, resulting in very sensitive methods, even when simple UV detection is employed.

CONTENTS

1. INTRODUCTION

A study of the literature published over the last few years suggests that chromatographic methods have become an indispensable tool for the rapid and specific determination of vitamins in foods.

Vitamins are diverse compounds with regard to structure, biological activities and their chemical properties. The water-soluble vitamin group includes thiamine and its derivatives (vitamin B_1), riboflavin (vitamin B_2), niacin (nicotinic acid) and its amide (nicotinamide, vitamin PP), vitamin B_6 vitamers (pyridoxal, pyridoxol and pyridoxamine and their derivatives), pantothenic acid, folic acid and its derivatives (folacin), biotin (vitamin H) and cyanocobalamin (vitamin B_{12}), in addition to L-ascorbic acid and L-dehydroascorbic acid (vitamin C) and other biologically active compounds, classified as pseudo-vitamins, such as orotic acid (vitamin

B_{13}), 4-aminobenzoic acid, inositol (myo- and mesoinositol), rutin (vitamin P) and S-methylmethionine (vitamin U).

The fat-soluble vitamins fall into four groups of vitamin-active compounds: vitamin A (retinoids and carotenoids), vitamin D [ergocalciferol (D_2) and cholecalciferol (D_3)], vitamin E (tocopherols and tocotrienols) and vitamin K (phylloquinone and menaquinones). This means that only very rarely can they be extracted from a complex matrix such as food by a simple and rapid procedure. Hence the extraction and clean-up steps represent the rate-limiting stage in almost all vitamin analyses. Further, the extraction has to be optimized not only both for vitamin degradation evaluation and for the specific food matrix, but also by checking the recovery of added vitamins.

Undoubtedly the continuing development of HPLC, LC–MS, GC and SFC methods applied to

vitamins suggests that their positive characteristics in resolving complex analytical problems are being confirmed; there has been a continuous improvement in these techniques with respect to the optimization of both stationary phases and of hardware, including the introduction of new detectors.

In this review we present a critical assessment of chromatographic methods developed for the determination of vitamins in foods, paying particular attention to sample handling and detection problems. Even if not applied to food matrices, but to multivitamin tablets or other pharmaceutical products and standards, some new techniques such as MECC, SFC and isotachophoresis, are included in view of their potential application to foods.

For each vitamin, surveyed methods are tabulated in order to assist the evaluation for method selection.

2. SIMULTANEOUS DETERMINATIONS

The great improvement in the simultaneous determination of different vitamins is due to the application of chromatographic methods; however, the principal problem to be taken into account should be the standardization of the conditions of extraction, paying attention to the chemical/physical characteristics of the food and the stability of the vitamins to be determined. When simultaneous determinations can be carried out, the analysis time is decreased and there is a less extensive use of materials and equipment.

2.1. Fat-soluble vitamins

HPLC has been the most often applied technique over the last 5 years, even though SFC has been developed and improved.

2.1.1. High-performance liquid chromatography

Ball [1] surveyed simultaneous HPLC methods up to 1984. However, there have been further developments with the application of microcolumn (narrow-bore) HPLC with multi-channel UV–VIS detection (Tables 1 and 2) for the determination of vitamin A, E and D and β-carotene in milk, animal feeds and vegetable oils [2,3], and of microcolumn HPLC with UV detection of vitamin A, D, E and K ·in margarine and butter [4]. NPC with UV and FL

detectors in series was used by Kneifel et al. [5] to determine vitamin A and D in butter and infant formula. To eliminate the need for time-consuming and laborious solvent extraction procedures, Brown-Thomas et al. [6] proposed a multi-dimensional HPLC method using a 10-μm gel column, to eliminate the bulk of the lipid material, which was in-line with a semi-preparative aminocyano column for NPC. This HPLC system was arranged with a switching valve to direct the solvent flow towards one or another column. In addition, to make the determination of dl-α-tocopheryl acetate possible, a final off-line RP-HPLC step was included.

Singh and Bradbury [7] developed an HPLC method for the simultaneous determination of α-carotene, β-carotene, retinol and vitamin D_2 in South Pacific root crops. Hot saponification was followed by hexane extraction, then for vitamin D_2 a clean-up procedure on an open silica gel column was applied. After testing five mobile phases, methanol–ACN–CHCl$_3$ (40:40:20) showed the best resolution in RP-HPLC, achieving the separation in the order retinol, vitamin D_2, α-carotene and β-carotene within 7–8 min. UV detection was performed with a dual-wavelength detector. Average recoveries were 87%, 91%, 93% and 94% for vitamin D_2, β-carotene, retinol and α-carotene, respectively.

The replacement of liquid–liquid partitioning with liquid–solid extraction of retinol and α-tocopherol from fatty samples of feedstuffs was studied by Bourgeois and Ciba [8]. By replacing methanol with methanol–ethanol-n-butanol in the saponifying solution, the two vitamins were de-esterified and extracted together from the same sample by using a disposable Kieselguhr cartridge. This procedure is faster than conventional liquid repartition methods, but it gives the same quantitative recovery (100.3% retinol and 98.5% α-tocopherol).

Kim and Kim [9] evaluated alkaline and enzymatic hydrolyses and both liquid–liquid and liquid–solid extraction for the HPLC determination of vitamin A, D, E and K in foods. No difference was found between alkaline and enzymatic hydrolysis for vitamin A, D and E, but enzymatic hydrolysis gave better results for vitamin K. Diethyl ether, pentane and hexane gave the best recovery for liquid–liquid extraction and a silica cartridge was used for liquid–solid extraction. The HPLC analysis was performed on a C_{18} column with methanol–

TABLE 1

SAMPLE PREPARATION FOR THE SIMULTANEOUS HPLC DETERMINATION OF FAT-SOLUBLE VITAMINS

For analytical HPLC conditions see Table 2.

Extraction	Chromatographic clean-up		Food	Analyte	Ref.
	Column	Mobile phase			
Dilution with n-hexane, in-line clean-up: (1) GPC to discharge lipids; (2) switching valve to HPLC column A; (3) fraction collected from (1), evaporate to dryness, dissolve in mobile phase, RP analysis with column B	GPC on PVB-PDVB polymer, 50 Å, 1 μm	30% methyl tert.-butyl ether–CH$_2$Cl$_2$ (30:70) in hexane	Fortified coconut oils	Vit. D$_2$, retinyl acetate	6
Saponify in ethanol–KOH (80–85°C, 1 h), extract with hexane, for vit. D$_2$ clean-up on open column, 2nd fraction collected, evaporate to dryness, dilute in hexane, RP analysis	Open column, silica gel 60 (300 × 15 mm I.D.)	75 ml benzene, 250 ml ethyl acetate–benzene (1:99), collect last 200 ml	South pacific root crops	Retinol, α-carotene, β-carotene, vit. D$_2$	7
KOH hot saponification in methanol–ethanol–n-butanol (4:3:1). Solid-phase extraction	Open column, Extralut 7, Kieselghur	Isooctane	Fatty samples, feedstuffs	Retinol, α-TOC	12

TABLE 2

ANALYTICAL CONDITIONS FOR SIMULTANEOUS HPLC DETERMINATION OF FAT-SOLUBLE VITAMINS

For sample preparation see Table 1.

Stationary phase	Mobile phase	Detection	Analyte	Food	Ref.
On-line with GPC: semi-preparative (A) NH_2-CN phase	Linear gradient: 0 min, 30% methyl tert.-butyl ether–CH_2Cl_2 (30:70) in hexane; 15 min, 100% methyl tert.-butyl ether–CH_2Cl_2 (30:70); flow, 2 ml/min	UV, 292 nm	Vit. D_2, retinyl acetate	Fortified coconut oils	6
Off-line: (B) Polymeric RP-C_{18}	Methanol–propanol–water (A) 60:10:30 (B) 89.5:10:0.5 Gradient: 0 min, 50% A; 18 min, 95% B; flow, 1.5 ml/min	UV, 284 nm	dl-α-Tocopheryl acetate		
5 μm C_{18} RCSS Guard-Pak C_{18}	ACN–methanol–$CHCl_3$ (40:40:20)	UV, 325 nm 280 nm 452 nm Dual wavelength	Retinol Vit. D_2 α-Carotene β-Carotene	South Pacific root crop	7
(1) 5 μm Spheri-5-RP18 guard column Aquapore ODS 7 μm	ACN–CH_2Cl_2–methanol (70:20:10), 1.8 ml/min	Programmable UV–VIS:	(a) Carotenoids (b) Retinol,	Vegetables	12
(2) 5 μm Spheri-5-ODS	ACN–methanol. Flow gradient: 0 min, 1.8 ml/min, 5 min, 3.5 ml/min	(a) 450 nm (b) 313 nm (c) 280 nm	Retinyl acetate (c) α-TOC, α-TOC acetate		

water (95:5) as eluent and detection at the highest absorption wavelength of the vitamins with a variable-wavelength UV detector.

Staroverov et al. [10] did not presaponify vegetable oils fortified with vitamin A, E and K_3; they separated the three vitamins on microcolumns filled with Silasorb using 6% diethyl ether in hexane as eluent and detection at 320 nm for vitamin A and K_3 and 220 nm for vitamin E.

Micali et al. [11] reported a procedure for the determination of α-, β-, γ- and δ-tocopherols, α-, β- and γ-tocotrienols, β-carotene, all-trans-retinol and retinyl palmitate in the unsaponifiable fraction of butter and margarine involving HPLC on an HS-Silica column with hexane–isopropanol (99.8:0.2) as eluent and programmable FLD ($\lambda_{ex.}$290–360 nm $\lambda_{em.}$ 330–480 nm). Olmedilla et al. [12] developed

two NARP methods for the rapid separation of ten carotenoids, three retinoids, α-tocopherol and α-tocopheryl acetate in vegetable samples using a single sample preparation and RP-HPLC separation on a C_{18} column with two-channel UV detection. Two eluent systems were tried and discussed for their efficiency to separate standards of the carotenoid, retinoid and tocopherol classes. ACN–methanol (85:15) as eluent at two flow-rates gave the best resolution among carotenes and retinyl palmitate in tomato extracts. The DL was found to be 1 ng for β-carotene, 0.3 ng for retinol and 15 ng for α-tocopherol.

The retention behaviour of fat-soluble vitamins on silica gel was investigated by Hara et al. [12] with binary solvents each containing ethyl acetate, THF or 2-propanol in hexane. A linear relationship be-

tween the log (capacity factor) and log (concentration of polar solvents) was confirmed. The retention sequence of the vitamins was retinol > ergocalciferol = cholecalciferol > δ- > γ- > β- > α- tocopherol > menadione > phylloquinone. They explained this retention sequence on the basis of hydrogen bonding interactions between the active functional group on the solute molecule and the silanol groups on the silica gel surface. The 2-propanol binary systems better resolved fat-soluble vitamins using a silica gel column.

A similar study was carried out by Ando et al. [14] on the retention behaviour of ten fat-soluble vitamins on aminopropyl- and cyanopropyl-bonded silica columns using binary solvents containing or ethyl acetate, THF or 2-propanol in hexane. A linear relationship between log(capacity factor) and log(solvent composition) was ascertained. The retentivity of the amino-bonded phase was stronger and that of the cyano-bonded phase was weaker than that of the silica phase when hexane–ethyl acetate or THF binary systems were employed. However, silica gel and 2-propanol binary solvents generally gave a superior peak shape for all vitamin samples.

2.1.2. Supercritical fluid chromatography

White et al. [15] reported a rarely used application of SFC for the separation of a group of fat-soluble vitamins in foods. Capillary SFC–FID was performed with an SFC system capable of linear pressure programming of the mobile phase while operating with either capillary or packed columns, with FID at 385°C. Two columns were evaluated: DB-5 (10 m × 50 μm I.D.) fused silica at 140°C with a CO_2 mobile phase held at 150 atm for 10 min then a linear pressure programme to 200 atm at 5 atm/min and held at 200 atm; and DB-WAX (10 m × 100 μm I.D.) at 150°C with CO_2 held at 175 atm for 25 min then a linear pressure programme to 350 atm at 4.17 atm/min and held at 350 atm. The first column resolved vitamin K_3, A, E and K_1 and provitamin D within 40 min and the second resolved vitamin K_3, vitamin A acetate, vitamin E acetate and vitamin K_1, A, E and D_3 within 48 min.

2.2. Water-soluble vitamins

The simultaneous separation and determination of water-soluble vitamins have been developed over the last few years especially for HPLC techniques.

2.2.1. High-performance liquid chromatography

Most of the HPLC methods (Table 3) have been developed to determine from two to four vitamins together in standard solutions and/or simple food

TABLE 3

CONDITIONS FOR SIMULTANEOUS HPLC DETERMINATIONS OF WATER-SOLUBLE VITAMINS

Sample preparation	Stationary phase	Mobile phase	Detection	Food	Ref.
Add I.S. solution (acetanilide in mobile phase), dilute with mobile phase, filter	Nucleosil 7 C_{18}, 40°C	Acetonitrile 0.01 M KH_2PO_4–triethylamine (8:91.5:0.5) with 5 mM Na octanesulphonate (pH 2.8)	UV, 254 nm	Oral liquid tonics	16
Dilute with water	RP-MPS	0.1 M H_3PO_4–0.1 M Na_2HPO_4 buffer (pH 5) with 10 mM Bu_4NBr	UV, 270 nm	Enriched pasta	21
Defat with hexane, extract in 0.1 M HCl (75°C, 15 min) with sonication, adjust pH to 4.6, filter, clean-up on SCX disposable column	Supelcosil LC-8-DB, 35°C	5 mM Na hexanesulphonate with 0.1% triethylamine (pH 2.8)–methanol (85:15)	UV, 200 nm	Almonds	20
Centrifuge, dilute 1:10 with water, filter through 0.20-μm membrane	Spherisorb ODS-2	5 mM octylamine orthophosphate	UV, 254 nm	Milks	18

matrices, such as drinks [16–18], even though more complex food matrices have been studied [19–23]. Dong et al. [24] systematically evaluated the factors controlling the separation in the IP-RPC mode of seven water-soluble vitamins (ascorbic acid, niacin, niacinamide, pyridoxine, folic acid, thiamine and riboflavin (Fig. 1) while Dai et al. [25] optimized the operating parameters in the RP mode using a polynomial fitting least-squares method, studying the relationship between retention time and peak width and methanol concentration in the mobile phase, which is the main factor affecting the RP separation of water-soluble vitamins.

One of the critical stages of the simultaneous methods is the extraction and the chemical/physical characteristics of the food and as the stability of the vitamins to be assayed have to be taken into ac-
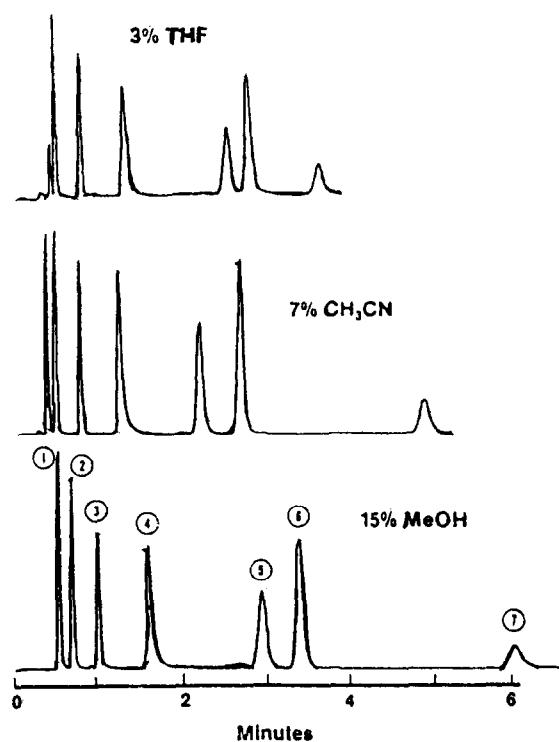

Fig. 1. Chromatograms showing the separation of water-soluble vitamins on a 3-μm end-capped spherical C_8 Pecosphere-3CR column (83 mm × 4.6 mm I.D.), using different organic modifiers + water containing 5 mM sodium hexansulphonate and 1% acetic acid. Peaks: 1 = ascorbic acid; 2 = niacin; 3 = niacinamide; 4 = pyridoxine; 5 = folic acid; 6 = thiamine; 7 = riboflavin [24].

count. Most of the simultaneous methods have been developed for those vitamins which can be extracted in a single procedure from food, without suffering losses; for example, to determine thiamine, riboflavin and niacin together, the extraction is carried out mainly with acidic and enzymatic treatments, sometimes followed by clean-up on C_{18} disposable columns [21–23]. On the other hand, Rizzolo et al. [20] extracted six water-soluble vitamins (biotin, niacin, riboflavin, thiamine, pyridoxine and folic acid) from raw almonds using an extraction procedure involving heat sonication of defatted materials and removal of interfering compounds by strong cation-exchange column clean-up.

Further, some published methods are not truly simultaneous, because the separation is not performed in a single chromatographic run, even though it is on the same column. For example, to determine riboflavin and thiamin in dietetic foods, Hasselmann et al. [22] performed two successive runs of the same extract on the same C_{18} column with an FL detector set at the proper wavelength (riboflavin $\lambda_{ex.}$ 422 nm, $\lambda_{em.}$ 522 nm and thiamine as thiochrome at $\lambda_{ex.}$ 366 nm and $\lambda_{em.}$ 435 nm).

To avoid successive runs on the same column, two or more detectors can be connected in series. To determine niacin and pyridoxine in fortified food products [26], the two vitamins were separated after acid extraction on an ODS phase and detected with UV and FLD instruments in series. To determine thiamine, riboflavin and niacin in heat-processed meats, Dawson et al. [23] set up an RP method in which an aliquot of the sample was directly separated on a C_{10} column, and riboflavin and niacin were detected using FLD and UV instruments in series. After derivatization of thiamine to thiochrome, the sample was separated under the same conditions and thiochrome was detected by FLD.

IP-RPC has been widely used, with different mobile phases and counter ions. However, to determine ascorbic acid and orotic acid in milks Gennaro and Abrigo [18] developed an RP ion-interaction HPLC method, using octylamine orthophosphate as the interaction reagent, without addition of an organic modifier or buffer to the mobile phase.

The RP separation on a C_{18} column of nicotinamide, pyridoxine, thiamine, riboflavin, folic acid and cyanocobalamin in oral liquid tonics, as affect-

ed by the ion-pair reagent chain length, concentration of triethylamine, pH and concentration of acetonitrile in the mobile phase, was extensively studied by Maeda et al. [16]. The capacity factor of thiamine was greatly influenced by the length of the alkyl chain of the ion-pair reagent, the concentration of triethylamine and pH. On the other hand, the same factors had only a slight influence on folic acid and riboflavin.

Generally, standard ODS columns are used, even though the fully end-capped columns provide a better peak shape and a better separation of the vitamins than the standard columns [17,20,26].

To shorten the time required for the simultaneous determination of ascorbic acid, nicotinic acid, thiamine, pyridoxine, hydroxycobalamin and FAD contained in beverages, Akiyama et al. [17] proposed three newly prepared bonded silica column packing materials [3-morpholinopropyl- (MPS)3- (1-piperazinyl)propyl- (PZS) and 3-piperidinopropyl- (PDS)silica gels], which run with one buffer solution throughout the analysis without gradient elution.

Using parallel dual-electrode ED at two different oxidative potentials, only selected vitamins could be determined [27,28]. For example, with one electrode controlled at +0.8 V (vs. SCE) and the other at +1.2 V (vs. SCE), Hou and Wang [28] successfully determined ascorbic acid, pyridoxine, pyridoxamine, p-aminobenzoic acid and folic acid in multivitamin tablets without interference from other water-soluble vitamins. Their HPLC–ED system offered superior selectivity and improved sensitivity, the DL being 20 pg (ascorbic acid), 40 pg (p-aminobenzoic acid), 0.2 ng (folic acid), 0.6 ng (pyridoxine) and 1 ng (pyridoxamine). Not all the methods are suitable for determining all the vitamins extracted, owing either to the too low concentration of one vitamin with respect to the others in the extract, as in the case of cyanocobalamin in oral liquid tonics [16], or to the presence of interfering substances in the extracts even after extensive clean-up from complex food matrices, as in the case of almonds, where only two vitamins out of six could be correctly determined [20].

2.2.2. Thin-layer chromatography

Zang and Ma [29] proposed a TLC method to determine thiamine, riboflavin and vitamin B6 si-

multaneously in various plant materials, using highly sensitive FLD. They claimed that the addition of Triton X-100 to the extractant (n-butanol) improved the effectiveness of the extraction media. Shrivastava and Prakash [30] used scolecite (a type of zeolite) in its pure form as an adsorbent for the TLC of a few vitamins.

2.2.3. Micellar electrokinetic capillary chromatography

To date MECC has not been applied to simple or complex food matrices. Nishi et al. [31] studied the retention behaviour of eleven water-soluble vitamins in MECC, using a 65 cm × 0.05 mm I.D. fused-silica capillary tube and UV detection at 210 nm, with 0.05 M SDS and/or 0.2 M sodium lauroylmethyltaurate (LMT) as the anionic surfactant in the electrophoretic medium. To clarify the effect of the micelle, they employed an ion-pairing reagent (sodium pentanesulphonate) that does not form a micelle structure. They separated all solutes within 15 min using a 0.05 M SDS solution (pH 9).

Fujiwara et al. [32] employed the same surfactant (SDS) and on-column UV detection at 254 nm, with ethyl p-aminobenzoate as the internal standard, to determine seven vitamins (thiamine, nicotinamide, nicotinic acid, pyridoxine, cyanocobalamin, L-ascorbic acid and riboflavin phosphate) obtaining DLs of 0.5 pmol for riboflavin phosphate and cyanocobalamin, 1 pmol for thiamine and ascorbic acid and 4 pmol for the other vitamins.

Ong et al. [33] separated a mixture consisting of dansylamino acids and two vitamins (riboflavin and pyridoxine) in a single run, using a 60 cm × 50 μm I.D. fused-silica capillary as the separation tube and 40 mM SDS in 0.1 M borate–0.05 M phosphate buffer (pH 7.56) as the electrolytic medium. The detection was carried out with an on-column FLD system with a wavelength programme [0.1 min, 340/400 nm (pyridoxine); 6.1 min, 325/550 nm; 7.1 min, 370/440 nm (riboflavin); and 7.4 min, 325/550 nm] in order to have the maximum sensitivity for each of the species in the mixtures.

2.3. Water-soluble and fat-soluble vitamins

Until now, the simultaneous chromatographic separation of both fat- and water-soluble vitamin groups in a single analysis has not been reported for food matrices; however, recently, a first approach

to this type of determination has been attempted for pharmaceutical products and standard solutions, using fairly new analytical techniques.

For example, to separate fourteen vitamins in drugs, Arai and Hanai [34] used an HPLC–DAD system, equipped with a helium–cadmium laser for fluorescence and a deuterium lamp for absorption detection. They separated the vitamins at 40°C on an Inertsil ODS column with gradient elution from 3% ACN in 0.1% phosphoric acid with 5 mM 1-pentanesulphonate to 97% ACN over a period of 20 min, with detection wavelengths ranging from 220 to 400 nm, selecting the maxima for each vitamin. They claimed that the DL with the laser FLD system was about 2000 times less than that with conventional FLD using a photomultiplier. They also stressed that when RP in gradient elution is used, the comparison of retention times is not sufficient for the qualitative analysis of vitamins. They also confirmed that standard ODS columns are not suitable for the determination of some vitamins (such as thiamine and pyridoxamine), owing to peak broadening and absorption phenomena on the packing material. Hence the use of DAD monitoring of fluorescence and absorption spectra and an end-capped ODS column should solve the difficulties with the simultaneous analysis of vitamin mixtures.

Another solution to the problem could be an apparatus consisting of a sample introducer, a switching path connected to a lipid-soluble vitamin trap, positioned between two flow paths (one for each group of vitamins) and the sample introducer, an RP column and a suitable detection system. Mikami [35] using this type of device separated seven water-soluble and five fat-soluble vitamins with a single run.

Another interesting approach to solve this analytical problem could be MECC. Ong et al. [36] successfully separated a mixture of seven water- and two fat-soluble vitamins in a single analysis by MECC, using a 50 cm × 50 μm I.D. fused-silica capillary as the separation tube and a micro UV spectrophotometer set at 210 nm as detector. In addition to SDS, they studied the effect of the introduction into the electrophoretic media of modifiers such as γ-cyclodextrin, β-cyclodextrin and 2-propanol on the overall separation of the nine vitamins. Among these modifiers, the combination of 3 mM

γ-cyclodextrin with 30 mM SDS in the electrophoretic medium (0.1 M borate–0.05 M phosphate, pH 7.6) provided the best selectivity (Fig. 2).

3. FAT-SOLUBLE VITAMINS

3.1. Vitamin A

Vitamin A in its various forms (retinol, retinyl esters, etc.) is present in food of animal origin (e.g., liver, milk, dairy products, fish, poultry, meat), while its precursors (provitamin A) in form of carotenoids are present in both animal and plant foods.

As provitamin A can be biologically converted into vitamin A in the human body, plant foods are an important dietary source of this vitamin, especially for people in developing countries (82% of the total dietary vitamin intake) [37].

3.1.1. Retinoids

The classical spectrophotometric and fluorimetric methods are still official [38], but they cannot discriminate molecular species with different vitamin A activity.
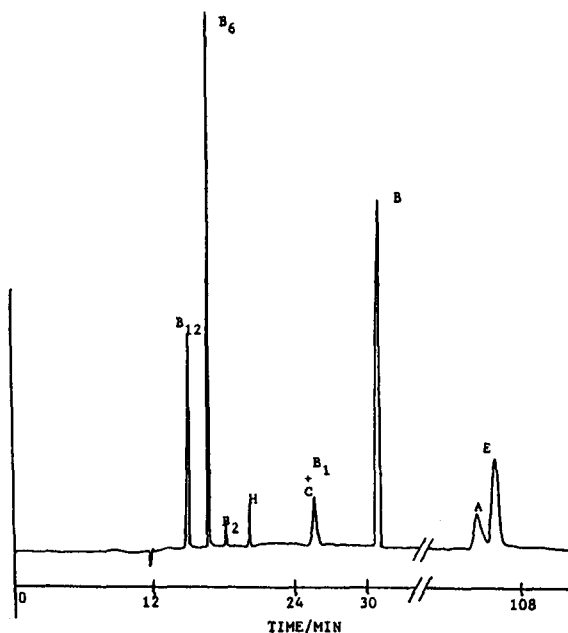

Fig. 2. Electrokinetic chromatogram of the vitamins with 2-propanol. Electrophoretic solution, 30 M SDS in 0.1 M borate–0.05 M phosphate with 3% isopropyl alcohol (pH 7.6); separation tube, 50 cm × 50 μm I.D. fused-silica capillary; voltage, 15 kV; amount injected, 0.75 nl [36].

The naturally occurring compounds having vitamin A activity and the synthetic analogues of retinol, with or without vitamin A activity, are included in the term 'retinoids'. Some retinol metabolites, such as retinal and retinoic acid, play an important role in the human organism.

The problems encountered in the determination of vitamin A in food are similar to those with biological samples. Wyss's review [39] on retinoid chromatography in drug and biological samples pointed out that there had been little progress because of the lack of suitable techniques for the determination of these extremely labile compounds, and stressed the success of HPLC in isolating and separating retinoids and in the identification of new metabolites, improving drug assay. De Leenheer et al.'s general review [40] on the fat-soluble vitamins in clinical chemistry pointed out that HPLC was the most applied technique. A review specifically dedicated to food was recently published by Tee and Lim [41], reporting general methods for carotenoids and retinoids.

Retinoids are very sensitive to light and oxidation and they are subject to isomerization and degradation during processing. As a consequence, several processed foods, namely milk infant formula, are fortified with synthetic vitamin A (usually retinyl palmitate) during or after processing. This aspect has to be pointed out because low or not active retinoids are formed.

However, while HPLC is now generally recognized [1] as the best technique for retinoid determination, for official purposes the HPLC methods must be compared with the official ones; for example, Mills [42] compared the Carr–Price colorimetric method of the AOAC [43] and their NP-HPLC method for milk. Thompson [44] discussed the problems with official methods for the determination of vitamin A in food and feeds.

3.1.1.1. Sample preparation. Saponification followed by solvent extraction and direct extraction are the main techniques used to prepare samples for vitamin A analysis (Table 4). Generally, the former is used for total retinol determination according to the AOAC [43] and it was applied, *e.g.*, by Al-Abdulaly and Simpson [45], while many workers [46–50] added an antioxidant such as ascorbic acid, hydroquinone or pyrogallol. Zahar and Smith [51] set up a rapid method where saponification and extrac-

tion stages are performed in the same centrifuge tube.

Direct extraction without saponification was used by Woollard and Woollard [52] for HPLC direct injection, by Woollard and Indyk [53] according to a previous method [54] to study the distribution of retinyl esters in milk and by McNeill et al. [55] to evaluate the addition of retinyl palmitate to skimmed milk powder as a fortifier during processing. The saponification method allows the determination of free retinoids [45–49,51], whereas the saponification step is omitted when the separation and determination of the different retinyl esters in food are required [52,53,56].

All these methods claim high recovery and efficiency of the selected procedures in preventing isomerization and oxidation of the vitamin.

3.1.1.2. Open-column chromatography. Despite its high specificity and sensitivity, HPLC has the disadvantage of being expensive to set up and maintain, so it is out of reach in most developing countries.

Al-Abdulaly and Simpson [45] developed an inexpensive method using an RP-FC with an open column pressurized with nitrogen and spectrophotometric determination of the eluted vitamin A band. Milk infant formula, margarine, egg yolk, chicken and lamb liver were analysed using this method and compared with the AOAC official method [43] and with an HPLC method [45]. The vitamin A recoveries were 98% with RP-FC, 99% with HPLC and 97% with the AOAC 1984 method. The mean value obtained by RP-FC did not differ significantly from those for the other two methods. The time of analysis of saponified extracts was within 15 min, comparable to that for HPLC, while that for the AOAC method was 35 min, with a lower cost than HPLC.

3.1.1.3. High-performance liquid chromatography. HPLC methods have been developed over the last 5 years according to both the well established modes of RPC and NPC (Table 4). Concerning RP methods, some papers report the application of recommended methods; e.g., Pepping et al. [46] used an EEC Cost 91 recommended method [57] for the RP-18 separation of vitamin A in fish oils. RPC on C_{18} was also used by Al-Abdulaly and Simpson [45] for a comparison between HPLC and RP-FC methods, and by Ötles and Hisil [50] for egg analysis, while Coverly and Macrae [47] used a C_8 phase in

TABLE 4

CONDITIONS FOR HPLC DETERMINATION OF RETINOIDS

Sample preparation	Stationary phase	Mobile phase	Detection	Food	Ref.
Milk: saponify with 15% KOH in methanol (70°C, 30 min). Extract with diethyl ether, dissolve in LP.	Open column: 20 × 1.5 cm I.D., 50-μm C_{18}, N_2 (10 p.s.i.)	Water–methanol (10:90), 100 ml	325 nm in LP	Milk, infant formula, margarine, egg,	45
Margarine: dissolve in hexane, filter to dryness, saponify with 80% KOH in methanol 70°C, 30 min), then as above. Egg, liver: dry sample over Na_2SO_4, saponify with 70% KOH, then as above	HPLC, RP C_{18}	ACN–CH_2Cl_2–CH_3OH (70:20:10)	325 nm	chicken and lamb liver	
Alcoholic saponification (hydroquinone as antioxidant)	C_{18}	Water–methanol (96:4); flow, 1.5 ml/min	294 nm	Fish	46
Automatic continuous Technicon sampling, on-line KOH saponification, clean-up	Supelco LC-8	87% aqueous CH_3OH containing 0.1% CH_3COOH	325 nm	Milk, infant formula	47
Saponify with 10% ethanolic KOH with pyrogallol at room temperature for 18 h in centrifuge tube	Silica Apex	Heptane–2-propanol	DAD	Milk, infant formula	48
Saponify with hot water–alcoholic KOH (30 min) with ascorbic acid as antioxidant, extract with LP	μPorasil	Hexane–$CHCl_3$ (60:40)	Fluorescence, 350/470 nm (ex./em.)	Premixes	49
In a centrifuge tube saponify with KOH (ascorbic acid and pyrogallol as antioxidant, 80°C, 20 min), add LP, shake, evaporate supernatant to dryness, dissolve in methanol	Nova-Pak C_{18}	Methanol–water (95:5); flow, 0.8 ml/min	325 nm 313 nm	Milk, dairy products	51
Saponify with aq. ethanol and ascorbic acid (boil with water, 30 min) extract with hexane, evaporate to dryness, dissolve in ethanol	μBondapak C_{18}	CH_2Cl_2 containing 0.001% trimethylamine–ACN–methanol (300:700:0.5); flow, 0.3 ml/min	280 nm	Eggs	50
Extract with hexane, clean up on PTFE (0.45 μm)	Radial silica	2-Propanol–hexane (0.07:99.93); flow, 2 ml/min	Multi-wavelength UV, fluorescence 325/465 nm (ex./em.)	Vegetable oils, ghee, butter, margarine	52
Milk: dilute with ethanol, extract with LP.	Novapak RPC	ACN–CH_2Cl_2 (80:20)	325 nm, fluorescence 325/470 nm (ex./em.)	Milk, milk products, shark and cod liver oils	53
Powder: extract with DMSO–DMF–$CHCl_3$ (2:2:1). Oils: dilute with ACN–CH_2Cl_2 (1:1)	Porasil NPC	Hexane–2-propanol (99.93:0.07)			
Extract from model system with CH_3OH–C_2H_5OH–acetone (6:3:10) and 200 ppm BHT, evaporate to dryness, dissolve in methanol	NPC LiChrosorb Si 60, 45°C	Hexane–2-propanol (99.93:0.07); flow, 1.5 ml/min	325 nm	Model system	56
	RP Spherisorb ODS	Methanol–water (90:10); flow, 1.5 ml/min			

an automatic continuous-flow method. The main elution system was aqueous methanol (range 87–96%) but a NARP system, such as with ACN–CH$_2$Cl$_2$–MeOH [45], was also used too.

Woollard and Indyk [53], using ACN–CH$_2$Cl$_2$ as the eluent, evaluated the distribution of retinyl esters in milk of different animal origin. May and Koo [58] discussed the behaviour of retinyl esters on an ODS column and observed that a plot of log k' versus the percentage of water in the eluting solvent was very close to linear in the range 1–3.5% of water in methanol. Measuring the effective carbon number (ECN) is useful for evaluating the effect of the introduction of double bonds into a molecule; some monounsaturated acyl esters have an ECN = 1.85. Hence the resolution of retinyl ester homologues can be evaluated.

NPC has been adopted in many methods using silica gel as a stationary phase, as carried out by Thorpe [49] for total retinol determination and by Thompson and Duval [48] and Woollard and Woollard [52] for the isocratic separation of cis isomers and retinyl esters. The latter workers applied a previously tested method [54] demonstrating the formation of several cis isomers, which were determined in manufactured UHT milk and milk powders. Skurikhin et al. [59] also applied NP in microcolumn HPLC using a 120 × 2 mm I.D. column filled with Silasorb with UV detection to analyse milk and colostrum. Studying the degradation of retinol during storage for low water activity model systems simulating dehydrated food, Manan et al. [56] found that the mode of HPLC analysis (NPC or RPC) influence the kinetic interpretation. With NPC the degradation could be adequately described by first-order kinetics, whereas with RPC better results were achieved using a second-order model. This discrepancy was attributed to the formation of a cis isomer, which was not resolved from all-trans-retinol using RPC; hence NPC showed a better resolution of cis isomers. On the other hand, the RPC and NPC modes are equivalent for total vitamin A analysis.

UV detection is widely used, with either fixed- or multi-wavelength instruments, even though DAD was employed by Thompson and Duval [48] and FLD by Thorpe [49]; Woollard and co-workers [52,53] used both modes of detection, and claimed that FLD is preferable because it allowed the vi-

sualization of the vitamin A components without the interference of other lyposoluble compounds.

To date, non-aqueous ED for LC analysis of vitamin A in food has not been applied. Hart and Jordan [60] for multivitamin tablets used ED with an inexpensive low-pressure RPC ststem, and Bryan et al. [61], studying the use of non-aqueous ED for retinoids after their NP separation on either silica or PVA-sil columns, found a DL of about 1 ng on-column compared with 2 ng with UV detection.

3.1.1.4. Other chromatographic techniques. Retinoids are sensitive to the hot injector port surface and to incompletely inactivated column packings. Smidt et al. [62] used cGC to determine underivatized retinol on a bonded-phase methylsilicone column by using a cold on-column injector and FID and claimed a DL of 3.5 ng for retinal. Fürr et al. [63] determined the Kováts retention indices of some retinoids for various bonded phases, confirming that the underivatized retinoids can be determined by cGC with on-column injection and either FID or MSD when identification is required. The universal nature and sensitivity of FID and MSD are also advantageous over the more specific detection methods available for LC. Hence this technique can be a valuable tool also in food analysis. So far HPLC–MS has been reserved for the determination of subnanogram amounts of retinoids in clinical chemistry [64].

3.1.2. Carotenoids

Rather than studying quantitatively the provitamin A activity, there have been extensive studies of the qualitative aspects concerning structure elucidation, which is of taxonomic and phytochemical importance. This was pointed out in Rodriguez-Amaya's recent critical review on provitamin A in plant food [65]; she listed four factors that make it difficult to obtain reliable provitamin data: the number of carotenoids present in plant foods; the variability of the qualitative and quantitative composition of food samples; the identification of provitamin A-active compounds and the variations in their activity in different foods; and the formation of artefacts during analysis.

3.1.2.1. Open-column chromatography. Conventional column chromatography (Table 5), often referred to as open-column chromatography, coupled with visible absorption detection has long been used

TABLE 5

OPEN-COLUMN METHODS FOR CAROTENOIDS

Extraction	Column	Eluent	Detector	Food	Ref.
Blend with acetone and Celite, extract with LP and water, saponify with 10% NaOH in methanol overnight at room temperature	MgO–Hyflo Supercel	1–15% acetone in LP	Visible	Vegetables	68
Blend with acetone, extract with LP and water, saponify with 60% KOH in methanol overnight at room temperature under nitrogen	RP-18	Acetonitrile–CHCl$_3$ (92:8), 10 p.s.i., under N$_2$ flow	Stopped-flow visible spectra	Vegetables	70

in carotenoid research [65]. Most published work, however, deals with the identification of carotenoids, with only a rough estimation of the quantitative composition.

The AOAC [66] and COST 91 [67] quantitative methods for carotenes do not attempt to separate the individual carotenoids, whereas HPLC methods give better separations, identification and quantification.

A modified method for the extraction and determination of individual provitamins used an MgO–Hyflo Supercel column eluted with 1–15% acetone in LP [68]. The results of this method were comparable to those of HPLC methods. To avoid provitamin degradation during hydrolysis of xanthophyll esters, an assessment study by Kimura et al. [69] recommended that saponification be carried out overnight at room temperature in LP with an equal volume of 10% methanolic KOH under a nitrogen atmosphere or with an antioxidant. Tsai et al. [70] proposed an alternative method based on RP-FC which separates β-carotene from β-cryptoxanthine, but did not succeed in separating β-carotene from lycopene; they claimed more than 97% recovery, calculated by spiking fruits with standards.

3.1.2.2. Thin-layer chromatography. There are few examples of TLC applications for quantitative analysis in the recent literature. Generally, TLC of carotenoids is carried out by NPC on alumina or silica layers eluted with a mixture of hydrocarbon carrier (hexane, LP or benzene) and a polar organic

modifier (acetone, chloroform or methanol). The NP method using acetone–LP mixtures for developing silica layers is still used in semi-preparative separations prior to HPLC analysis [71,72].

Francis and Isaksen [73] improved the qualitative separation of oxygenated carotenoids using LP containing tertiary alcohols, which are unaffected by the amounts of lipids found in carotenoid extracts.

3.1.2.3. High-performance liquid chromatography. Numerous reviews have been published over the last few decades, dealing especially with HPLC analysis [41,65,74–77].

Rodriguez-Amaya [65] reviewed the HPLC methods set up to determine the provitamin A content of foods up to 1987, and pointed out the difficulties in separating and determining isomers with a real provitamin activity. NPC (Table 6) has seldom been used in recent years. NPC analyses for carotenes have been developed using a variety of stationary phases. Magnesium oxide and alumina have frequently been used, but active alumina decomposes some carotenoids such as lycopene [78], depending on the retention time in the column and on alumina activities. The cis and trans isomers of α- and β-carotenes were separated on lime [79]. Silica has been less useful for carotenes, giving long retention times with poor resolution [80]. Rhodes et al. [81] suggested using an isocratic mobile phase with a definite water content and silica as stationary phase; they claimed quantitative recoveries and a DL of 20 ng for β-carotene. NPC has been judged inadequate

for carotenoid separations [82]. Although capable of resolving the hydrocarbons, the polar xanthophylls are highly retained on silica unless relatively complex gradient programmes are employed. Further, the carotenoids easily degrade on the slightly acidic surface of silica.

RPC is widely utilized, but carotene separation is difficult and may require the use of gradients or

TABLE 6

CONDITIONS FOR HPLC DETERMINATIONS OF CAROTENOIDS

Sample preparation	Stationary phase	Mobile phase	Detection	Food	Ref.
Extract with Na_2SO_4, $MgCO_3$, THF in a Waring blender at moderate speed for 5 min	Partisil 5 ODS	Acetonitrile–THF–water (85:12.5:2.5); flow, 2 ml/min	470 nm	Fruit and vegetables	108
Blend with Na_2CO_3 (pH 8–9) and acetone (10 min, 0–5°C) extract with diethyl ether and water, TLC on silica layer developed with LP–acetone (1:1)	Hypersil ODS	(A) Methanol–water (75:25) (B) ethyl acetate. Gradient: 0 min, 100% A; 10 min, 70% B; 14 min, 100% B; flow, 1.7 ml/min	430 nm	Kiwi	72
Extract with CCl_4–methanol (2:1)	Chromsil C_{18}	Acetonitrile–2-propanol–water (39:57:4); flow, 1 ml/min	438 nm	Tomato, paprika	86
Saponify with ethanolic KOH in the presence of ascorbic acid, extract with diisopropyl ether	Hypersil ODS	Methanol–water–acetonitrile–$CHCl_3$ (200:11:250:50); flow, 1.5 ml/min	445 nm	Vegetables	109,110
Extract with DMF, filter, extract with hexane	Spherisorb ODS-2	Gradient from A to B. A = water–IP reagent–methanol (1:1:8) [IP = 0.05 M tetrabutylammonium acetate –CH_3COONH_4, 1 M in water]. B = acetone–methanol (1:1)	DAD	Olive	71
Extract at 100°C with hexane–acetone–diethyl ether (30:50:20)	ODS RP-18, 22°C	CH_2Cl_2–methanol–acetonitrile (10:27:63); flow, 1 ml/min	DAD	Dried carrots	100
Saponify with 50% KOH in ethanol and 0.1% ascorbic acid, extract with LP–diisopropyl ether (75:25) and water	RP C_{18}	Acetonitrile–CH_2Cl_2–methanol–2-octanol (70:20:10:0.1); flow programme, 0.7–3.5 ml/min	436 nm 313 nm	Milk	112
Blend with acetone–hexane (40:60), column chromatography on deactivated alumina	ODS C_{18}	Methanol–ACN–$CHCl_3$ (47:47:6); flow, 2 ml/min	461 nm	Raw and cooked vegetables	111

TABLE 6 (*continued*)

Sample preparation	Stationary phase	Mobile phase	Detection	Food	Ref.
Extract with acetone, Na_2SO_4, $MgCO_3$, saponify with KOH in ethanol and water overnight at room temperature, extract with hexane–diethyl ether (70:30) with BHT (0.1% in hexane)	Zorbax ODS Spherisorb ODS-2, 30°C	ACN–CH_2Cl_2–methanol (70:20:10); flow, 2 ml/min ACN–methanol–CH_2Cl_2–hexane; (A) 75:15:5:5; (B) 40:15:22.5:22.5. Gradient: 0–6 min A, 6–20 min A to B; flow, 1 ml/min	450 nm	Vegetables, fruits, berries	113
Dissolve in CH_2Cl_2, saponify with KOH–methanol extract with LP	Rainin Microsorb ODS	ACN–methanol–CH_2Cl_2 (60:35:5); flow, 2 ml/min	DAD	Palm oil	99
Blend with H_4folic acid, Na_2SO_4, $MgCO_3$ and I.S, extract with LP–water, saponify with 30% KOH–methanol, purify on C_{18} TLC plates	Microsorb C_{18}	Methanol–ACN–CH_2Cl_2–hexane (1:1): (A) 10:85:5; (B) 10:45:45. Gradient: 0–10 min A, 10–40 min A to B; flow, 0.7 ml/min	DAD	Fruit and vegetables	91
Extract with CS_2–acetone–hexane (2:2:1), saponify with ethanol–KOH solution	Supelcosil LC 18 S	(A) 2-propanol–1,2-dichloroethane (2:1); (B) acetonitrile. Gradient: 0 min, 5% A; 10 min, 7.5% A; 15 min, 30% A; 30 min, 50% A; flow, 1.5 ml/min	458 nm	Shellfish	107
Blend with acetone–ethanol (1:1), extract with LP without saponification	Ca(OH)$_2$	Hexane–acetone (99:1); flow, 0.25 ml/min	DAD	Vegetables	79

complex ternary or even quaternary mixtures. There are some disadvantages of RP analyses, such as long run times and the use of less volatile solvents, which make solvent removal difficult in preparative work. C_{18} column selection must be carefully chosen, as the performance varies considerably from batch to batch.

RP separations with water are still in use according to Schwartz *et al.* [83] and Bushway and Wilson [84]; the addition of water greatly improved the resolution of the xanthophylls and revealed a greater complexity in the mixtures of these components. On the other hand, the retention times of all compounds increased up to 300 min for carotenes [85].

Biacs and co-workers [86–88] conducted some studies on mobile phase composition and claimed that using ACN instead of acetone prevents column deterioration and sample degradation [88].

Nelis and De Leenheer [89] proposed NARP chromatography as a useful approach for the isocratic separation of non-polar compounds. In order to cover the whole range of polar and non-polar carotenoids, many systems employ gradient elution (Table 6).

The choice of the right C_{18} phase has been widely investigated. Khachik and co-workers [90,91] compared Rainin Microsorb C_{18} (with a high carbon loading and small pore size) and Vydac C_{18} (with a

low carbon loading and a large pore size); the latter gave shorter retention times. A qualitative study showed that, among the various classes of carotenoids, the separation of the stereoisomers of the hydrocarbon carotenoids could be better accomplished on a Vydac column; on the other hand, the separation of the oxygenated carotenoids (xanthophylls and their stereoisomers) was better on a Microsorb C_{18} column. The same group [92] developed a method to separate and identify fatty acid esters which have different fatty acid side-chains. Khachik et al. [93] were concerned about possible artifacts and peak distortion caused by the different solubilities of carotenes in the injection solvent (THF and CH_2Cl_2) with respect to a mobile phase containing a polar modifier.

Recently, Epler et al. [94] evaluated 65 HPLC columns under standardized conditions for carotenoid separation and recovery. They agreed with Quackenbush's [95] observations that polymeric C_{18} phases showed excellent selectivity of structurally similar carotenoids, whereas the most commonly used monomeric C_{18} phases did not. Epler et al. found that mobile phases containing ACN gave lower recoveries than those containing methanol.

From optimization studies on isocratic separations using a polymeric C_{18} column, the best separation in the shortest analysis time was achieved by using THF as modifier in the methanolic eluent [96] (Fig. 3). Sander and Craft [97] gave an example of enhanced shape discrimination at sub-ambient temperatures, separating 9-cis- and-trans-carotenoids at $-13°C$.

Granado et al. [98] compared monofunctional and polyfunctional C_{18} columns with a series of vegetable samples; they observed a marked increase in the resolution, especially for polar carotenoids, and a decrease in the separation factors. They reported DLs ranging from 0.1 ng for canthaxanthin to 2.4 ng for phytoene using dual-channel dispersive UV detection.

Ng and Tan [99] developed a NARP–DAD method to identify palm oil carotenoids; they found that the β-carotene DL was 31 ng, confirming that DAD has a lower sensitivity than fixed-wavelength monochromator detectors currently being used; on the other hand, DAD is necessary for identifying and characterizing the various carotenoid isomers.

Lesellier et al. [100] developed NARP chroma-
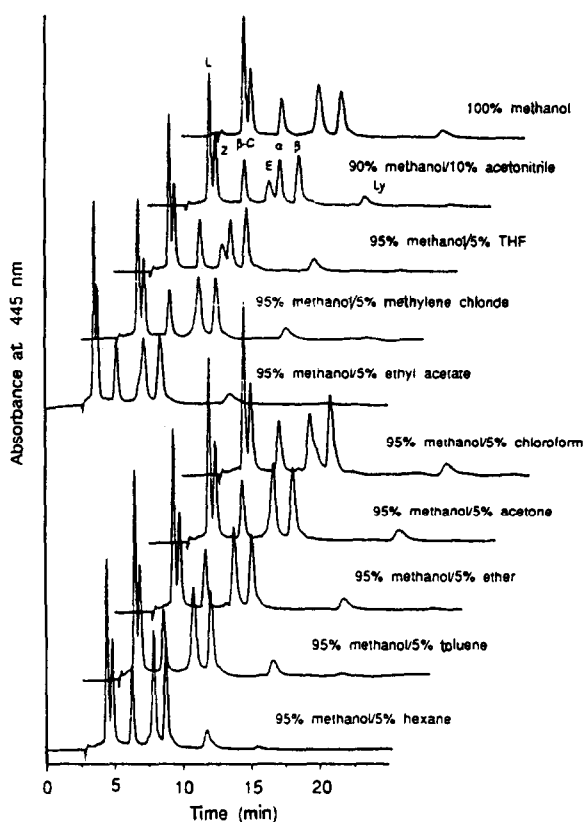
Fig. 3. Effect of nine solvent modifiers on the separation of seven carotenoids. Chromatographic conditions: Vydac 201TP C_{18} (5 μm) column (250 × 4.6 mm I.D.); mobile phases as shown; flow-rate, 1 ml/min; UV–VIS detection at 445 nm; column temperature, 25°C. Peaks: L = lutein; Z = zeaxanthin; β-C = β-cryptoxanthin; E = echinenone; α = α-carotene; β = β-carotene; Ly = lycopene [96].

tography to optimize the separation of trans- and cis- α- and -β-carotenes, studying the effects of the elution strength of the solvents, their solubility properties, the influence of temperature on the capacity factor and selectivity. They stressed the need for a polymeric bonding on the stationary phase to separate trans–cis isomers. The same group [101,102] assessed an SFC method with a C_{18} column and CO_2 containing a polar modifier; this supercritical fluid has a solvent polarity similar to that of hexane, but it has a lower viscosity; therefore, solute diffusion coefficients are higher than in conventional solvents. The relationships between capacity factors and operating parameters such as

temperature, pressure, nature and concentration of the polar modifier were investigated. These studies have led to a fivefold reduction in the analysis time together with an improvement in the resolution of these compounds.

An interesting study by O'Neil et al. [103] compared methods for the determination of cis–trans isomers of β-carotene. Although both Vydac C_{18} and $Ca(OH)_2$ were effective in resolving diastereoisomers of β-carotene, they found that the lime method had fewer interferences and higher selectivity when used to analyse real vegetable samples. The only possible way to separate a complex mixture of esters and carotenes is the use of an NP and an RP in series: on NP, cryptoxanthin esters are well separated from the diesters. NP provides quantitative data on the different classes of carotenoids and RP determines the individual elements.

The choice of the correct method for quantitative analyses was discussed by Philip and Chen [104]; owing to the limited availability of pure carotenoids as standards, the use of external standards is rare, whereas the use of an I.S. is affected by the shifts in absorption maxima in different solvents during gradient elution. They claimed that Sudan I is suitable as an I.S. for both NP and RP carotenoid analysis.

There are other techniques besides NP and RP chromatography, but they are less frequently used. Minguez-Mosquera et al. [71] presented a method using IP-RP chromatography to study olive fruit lactic fermentation; pigment concentrations were evaluated from an extension of Beer's law. This procedure was compared with the external standard method; an analysis of variance showed no significant differences between the two methods.

Stalcup et al. [105] proposed a cyclodextrin bonded phase used in the NP mode to separate and identify a complex standard mixture. An improvement in the identification and characterization of various carotenoids could be the use of MSD in LC analysis. This topic was reviewed by Taylor et al. [106].

3.2. Vitamin D

HPLC is the most commonly used chromatographic method for the determination of vitamin D vitamers [ergocalciferol (D_2) and cholecalciferol (D_3)], which have very similar biological activity in man. Usually, total vitamin D evaluation is re-

quired, even if the knowledge of the content of each vitamer in fortified food is important.

Although in the past other techniques, such as open-column chromatography, TLC and GC, were applied [114], over the last 5 years these techniques have not been improved. In contrast, HPLC techniques have been extensively studied over the last decade [1] and two methods, which were tested by collaborative trials by de Vries and co-workers [115,116], were accepted as official by the AOAC [117,118].

3.2.1. Sample preparation

Usually, sample preparation is carried out in two steps: saponification and clean-up (Table 7). The most commonly applied mode is saponification with refluxing alcoholic KOH and it was included in AOAC methods, with some precautions to reduce thermal isomerization [119–122] and to prevent oxidation by adding some antioxidants [117,118,123–126]. Agarwal [127] used an overnight cold saponification, while Hung [126] did not saponify the samples before extraction.

The clean-up stage is the critical point of the methods and it appears rather complex. This is still a matter of major development, as a well prepurified extract is needed for analytical HPLC. To avoid these clean-up procedures, Agarwal [127] converted both vitamers present in the unsaponifiable matter from fortified milks into isotachisterol by reaction with $SbCl_3$; the derivative was measured at 301 nm without interference from the other substances present.

The clean-up procedure varies according to the type of food matrix. For example, for fortified milk and milk powders [117], semi-preparative HPLC on a CN-bonded phase prior to the analytical HPLC is suggested, whereas for feedstuffs [118] a two-step clean-up is necessary, involving open-column chromatography on alumina to separate vitamin D from vitamin E and carotenoids, followed by purification of the vitamin D fraction by semi-preparative HPLC on an RP-18 column, prior to the analytical HPLC.

Clean-up by open-column chromatography on alumina was also used by Villalobos et al. [122] for corn flakes and sardines and by Romanov and Osipova [121] on plant materials. Bekhof and Van den Bedem [119], using a two-dimensional HPLC sys-

TABLE 7

SAMPLE PREPARATION FOR HPLC DETERMINATION OF VITAMIN D

For analytical HPLC conditions see Table 8.

Extraction	Chromatographic clean-up		Food	Analyte	Ref.
	Column	Mobile phase			
Saponify in ethanol–KOH extract with diethyl ether, (1) open-column clean-up, eluate evaporation; (2) semi-preparative HPLC, evaporate solvent, dissolve in hexane, analytical HPLC	*(1) Open column* Al_2O_3 *(2) Semi-preparative HPLC* Lichrosorb RP-18	Diethyl ether– hexane (40:60) ACN–methanol– water (50:50:5); flow, 1 ml/min	Various foods and feeds	Total vit. D	118
Extract with CH_2Cl_2–BHT– Na_3PO_4, shake for 1 h, filter, evaporate, dissolve and 4-step clean-up	*(1) Sep-pak* Silica *(2) Filtration* Teflon F.H.O., 5 μm *(3) GPC* Sephadex LH-20 *(4) Preparative HPLC* Partisil 10 PAC	1% Ethyl acetate in benzene, evaporate, dissolve in CH_2Cl_2 Evaporate, dissolve in $CHCl_3$ $CHCl_3$–isooctane (98:2), collect fraction at 29–56 min, evaporate, dissolve in CH_2Cl_2 Methanol–water (90:10)	Various feeds	Vit. D_2, D_3	126
Saponify in ethanol–KOH (water-bath, 30 min), extract with pentane. light coloured extracts: direct injection; coloured extracts, open column clean-up, evaporate and dissolve in hexane (column A), mobile phase (column B), methanol (column C)	*Open column* Al_2O_3 neutral	4% Acetone in hexane; 15% acetone in hexane to elute vit. D + vit. A band with fluorescence monitoring	Corn flakes, sardines	Vit. D_2, D_3	122
Saponify in ethanol–KOH (pyrogallol, 80°C, 30 min), extract with benzene, evaporate, dissolve in methanol–ACN (1:1), HPLC	*HPLC* Lichrosorb RP-18	Methanol–ACN (1:1)	Mushrooms	Vit. D_2	123
Saponify in ethanol–KOH (pyrogallol, 80°C, 30 min), extract with benzene, (1) TLC clean-up, $CHCl_3$ extract, (2) HPLC clean-up, HPLC analysis, (3) identification by MS	*(1) TLC* Silica gel *(2) HPLC* Lichrosorb RP-18	Benzene–acetone (95:5) Methanol–ACN (1:1)	Mushrooms	Vit. D_2 identification	124

TABLE 7 (continued)

Extraction	Chromatographic clean-up		Food	Analyte	Ref.
	Column	Mobile phase			
Saponify in ethanol–KOH (pyrogallol, overnight, room temperature), extract with hexane, evaporate, dissolve in $CHCl_3$, isomerization with $SbCl_3$ in $CHCl_3$	No clean-up		Milks	Total vit. D	127
Saponify, extract with benzene, HPLC clean-up, collect vit. D fraction, analytical HPLC	*HPLC* Nucleosil 5-C_{18}	ACN–methanol (3:2)	Baby foods	Vit. D_2	120
Saponify in ethanol–KOH extract with hexane	*Open column* Al_2O_3	2-Propanol– hexane	Plant materials	Vit. D_2	121
Saponify in ethanol–KOH (Na ascorbate, water-bath, 40 min) extract with pentane– BHT, evaporate to dryness, dissolve in the semi-pre-parative HPLC mobile phase	*HPLC* Si-60 D 10-CN	0.35% Pentanol in hexane, collect vit. D fraction, add BHT, dilute with 5% toluene in hexane	Milk	Vit. D	117
Saponify in ethanol–KOH (water-bath under N_2, + BHT, 30 min) extract in diethyl ether, evaporate, dissolve in methanol, semi-preparative HPLC, collect 24–32-min fraction, dry, dissolve in hexane	*Semi-preparative HPLC* LiChrosorb RP-8, 7 μm	Methanol–water (90:10), detection at 264 nm	Feeds	Vit. D	125

tem, carried out the clean-up on a CN-bonded phase, which was directly coupled to the analytical column via a six-way valve. Preparative RP-18 HPLC was used to separate the vitamin D fraction from the unsaponifiable matter of baby foods [120] and from mushrooms [123], while Laffi [125] used an RP-8 column for feeds. Takamura et al. [124], in order to identify vitamin D_2 in mushrooms by HPLC–thermospray MS, carried out a preparative TLC prior to the preparative RP-HPLC step.

When the saponification step is omitted to prevent isomerization, a more extensive clean-up procedure is necessary [128]. Hung [126] carried out a four-step clean-up procedure: a silica cartridge clean-up, followed by filtration through a Teflon membrane and then by GPC and semi-preparative

NP-HPLC. This procedure was claimed to be especially useful for determining low levels of vitamin D_2 and D_3 in complex matrices.

3.2.2. High-performance liquid chromatography

Both NPC [117,118,120,121,125,127] and RPC [126] have been used to determine total vitamin D (Table 8). The results obtained with AOAC official methods [117,118] by NPC have to be corrected for the effect of isomerization due to the high temperature applied during the saponification stage (factor: 1.25). However, vitamin D_2 and D_3 are resolved only by using RPC [126]; in fact, comparing the separation efficiency for vitamin D_2 and D_3 for three types of packing materials, using eight selected types of matrix after saponification and the

AOAC clean-up procedure [117,118], Villalobos *et al.* [122] found that RP columns separate vitamin D_2 and D_3, whereas an NP silica column does not resolve the two vitamers. Bekhof and van den Bedem [119] detected simultaneously the two vitamers using an NH_2-bonded phase. Generally a UV detector was used, either at a fixed wavelength of 254 nm or with multi-wavelength models set at 260 nm

[119] or at 301 nm, as carried out by Agarwal [127] in an isomerization method.

Takamura *et al.* [124] used MSD to identify vitamin D_2 in mushrooms. The choice of HPLC mode (RP or NP) depends on the aim of the analysis: for total vitamin D content NP is very efficient, whereas to separate vitamin D_2 from D_3 an RP phase is necessary, although an NH_2-bonded phase is re-

TABLE 8

CONDITIONS FOR HPLC ANALYTICAL DETERMINATION OF VITAMIN D

For sample preparation see Table 7.

Stationary phase	Mobile phase	Detection	Food	Analyte	Ref.
5-μm Partisil 5	0.35% Pentanol in hexane	UV 254 nm	Various foods and feeds	Total Vit. D	118
3 M C_{18} Rainin Accupack Microsorb		UV 254 nm	Feeds	Vit. D_2, D_3	126
(A) LiChrosorb Si 60, 5 μm	Hexane–2-propanol (98.3:1.7); flow, 1.5 ml/min	UV 254 nm	Corn flakes, sardines	Vit. D_2, D_3	122
(B) Vydac 201 TPB C_{18}, 10 μm	Methanol–$CHCl_3$– ethyl acetate (88:4:8); flow, 1.5 ml/min				
(C) Baker ODS, 5 μm	Methanol–water (98:2); flow, 1 ml/min				
Nucleosil 100-5, 5 μm	Hexane–1-pentanol– 2-propanol (99.5:0.1:0.4); flow, 1 ml/min	UV 254 nm	Mushrooms	Vit. D_2	123
Nucleosil 100-5, 5 μm	Hexane–1-pentanol– 2-propanol (99.5:0.1:0.4); flow, 1 ml/min	UV 254 nm, dissolve in CH_3COONH_4– methanol, thermospray MS	Mushrooms	Vit. D_2 identification	124
Silica Spherisorb, 3 μm	Hexane–ethyl acetate–methanol (97:2.5:0.5); flow, 0.7 ml/min	UV 301 nm	Milks	Vit. D	127
Zorbax Sil	2-Propanol–hexane (0.8:99.2)	UV	Baby foods	Vit. D_2	120
Silica gel	Hexane–$CHCl_3$	UV	Plant materials	Vit. D_2	121
5-μm Partisil	0.35% 1-Pentanol in hexane	UV 254 nm	Milk	Vit. D	117
LiChrosorb Si 60, 5 μm	7% Dioxane in hexane; flow, 1.0 ml/min	UV 264 nm	Feeds	Vit. D	125

ported also to be efficient in resolving the two vitamers.

All methods claimed a high recovery and high sensitivity for the different samples tested.

3.3. Vitamin E

Vitamin E is an essential nutrient and it is also added to some foods as an antioxidant. There are eight naturally occurring vitamin E vitamers: four tocopherols (α-, β-, γ- and δ-TOC) and the corresponding tocotrienols. They each have a different biological activity, with α-TOC being the most active and α-TOC and δ-TOC being the predominant forms occurring in foods.

Analysis for vitamin E in foods involves separation and individual determination of all the tocopherols, tocotrienols and, if supplemented, tocopheryl acetate. To measure the total vitamin E activity, it is not sufficient to determine α-TOC, as other tocopherols and tocotrienols, such as γ-TOC in vegetable oils and β-TOC in wheat bran, may be present in large amounts and can contribute significantly. In many methods β- and γ-TOC are measured as a combined fraction, whereas the tocotrienols are excluded completely.

Recent reviews [129–131] have discussed various methods for analysing vitamin E isomers, ranging from the spectrophotometric methods of Emmerie and Engel [286] to TLC, GC and HPLC. The last technique has predominated over the last few years, especially as specific detection methods can be employed and exposure to air and handling of the tocopherols can be minimized.

3.3.1. Gas chromatography

GC has long been a useful approach for the qualitative and quantitative determination of tocopherols in natural fats (Table 9). Tocopherols and sterols are found in the unsaponifiable fraction and both have a hydroxyl group, so they are separable by GC under similar conditions. Their GC analysis has been extensively studied, although few papers have been published recently.

Ulberth [132], comparing derivatization systems such as silylation and esterification, claimed that the use of heptafluorobutyril (HFB) esters gave an optimum separation between cholesterol and α-TOC with a capillary column and ECD and proposed this method as an alternative to HPLC for the determination of the vitamin E content in foods of animal origin.

A collaborative study [133] evaluated the feasibility of GC procedures for assaying α-tocopheryl acetate in supplemental vitamin E concentrate. The GC method was more specific and precise, because it separates α-tocopheryl acetate and α-TOC, whereas spectrophotometric methods measure total saponifiable isomers of vitamin E. This method was accepted as official by the AOAC in 1990 [134].

3.3.2. Thin-layer chromatography

TLC methods are usually employed for sample purification [135]. A comparative study [136] on the determination of tocopherols between direct spectrophotometric analysis and TLC with UV detection at 295 nm showed some differences in the results, especially for low tocopherol concentrations. Askinazi et al. [137] compared a modified TLC

TABLE 9

CONDITIONS FOR GC DETERMINATION OF TOCOPHEROLS

Compound	Detector	Column	Stationary phase	Solid support	Temperatures (°C)			Internal standard	Ref.
					Injector	Column	Detector		
α-Tocopheryl acetate	FID	2 m (glass)	SE-30	80–100 mesh	275	265	275	Hexadecyl palmitate	133,134
α-TOC, β-TOC, γ-TOC, δ-TOC Cholesterol	FID	20 m × 0.25 mm I.D. (silica)	DB-5	–	300	260 (60 s), 4°C/min to 300	300	Cholestane	132

TABLE 10

CONDITIONS FOR ANALYTICAL HPLC DETERMINATION OF VITAMIN E

Sample preparation	Stationary phase	Mobile phase	Detection	Food	Analyte	Ref.
Saponify with ethanol–KOH (26°C, 10 min, pyrogallol), dilute with water, extract with diethyl ether, wash organic layer with dilute HCl, evaporate to dryness, dissolve in hexane	Silica	2-Propanol–hexane (0.5:99.5); flow, 1 ml/min	Fluorescence, 290/330 nm (ex./em.); UV 292 nm	Vegetable oils, fats	All tocopherols, tocotrienols	148
Extract according to AOAC (1984) method, dilute with hexane–isopropanol (1:4)	Nucleosil 120-5 C_{18}, 5 μm	Gradient elution: (A) water–H_3PO_4, pH 3; (B) ACN–methanol (7:5); (C) 2-propanol; flow, 1 ml/min	UV, 215 nm UV, 280 nm	Sunflower oil	Phenolic antioxidants, tocopherols, triglycerides	131
Saponify with ethanol–KOH (reflux, 30 min, Na ascorbate) extract with LP, evaporate to dryness, dissolve in hexane	Partisil 5-Si, LiChrosorb Si-60	Isooctane–2-propanol (99:1); flow, 1 ml/min	Fluorescence, 293/326 nm (ex./em.)	Animal feeds	α-TOC	149
Saponify overnight with KOH, extract with hexane	Zorbax ODS, 5 μm	ACN–CH_2Cl_2–methanol (70:25:5)	Fluorescence, 290/330 nm (ex./em.)	40 food products	α-,δ-,γ-TOC	154
For RPC: dissolve in ethyl acetate. For NPC: dissolve in hexane. Chill overnight at −20°C, centrifuge at 4°C	Zorbax Sil Zorbax ODS	Hexane–2-propanol (99:1); flow, 2 ml/min ACN–methanol–CH_2Cl_2 (60:35:5); flow, 2 ml/min	DAD	Crude palm oil	All tocopherols, tocotrienols	147

Saponify in ethanol–KOH (pyrogallol, 8 min, 70°C), extract with hexane–diisopropyl ether (3:1), centrifuge, evaporate, dissolve in hexane	Radial Pak C_{18}, 5 μm	Methanol; flow, 1. ml/min	Various foods	UV, 210 nm; Fluorescence, 295/330 nm (ex./em.)	α-TOC, β-+γ-TOC, δ-TOC, cholesterol, phytosterol	139,146
Saponify with ethanol–KOH (30°C, overnight, under N_2), extract with hexane, evaporate to dryness, dissolve in methanol	Biosil ODS 5S	Methanol; flow, 1 ml/min	Chicken, meat	Fluorescence, 296/330 nm (ex./em.)	α-TOC, γ-TOC	155
Fully automatic extraction apparatus	LiChrosorb RP-18, 7 μm	Methanol; flow, 2 ml/min	Tablets	UV, 288 nm	α-TOC acetate	166
Extract fat according to AOAC (1984), saponify with ethanol–KOH and pyrogallol, extract with hexane	LiChrospher Si 60	1% 2-Propanol and 0.5% ethanol in hexane; flow, 1 ml/min	Infant formula	Fluorescence, 292/320 nm (ex./em.)	All tocopherols	156
Dilute with mobile phase	LiChrospher Si 100	Cyclohexane–H_4-folic acid (95:5); flow, 1.8 ml/min	Olive oil	UV, 295 nm	α-TOC, γ-TOC	157
Extract with $CHCl_3$–methanol (2:1), saponify with ethanol–KOH (30 min, 70°C), extract with NaCl solution and ethyl acetate–hexane (1:9)	Nucleosil 5-NH_2	Hexane–2-propanol (98.5:1.5); flow, 1 ml/min	Cereals legumes	Fluorescence, 295/325 nm (ex./em.)	All tocopherols	158

method for the determination of tocopherol vitamers with GC; both techniques gave consistent results.

3.3.3. High-performance liquid chromatography

3.3.3.1. Sample preparation. Tocopherols in animal and vegetable oils are directly determined after appropriate dilution of the sample with *n*-hexane. Andrikopoulos *et al.* [138] used 2-propanol–hexane (4:1) to prepare oil solutions. Other foods and animal feeds need a previous saponification step under a nitrogen flow. Indyk [139] described a simplified approach to saponification, which involves a single piece of glassware; the tediousness of conventional multi-step saponification was therefore overcome, sample preparation being completed within a single test-tube and the full procedure within 45 min.

Many factors affect the extraction ratio of tocopherols from the saponification medium, such as ethanol concentration in the medium, extraction solvents and co-existing fats. Ueda and Igarashi [140,141] surveyed the factors influencing *n*-hexane extraction from a saponified medium in food and biological samples. Takeyama *et al.* [142] and Ujie *et al.* [143] proposed *n*-hexane instead of diethyl ether as the extraction solvent, because the former is less volatile and contains fewer contaminating compounds. They found an improvement in the extraction ratio of tocopherols with the addition of up to 10% of ethyl acetate in hexane.

Håkansson *et al.* [144] proposed direct hexane extraction from wheat products using a Soxhlet apparatus.

Rizzolo *et al.* [145] evaluated the effect of the extraction technique on almonds, comparing overnight cold oil extraction under a nitrogen atmosphere with Soxhlet extraction followed by either dilution with mobile phase or cold saponification. They found that the cold extraction provided the best accuracy and precision for α- and γ-TOC, whereas it was not suitable for determining the small amounts of β-TOC.

3.3.3.2. Analytical high-performance liquid chromatography. Ball's review [1] covered HPLC methods up to 1987. There have not been many HPLC separations of just tocotrienols and tocotrienols with tocopherols. Methods from 1988 have reported in Table 10, where it can be seen that both NPC and RPC are commonly used.

The acknowledged stability and robustness of the ODS phases are considered to be advantageous for routine application; moreover, RPC is more efficient and versatile when we have to determine tocopherols with other lipids in the same run [138,146]. Unfortunately, the positional isomers β- and γ-TOC cannot be resolved, even if complex gradient elution is employed.

Tan and Brzuskiewicz [147] described an optimization study of seven tocopherol and tocotrienol standards using both NP and RP columns with various isocratic mobile phases. They compared C_{18} columns used in NARP chromatography, but none could separate the two positional isomers; on RP columns the less polar but more saturated tocopherols were retained in the stationary phase longer. NP columns provided separation based on the number and position of methyl substituents on the chromanol moiety, and succeeded in separating positional isomers. The study also considered cyano- and amino-bonded phases; the former was ineffective in the separation of all isomers, but the latter were comparable to silica columns in selectivity and resolution. In addition to the difficulty of separating the eight isomers, these compounds also have similar UV spectra; to characterize the isomers the use of DAD was proposed, with spectra recorded in second- and fourth-derivative form.

NPC allows the complete separation of all eight isomers; these methods were proposed as official methods of analysis from two comparative studies carried out by the IUPAC Commission on Oil, Fats and Derivatives [148] and by the Analytical Methods Committee of the Royal Society of Chemistry [149]. The IUPAC Commission examined also the potency of a Partisil PAC (amino- and cyano-bonded) column; this column is reported as acting as a highly polar stationary phase when used with a hydrophobic mobile phase [hexane–THF (9:1) was the proposed mixture for tocopherols analysis]. All of them achieved good results using this column and the Commission concluded that it was undoubtedly worth investigating the applications of this type of column when it becomes more widely available.

Both interlaboratory studies used FLD, because it allows selective and sensitive monitoring, without interference from artifacts, which can be a problem in some foods when UV detection alone is used.

Moreover, UV detection is at least ten times less sensitive then FLD, and needs an accurate choice of mobile phase composition and column packing material, because UV spectra are sensitive to environmental conditions. Using FLD, α-TOC is appropriate as the sole standard for the vitamin E congeners, as the fluorescence responses are essentially equivalent [139]. The only disadvantage is that, as esters of tocopherols are weakly fluorescent, fortified formulas containing tocopheryl acetate must be subjected to saponification before analysis, with possible degradation of vitamers.

An alternative to FLD could be ED, which needs aqueous RPC, as the supporting electrolyte is soluble in a semi-aqueous mobile phase. Ueda and Igarashi [150] evaluated ED for the determination of tocopherols in feeds; they found that the sensitivity was twenty times higher than with FLD and the DL for α-TOC by ED was 0.1 ng. Luscombe and Bond [151] claimed a DL for α-, γ- and δ-TOC down to 10^{-7} M using a surface-modified platinum microelectrode as detector in RPC analysis [methanol–water (95:5)]. MS can be used to allow the unequivocal characterization and determination of tocochromanols, both for detection in HPLC and as a direct analytical technique such as tandem MS, so minimizing the losses due to each manipulative stage [152].

Tri Wahyuni and Jinno [153] investigated the separation of tocopherols on various chemically bonded phases in microcolumn liquid chromatography. For NPC a naphthylethyl-bonded phase column with 0.1% hexafluoropropanol in n-hexane as the mobile phase gave the best separation. A polymeric C_{18} column with 8.5% n-hexane in ACN as the mobile phase gave the best results in RPC, the four tocopherols being well separated; microcolumn RPC could be developed as an optimum separation method in tocopherol analysis.

3.3.3.3. Preparative high-performance liquid chromatography. It is of commercial interest to isolate tocopherol isomers with a purity greater than 90%. Bruns et al. [159,160] achieved a direct scale-up from the analytical to the preparative mode by only a slight change in chromatographic conditions and isolated up to 4 g of 95.4% pure natural D-γ-TOC from 15 g of a vegetable oil extract injected each run (Table 11).

3.3.4. Supercritical fluid extraction and chromatography

SFE with CO_2 is now used in industry as a clean up, solvent-free separation process. A fundamental study was carried out to develop an extraction process for concentrating 18% tocopherols from soybean sludge using supercritical CO_2 [162]. Saito and co-workers [163,164] proposed directly coupled SFE–preparative SFC as a powerful method not only for analytical purposes but also for preparative separation. They achieved the isolation of tocopherols from wheat germ oil by recycling semi-preparative SFC using two 250 × 10 mm I.D. columns packed with 5-μm silica gel. This method has the potential capability of replacing conventional preparative HPLC because it effects extraction, preconcentration and chromatographic fractionation in a single run. The use of supercritical CO_2 allows the easy separation of solutes at a low temperature and in an oxygen-free environment, which is essential for separating labile compounds such as tocopherols. In addition, a non-flammable, non-polluting and inexpensive mobile phase is very helpful for safety and economy in the laboratory and in the production process.

Takeuchi and Saito [165] presented an example of the separation and identification of α- and β-TOC standards using on-line coupling of SFC to FAB-MS with a micropacked ODS column.

3.4. Vitamin K

Three forms of vitamin K vitamers are present in food: vitamin K_1 (phylloquinone, PK) of plant biosynthetic origin, vitamin K_2 (menaquinones, MK), a group of vitamers synthetized by bacteria ranging from MK-4 to MK-13, according to the number of isoprene units in the side-chain; and synthetic vitamin K_3 (menadione MD) and its water-soluble hydrogensulphite derivative (MSB). These compounds are very sensitive to light and are easily reduced to the hydroquinone forms. On this reactivity are based a number of chemical methods [167]. According to published reviews [167–169], there have been great developments in chromatographic techniques for vitamin K analysis in recent decades. However, whereas open-column methods, TLC and GC were widely applied until the 1980s [167,170], more recently HPLC has become more important

TABLE 11

CONDITIONS FOR PREPARATIVE HPLC SEPARATION OF VITAMIN E

Sample preparation	Stationary phase	Mobile phase	Detection	Food	Analyte	Ref.
Purify by silica open column, eluting with hexane	LiChroprep Si 60, 25–40 μm	Hexane–butyl methyl ether (97:3); flow, 450 ml/min	UV, 205 nm	Vegetable oils	α-TOC	159,160
Purify by silica open column, eluting with hexane–ethyl acetate (85:15)	HP Si 100	2–10% Ethyl acetate in hexane	UV, 254 nm	Palm fatty acid distillate	Tocotrienol	161

TABLE 12

SAMPLE PREPARATION CONDITIONS FOR HPLC DETERMINATION OF VITAMIN K

For analytical HPLC conditions see Table 13.

Extraction	Chromatographic clean-up		Food	Analyte	Ref.
	Column	Mobile phase			
Lipase treatment, extract with pentane, evaporate, dissolve in 2-propanol, semi-preparative HPLC, evaporate PK and MK-n fractions, dissolve in methanol, analytical HPLC	Nucleosil C_{18}, 5 μm, 35°C	Methanol–ACN (1:1), detection at 248 nm	Milks	PK, MK-4, MK-6, MK-7, MK-8, MK-9	171
Extract with $CHCl_3$ and aq.NH_3, neutralize with CH_3COOH, add Celite and Na_2SO_4, centrifuge, evaporate to dryness, dissolve in 1,2-dichloroethane	No clean-up		Premix	Menadione	173
SFE extraction, 15 min, CO_2, 8000 psi, 60°C, silica trap, elution with CH_2Cl_2 evaporate to dryness, dissolve in ACN	No clean-up		Animal feed	Menadione	180
SFE extraction, 15 min, CO_2, 8000 psi, 60°C, silica trap, elution with CH_2Cl_2– acetone (1:1), evaporate to dryness, dissolve in mobile phase	No clean-up		Infant formula	Phylloquinone	179
Preparative extraction using silica gel in N_2 atmosphere	Open-column SiO_2		Milk, colostrum	Vit. K	174
Homogenize in 2-propanol–hexane (66:34), centrifuge, evaporate hexane layer to dryness, dissolve in hexane, (1) Sep-Pak clean-up, evaporate to dryness, dissolve in hexane; (2) TLC, vitamin K band extracted with $CHCl_3$, evaporate to dryness, dissolve in ethanol	*(1) Sep-Pak* Silica *(2) TLC* Silica gel 60 F_{254}, 0.25 mm	Hexane–diethyl ether (96:4) LP–diethyl ether (85:15)	Various meats	Vit. K_1, MK-4, MK-5, MK-6, MK-7, MK-8, MK-9, MK-10, MK-13	175–178
Extract in water–methanol (60:40), centrifuge, MSB conversion to MD (5% Na_2CO_3 n-pentane), evaporate organic layer to dryness, dissolve in methanol	No clean-up		Animal feed	MSB	173

[1]. Open column and TLC are now sometimes applied as a clean-up procedure prior to HPLC injection. Hyphenated GC is preferably used for identification purposes.

3.4.1. Sample preparation

Owing to the high degradability of vitamin K, the samples cannot be submitted to alkaline saponification treatments [1,167]. Lipase hydrolysis for the extraction of PK and MK-5 from milk was chosen by Isshiki et al. [171] followed by a clean-up by preparative RPC, where vitamin K was monitored by UV detection (Table 12).

Mild alkaline hydrolysis of MSB extracted from animal feeds was carried out using ammonia solution [172] and Na_2CO_3 [173]. A preparative silica gel open-column method in a nitrogen atmosphere was used by Canfield et al. [174] to clean up the vitamin K extracted from human milk and colostrum.

K_1 and K_2 vitamers extracted from animal tissues [175] and vegetables [176] and K_3 vitamer from animal diets and food for livestock [177,178] were cleaned up in two steps: using a disposable silica column followed by a TLC on silica gel, collecting the vitamin K fraction by scraping off the band monitored by UV detection.

SFE extraction with CO_2 is a promising technique. Schneiderman and co-workers [179,180] developed a rapid, single-step sample preparation that allowed the quantitative extraction of PK from infant formula and of menadione from animal feeds within 15 min. No intermediate clean-up procedure was necessary, as the compounds trapped on silica gel were quickly eluted and then transferred to the HPLC mobile phase for direct injection.

3.4.2. High-performance liquid chromatography

NPC on a silica column was used only by Laffi et al. [172] for menadione determination, RPC mostly being applied [173,175–180] (Table 13). Two types of ODS columns were used by Isshiki et al. [171] for the separation of PK and MK-4 and for the MK vitamers, respectively. Canfield et al. [174] used a two-steps RP and two detectors.

The detection mode is the most interesting aspect of the development of newer HPLC methods. Three types of detectors have been reported. UV detection was used by Laffi et al. [172] for menadione determi-

nation and by Canfield et al. [174] in the first gradient step of HPLC analysis. This is a traditional detection mode [1]. The behaviour of the electrochemical reduction of 1,4-naphthoquinones was reviewed by Cadenas and Ernster [169], who pointed out the advantages of sensitivity and reliability of ED for K vitamers. Different types of ED were applied with either commercial or laboratory constructed detectors. The DL and the linearity were reported to be excellent: 2–5 ng for PK and 1–30 ng for MK-4 [171] and 125 ng for menadione [180]. The postcolumn formation of fluorescent reduced forms for FLD is a recent technique; the effluent from the analytical column is passed through a reduction cell and then reaches the detector in-line. Both chemical and electrochemical reduction were applied. Sakano et al. [176] for PK analysis in vegetables and Notsumoto et al. [177] for menadione in animal feed used $NaBH_4$ as the reducing agent, while Billedeau [173] used a column filled with zinc powder in-line between the HPLC column and the detector. Electrochemical reduction was used by Hirauchi et al. [175], coupling a commercial coulometric detector in line prior to the fluorescence detector. A simultaneous reduction and FLD of vitamin K_1 in a polyvitamin premix was investigated by Indyk [181]. The fluorescence was directly induced on the phylloquinone flowing through the flow cell of a recently developed commercial fluorescence detector which uses an intense (150 W) xenon source coupled with advanced electronics. This results in enhanced sensitivity (DL ca. 10 ng) which could help the development of FLD without postcolumn reduction devices.

3.4.3. Other chromatographic techniques

Haiduc et al. [182] determined vitamin K_3 in food supplements by GC on Chromosorb G with FID. They also used TLC on silica gel with a fluorescent indicator, followed by UV spectrophotometric detection at 267 nm of the extracted bands; they claimed a relative standard deviation of 5% in the range 4–40 μg/ml.

Coupled GC techniques (Table 14) have been applied to confirm the identification of the extracted vitamers from different matrices. Schneiderman et al. [180] identified menadione extracted from animal feeds by SFE by means of GC–MS. Billedeau [173] used GC–FT-IR for the identification of men-

TABLE 13

ANALYTICAL CONDITIONS FOR HPLC DETERMINATION OF VITAMIN K

For sample preparation see Table 12.

Stationary phase	Mobile phase	Detection	Analyte	Food	Ref.
For PK and MK-4: Partisil ODS 2 (5 μm). For MK-6–MK-9: Partisil ODS 3 (5 μm)	Methanol–ethanol–60% HClO$_4$ (60:40:0.12) containing 0.05 M NaClO$_4$; flow, 1 ml/min	ED, two GCE in series	PK, MK-4, MK-6, MK-7, MK-8, MK-9	Milk	171
LiChrosorb Si 60, 45°C	1,2-Dichloroethane; flow, 1.8 ml/min	UV, 251 nm	Menadione	Animal feeds	172
μBondapackC$_{18}$, 10 μm	ACN–0.25 M NaClO$_4$ (90:10)	ED (Ag electrode), −8.75V vs. SCE	Menadione	Animal feeds	180
μBondapack C$_{18}$, 10 μm	ACN–CH$_2$Cl$_2$–0.025 M NaClO$_4$ (90:5:5)	ED (Ag electrode), −1.1V vs. SCE	Phylloquinone	Infant formula	179
C$_{18}$, two-step elution	(1) Gradient elution; (2) isocratic elution, ethanol–hexane–water (90:6.5:3.5)	(1) UV; (2) ED	Vit. K	Milk colostrum	174
Nucleosil C$_{18}$, 5 μm	92.5% or 97.5% ethanol containing 0.25% NaClO$_4$; flow, 1 ml/min; post-column coulometric reduction	Fluorescence, 320/430 nm (ex./em.)	Vit. K$_1$, MK-4, MK-5, MK-6, MK-7, MK-8, MK-9, MK-10, MK-13	Various meats	175
Nucleosil C$_{18}$, 5 μm	92.5% ethanol, postcolumn alkaline NaBH$_4$ reduction	Fluorescence, 320/430 nm (ex./em.)	(a) Vit. K$_1$, K$_2$; (b) menadione	(a) Various foods; (b) premix	(a) 176; (b) 177
Chemcosorb ODS-NH	45% Dioxane containing 0.2% NaClO$_3$, postcolumn coulometric reduction	Fluorescence, 320/430 nm (ex./em.)	MSB	Food from livestock	178
Supelcosil LC-18, 5 μm	Methanol–water (75:25), in-line postcolumn reduction with 20 × 2 mm I.D. column filled with Zn	Fluorescence, 325/425 nm (ex./em.)	MSB	Feeds	173

adione in feed extacts to confirm the results of a previously carried out HPLC method. The GC–FT-IR method gave a DL of 2 mg/kg $versus$ 20 μg/kg in HPLC; therefore, it must be reserved for the qualitative analysis of menadione in synthetic feeds. GC coupled with automatic retrieval of spectra from computer libraries can be very useful in comparing analytical data and in evaluating the identification and purity of vitamin K extracts.

4. WATER-SOLUBLE VITAMINS

4.1. Thiamine (vitamin B$_1$)

4.1.1. High-performance liquid chromatography

The technique of extraction adopted (Table 15) is influenced by the mode of separation and detection selected. To detect thiamine and some of its derivatives, such as esters or coenzymes by using UV

TABLE 14

COUPLED GC METHODS FOR VITAMIN K IDENTIFICATION

Sample preparation	GC conditions	Detection	Analyte	Ref.
Extract in water–methanol (60:40), centrifuge, MSB conversion to MD (5% Na_2CO_3 in n-pentane), evaporate organic layer to dryness, dissolve in methanol	30 m × 0.32 mm I.D. DB-1701 column, 80°C (30 s), 16°C/min to 250°C (15 min); splitless injection, 200°C	FT-IR	MSB	173
SFE extraction, 15 min, CO_2, 8000 psi, 60°C, silica trap, elution with CH_2Cl_2, evaporate to dryness, dissolve in ACN	30 m × 0.25 mm I.D. DB-5 column, 150°C (2 min), 10°C/min to 250°C; splitless injection	Quadrupole MSD	Menadione	180

assay at 254 nm, a simple acid extraction followed by clean-up through disposable columns has been adopted. On the other hand, when the aim is to evaluate thiamine after derivatization to fluorescent thiochrome, an acid extraction with autoclaving, followed by enzymatic hydrolysis in the presence of diastatic and phosphorolytic enzymes and protein precipitation with trichloroacetic acid is used. The derivatization may be either pre- or postcolumn and consists of oxidation of the free thiamine with alkaline potassium hexacyanoferrate (III). This reaction is not stoichiometric, but it is reproducible under standard conditions.

Almost all the methods [19,183] employ RPC, using appropriate mixtures of methanol and either water or buffer solutions, with or without ion-pair modifiers. In most methods, however, the RP conditions chosen led to the co-elution of thiochrome monophosphate with thiochrome [184]. The DL depends on the method used: fluorimetric assay is more sensitive than UV assay. The latter technique could be suitable only for the determination of large amounts of thiamine (greater than 30 ng), because of the poor shape of the peak (broad with tailing), which further limits the sensitivity and increases the risk of interference from other components present in the extract [185]. The sensitivity and selectivity can be greatly increased by conversion to the fluorescent derivative thiochrome; in fact, FLD has a DL of 0.1 pmol [186]. On the other hand, Bertelsen et al. [184] found linear detector responses within the range 0.05–0.50 μg/ml of thiamine, with a DL of 0.5 μg per injection. They also reported that the

relative standard deviation for the conversion of thiamine to thiochrome was 1.6%. Fernando and Murphy [187] proposed the neutralization of the precolumn-oxidized thiamine with concentrated H_3PO_4 in order to ensure a pH level acceptable to the C_{18} column and to eliminate possible pH-dependent alkaline degradation of thiochrome to its disulphide.

Nicolas and Pfender [188] described a simple sample preparation procedure without clean-up, which involves a two-step pH adjustment to deproteinize the sample followed by filtration, which provides a pure thiamine peak when the extract is chromatographed by IP-RP. Minor changes in the amount of IP reagent strongly affect the retention of thiamine, allowing the separation of the thiamine peak from potential interferents. Moreover, they also maximized the sensitivity of the method, minimizing the dilution during sample preparation, optimizing the UV detector response and using a large injection volume (100 μl).

4.1.2. Gas chromatography

Thiamine is a heat-sensitive and non-volatile compound, hence it cannot be directly determined by GC. Early attempts to prepare volatile derivatives of thiamine include the O-benzoyl, trimethylsilyl and trifluoroacetyl derivatives [189], but all these compounds have low volatilities at low temperatures and decompose at temperatures above 250°C. A group of indirect methods have therefore been developed, involving pretreatment with sulphite, which splits thiamine into the 5(2-hydroxy-

TABLE 15

CONDITIONS FOR HPLC DETERMINATION OF THIAMINE

Sample preparation	Stationary phase	Mobile phase	Detection	Food	Ref.
Add 0.05 M H_2SO_4, autoclave for 30 min at 121°C, adjust pH to 4.6 with NaOH, dilute with water, filter through paper and 0.45-μm membrane	μBondapak C_{18}	Methanol 50 ml, acetic acid 5 ml, PIC B_5 12.5 ml, adjust to 1 l; flow, 1.2 ml/min	UV, 254 nm	IMF	191
According to AOAC [193] as modified by Ang and Moseley [194]. Conversion of thiamine to thiochrome	μBondapak C_{18} plus RP guard column	Water–methanol (70:30); flow, 1.5 ml/min	Fluorescence, 365/435 nm (ex./em.)	Chicken	184
Add 0.1 M HCl, heat for 30 min at 100°C, adjust pH to 4.5 with CH_3COONa, enzymatic digestion (β-amylase and takadiastase), filter through 0.2-μm cellulose acetate membrane. Conversion of thiamine to thiochrome. Purification on Sep-Pak	μBondapak C_{18}	Methanol–0.05 M CH_3COONa (pH 4.5) (60:40); flow, 1 ml/min	Fluorescence, 366/435 nm (ex./em.)	Dietetic foods	192
Add 5 M HCl, autoclave at 20 p.s.i. for 15 min, adjust pH to 4.5 with NaOH, centrifuge, filter through paper and 0.45-μm membrane. Thiamine conversion to thiochrome. Neutralization with H_3PO_4	Ultrasphere C_{18}	Acetonitrile–0.01 M acetate buffer (pH 5.5) (13:87); flow, 1.2 ml/min	Fluorescence, 366/435 nm (ex./em.)	Soybean, tofu	187
Dilute with water, adjust pH to 1.7–2.0 with 6 M HCl, add 5 M NaOH to pH>4.0, adjust to volume with water, filter through paper	μBondapak C_{18} 50°C	0.15% Na hexane-sulphonate, 1.5% CH_3COOH, 0.1% EDTA, 20% methanol; flow, 2.5 ml/min	UV, 248 nm	Milk, infant formula	188

ethyl)-4-methylthiazole (HEMT) derivative. Among them, the method of Velísek and co-workers [189,190] involves acid and enzymatic (takadiastase) hydrolysis, cleavage by sulphite, extraction of the resulting HEMT with chloroform and direct GC analysis of the concentrated extract on a Carbowax 20M column at 220°C with the injection port and detector (FPD) temperatures set at 250°C. The sensitivity of the determination was about 0.1 μg/ml, comparable to thiochrome method results.

Velísek et al. [189] claimed some advantages of their GC method over HPLC and thiochrome methods: any gas chromatography equipped with FPD can be used; the clean-up procedures on base-exchanger silicates may be omitted; the method is applicable even in the presence of materials which either absorb thiamine or affect thiochrome fluorescence. On the other hand, an important draw-back of this GC method is that the reaction cannot be performed in an "in-line" procedure, although this can be carried out for the HPLC analysis using the post-column thiochrome reaction.

4.2. Flavins (vitamin B_2)

Vitamin B_2 is a naturally fluorescent compound that occurs in foods in three principal forms: riboflavin (RF), flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). The chromatographic analysis provides a powerful tool for determining vitamin B_2 forms and for separating them from interfering substances, overcoming some of the drawbacks of the wet chemistry methods [196].

Early methods involved adsorption column chromatography, sometimes coupled with TLC, and fluorescence densitometric assay [197–199]. Nowa-

days, using HPLC we can determine simultaneously total riboflavin with other water-soluble vitamins [19,183].

4.2.1. High-performance liquid chromatography

Generally, HPLC methods for determining total RF are based on RPC both on C_{18} [200–203] and either on C_8 or on C_{18} in IP-RPC [187,204,205], although some procedures involving NPC have been proposed [206] (Table 16). Russell and Vanderslice [207] used polymer-based columns with an ACN–citrate–phosphate buffer gradient at 40°C in order to achieve the resolution of RF, FMN, FAD and 7-ethyl-8-methylriboflavin (I.S.). FLD is generally used, although some workers [202] applied UV detection. Even in this case, UV detection has lower sensitivity and specificity compared with FLD [207].

In several of the HPLC methods sample chromatograms suffer from poor resolution of the total RF peak, making its integration and quantification questionable.

TABLE 16

CONDITIONS FOR HPLC DETERMINATION OF FLAVINS

Sample preparation	Stationary phase	Mobile phase	Detection	Food	Ref.
Add aq. CH_3COOH to pH 3, stir, centrifuge for 30 min, filter through 0.22-μm membrane	Cosmosil 5 C_{18}	Water–methanol–CH_3COOH (65:45:0.1); flow, 1 ml/min	UV, 254 nm	Milks	215
Grind to smooth paste, acid hydrolysis (0.125 M H_2SO_4, 121°C for 30 min), adjust pH to 4.6, enzymatic digestion (takadiastase, 25 min; papain, overnight), add TCA, heat for 5 min at 50–60°C, filter	Silica, 10 μm	Acetate buffer; flow, 1 ml/min	Fluorescence, 425/525 nm (ex./em.)	Blanched soya beans	206
Acid–enzymatic hydrolysis according to AOAC [196], filter through 0.45-μm membrane. For high-fat samples: extraction with hexane prior to filtration	LiChrosorb RP-8	5 mM hexanesulphonic acid–methanol (60:40); flow, 1 ml/min	Fluorescence, 440/565 nm (ex./em.)	Various foods	204
Degas under vacuum, filter	μBondapak C_{18}	Methanol–water (40/60); flow, 1 ml/min	Fluorescence, 450/525 nm (ex./em.)	Wines, champagnes	200
Add 0.1 M HCl, heat (100°C for 30 min), adjust pH to 4.5, enzymatic digestion (β-amylase and takadiastase), filter through 0.2-μm cellulose acetate membrane	μBondapak C_{18}	Methanol–0.05 M CH_3COONa (pH 4.5) (60:40); flow, 1 ml/min	Fluorescence, 422/522 nm (ex./em.)	Dietetic foods	192
Autoclave at 121°C for 15 min in HCl, adjust pH to 4–4.5, enzymatic digestion (takadiastase, 48°C, 3 h), filter through paper. Purification on Fluorisil and on C_{18} disposable columns	μBondapak C_{18}	Methanol–CH_3COOH–water (32:1:67), 5 mM Na hexanesulphonate	UV, 254 nm	Legumes, milk powders	195, 205
Homogenize in methanol and CH_2Cl_2 after addition of 7-ethyl-8-methylriboflavin (I.S.) Add 100 mM citrate–phosphate buffer (pH 5.5) containing 0.1% NaN_3, homogenize, centrifuge, filter	Act-I 80 A or 2 PLRP-S 100 A in series	Acetonitrile–10 mM citrate–phosphate buffer (pH 5.5) in gradient	Fluorescence, 360/550 nm (ex./em.)	Various foods	211

CHROMATOGRAPHIC DETERMINATION OF VITAMINS   135

Generally, the method of extraction is similar to that for thiamine, *i.e.*, mineral acid/enzymatic extraction according to the AOAC official method [196], sometimes with some modifications [19,183]; this extraction method converts the protein-bound FMN and FAD into free forms and hydrolyses the phosphate forms to free RF, in order to measure the total vitamin B_2 activity. However, the hydrolysis step with takadiastase causes fluorescent interference in the sample extract, which could cause an overestimation of RF when FLD is used [187,205]. Hence a sample purification and concentration step using disposable cartridges, whose efficiency should be tested regularly and discharged if the amount of vitamin retained is less than 95% [208], is necessary.

To enhance the sensitivity of UV detection (DL 0.4 ng per injection), Vidal-Valverde and Reche [205] used a two-stage purification on Fluorisil and on a Sep-Pak C_{18} cartridge.

Hou and Wang [209,210] selectively detected RF and FAD utilizing a thin-layer amperometric detector with two electrodes in series. They found a DL of 40 ng for FAD and 4 ng for RF, and claimed that the dual-electrode detection offers superior selectivity over UV detection. Ollilainen *et al.* [203] compared the performances of FLD and UV detection and found a DL for RF of 20 pg per injection using FLD and 0.25 ng per injection using UV detection at 254 nm.

To determine simultaneously RF, FMN and FAD fluorimetrically in various foods, Russell and Vanderslice [211] developed a two-step non-degradative extraction procedure, using methylene chloride (to remove lipid artifacts and RF photodegradation products such as lumiflavin and lumichrome), methanol and 100 mM citrate–phosphate buffer (pH 5.5) containing 0.1% sodium azide (to maintain the pH within the range of flavin stability and to provide phosphate anion, as an inhibitor of phosphatase enzymes). They reported DLs of 0.21 ng for RF, 0.89 ng for FMN and 11.15 ng for FAD. To determine an RF content lower than 0.01 mg per 100 g, a trace enrichment technique could be used, by loading successive injections of the sample extract on to an RP guard column using water as mobile phase, the RF then being eluted as a sharp band by changing the mobile phase to methanol–water [208].

4.2.2. Other techniques

For qualitative studies, some advanced methods have been proposed recently. Asakawa *et al.* [212] developed an LC–MS system to overcome the problems imposed by the use of buffers as mobile phases. The RF peak is heart cut from the analytical column effluent, passed into sampling loops and adsorbed on a trapping column after dilution with the analytical mobile phase. Buffer constituents are washed out and the RF is eluted from the trapping column and re-chromatographed with a suitable mobile phase for its LC–frit-FAB-MS determination. The authors claimed that the system provides high sensitivity (300 ng per injection).

Kenndler *et al.* [213] developed a capillary zone electrophoretic (CZE) method to detect impurities (RF and RF mono- and diphosphates) in RF-5′-phosphate. They used a fused-silica separation capillary (100 cm × 100 μm I.D.), mounted in an HPLC–FLD system. This was modified by Kurosu *et al.* [214] in an immersed flow cell, improving the sensitivity of FLD in CE.

4.3. Niacin group

Niacin is found in foods as free acid (nicotinic acid) and the corresponding amide (nicotinamide); they exist as analogues in pyridine nucleotides (NAD and NADP). It is present also as nicotinoyl esters or NADH, which are not metabolically available. The determination of these compounds in foods is mainly carried out by using HPLC, although some workers prefer GC separations.

4.3.1. High-performance liquid chromatography

Depending on the form of the vitamin to be determined and the type of food, various methods of extraction have been developed (Table 17). Both acidic [216] and alkaline [217,218] extraction media have been used; however, it has been reported that the acidic extract could contain part of the niacin in bound form, which is therefore not available for the chromatographic analysis; in fact, HPLC values for the acid extract are consistently lower than those of alkaline extracts and of microbiological assay [208]. Therefore, when the purpose of the analysis is to determine the available niacin in foods, acid hydrolysis is preferred, as the alkaline procedure would release non-available niacin [219].

TABLE 17

CONDITIONS FOR HPLC DETERMINATION OF NIACIN

Sample preparation	Stationary phase	Mobile phase	Detection	Food	Ref.
Homogenize with ethanol, centrifuge, pass through Na_2SO_4 column, concentrate	LiChrosorb RP-18	Phosphate buffer–methanol–ACN–Bu_4NBr	UV, 260 nm	Meat	221
Homogenize in water, boil for 10 min, cool, filter through 0.45-μm membrane	Partisil SCX	50 mM phosphate buffer (pH 3)	UV, 260 nm	Meat	220
Add H_3PO_4, extract with methanol, homogenize, filter through paper and 0.45-μm membrane	FLC ODS, 50°C	1 mM Na dodecyl-sulphate–20 mM H_3PO_4–methanol (7:3) (pH 2.4)	UV, 261 nm	Meat, tuna	216
Extract in $Ca(OH)_2$, autoclave (15 min, 121°C), filter, adjust pH to 6.5 with oxalic acid, filter, clean-up on C_{18} column	LC-18-DB	Acetonitrile–0.1% H_3PO_4 and 0.1% Na dodecyl sulphate in water (23:77)	UV, 254 nm	Semolina, cheese, meat	218
Extract in 20% NaOH (100°C, 30 min), adjust pH to 7 with HCl, filter, clean-up on anion- and cation-exchange columns, filter through 0.45-μm membrane	Asahipak NH_2P-50	Acetonitrile–water (60:40) containing 0.075 M CH_3COONa	UV, 261 nm	Various foods	217
Acid hydrolysis (HCl, 121°C, 15 min), adjust pH to 4.5, enzymatic hydrolysis (takadiastase, 48°C, 3 h), filter through paper, clean-up on Dowex 1-X8	μBondapak C_{18} or Spherisorb ODS-2	Methanol–0.01 M CH_3COONa (pH 4.66) (1:9) containing 5 mM Bu_4NBr	UV, 254 nm	Legumes, meats	219

To determine both nicotinic acid and nicotin-amide, a simple extraction with water [220] or with ethanol [221], followed by clean-up and/or filtration, is used. According to Vidal-Valverde and Reche [219], enzymatic hydrolysis after acid treatment is essential with legume samples, because of the high starch content of the hydrolysate. Further, the same workers stressed the need for the purification step prior to the chromatographic determination. They tested several purification methods and found that, using Dowex 1-X8 acetate resin conditioned with 1 M HCl, an optimum purification was achieved. The separation is generally carried out by IP-RP. As niacin has both acidic and basic groups, the IP reagent may be either an alkyl sulphate [216,218] or a quaternary ammonium salt [221].

However, some drawbacks have been reported: NP requires a long analysis time, whereas in the RP mode niacin moves with the solvent front or yields poor peaks, with tailing [222]. The IP-RP separation mode does not always ensure good retention time stability, and UV detection shows low sensitivity and selectivity in the presence of interfering compounds. To overcome these problems, Balschukat and Kress [223] proposed for feed products, a column-switching procedure in order to have an adequate separation of niacinamide from interferents; it performs the preparation of the niacinamide fraction on an RP column, followed by switching of the vitamin band on to a cation-exchange column for the determination, obtaining a complete chromatographic separation within about 30 min. The problems of low sensitivity and interferences could be overcome by using RP conditions with a gradient elution programme [208].

To improve the DL, a new approach, proposed for pharmaceutical products, could be the derivatization of niacin with N,N'-dicyclohexyl-O-7(meth-ylcoumarin-4-yl)methylisourea (DCCI) to give a

highly fluorescent derivative; according to Finglas and Faulks [208], however, the use of this technique with food extracts could produce co-fluorescent compounds, which can require extensive sample clean-up and extract purification.

Using UV detection at 261 nm, Tsunoda et al. [216] found for both nicotinic acid and nicotinamide a DL of 0.1 mg per 100 g, while Balschukat and Kress [223] with UV detection at 264 nm coupled to a switching technique found for nicotinamide a DL of 0.5 ppm. A smaller amount of niacin (0.01 mg%) could be detected only when impurities arising from the hydrolysis process are removed by a proper clean-up procedure. For example, Hirayama and Maruyama [217] proposed a general procedure that can be used for various types of foods (high fat or protein contents, liquid or solids) consisting of alkaline hydrolysis, neutralization with acid, addition of the same volume of methanol to perform effective sample filtration for high-protein or carbohydrate-rich samples, elution from an AG 1-X8 anion-exchange column and additional clean-up on an IC-SP M cation-exchange column.

Tyler and Genzale [218] improved the UV detection limit to 0.004 mg per 100 ml by injecting niacin in a large volume (200 μl) of dilute H_3PO_4 which has a peak-sharpening effect and results in an increase in plate count approximate twenty times more than would be expected with an RP-18 phase.

4.3.2. Gas chromatography

The analytical approach differs according to the form of the vitamin to be determined (nicotinic acid or nicotinamide) (Table 18).

To detect nicotinamide, the simplest method involves its direct determination by GC with FID [224], but it lacks sensitivity. Another attempt involves the determination of nicotinamide as methyl nicotinate [225] by GC–FID, after ion-exchange separation, decomposition of nicotinamide and methylation in the presence of methanol and HCl. This method, however, is not specific and it is time consuming owing to the long time required for the methylation step.

Tanaka et al. [226] proposed a simpler procedure, which determines nicotinamide in meats and meat products after dehydration to 3-cyanopyridine with heptafluorobutyric anhydride. The product is over six times more sensitive than nicotinamide in GC

and, after the reaction, no clean-up stage is necessary. They reported that the various possible interfering compounds present in the sample do not hinder the production and determination of 3-cyanopyridine. With this procedure they found a DL of 5 ppm and an average recovery of 98%.

To detect the total niacin content in various vegetables and animal products, Velísek et al. [227] esterified the free nicotinic acid with ethanol and after extraction, determined the ester by cGC on OV-1 using selective nitrogen–phosphorus detection (NPD).

4.4. B_6 vitamers

There are various biologically forms of vitamin B_6 usually present at very low levels in foods: pyridoxal (PL), pyridoxamine (PM), pyridoxine (PN), pyridoxal-5'-phosphate (PLP), pyridoxamine-5'-phosphate (PMP), pyridoxine-5'-phosphate (PNP) and the inactive pyridoxic acid (PA). Most of the methods proposed for the determination of vitamin B_6 have been based on HPLC, although other chromatographic techniques, such as GC [190] and MECC, have been used.

4.4.1. Sample preparation

Acidic media coupled with high temperatures (0.1 M H_2SO_4–HCl, 121°C) are required to denature proteins and to disintegrate the sample matrix, in order to promote extraction [19,183, 228–231]. However, these conditions cause the complete hydrolysis of the phosphate esters PNP, PLP and PMP to PN, PL and PM [232], respectively, and of PN-glucoside [233].

To determine all the vitamers individually, milder conditions, which do not cause dephosphorylation or deconjugation, such as an extraction medium containing a deproteinizing agent, has to be used [19,233]. The most commonly used deproteinizing compounds are perchloric acid [234], trichloroacetic acid (TCA) and sulphosalicylic acid (SSA). TCA can be removed from the extract by extraction with diethyl ether, while perchloric acid, which also extracts PA, can be precipitated by neutralization with KOH [19,233]. SSA is the most effective in deproteinizing samples and extracting B_6 vitamers, preventing enzymatic interconversion, but it is highly fluorescent so it must be removed by ion-

TABLE 18

CONDITIONS FOR GC DETERMINATION OF NIACIN

Compound	Detection	Column	Stationary phase	Solid support	Temperatures (°C)			Derivatization	Ref.
					Injector	Column	Detector		
Nicotinic acid	NPD	25 m × 0.32 mm I.D. (silica)	OV-1 (0.25 μm)	–	230	50 (36 s), 20°C/min to 200	300	Esterification with ethanol	227
Nicotinamide	FID	2 m × 3 mm I.D. (glass)	5% OV-17	Chromosorb W AW DMCS (80–100 mesh)	190	130	190	Dehydration with heptafluorobutyric anhydride to 3-cyanopyridine	226

exchange chromatography prior to FLD [228,235]. This procedure leads to sample dilution, which makes the detection of low concentrations of B_6 vitamers difficult. To verify the presence of PLP and PMP, Bitsch and Möller [234] treated the samples with alkaline phosphatase (30 min, 25°C).

To determine all forms of vitamin B_6 in various food samples, Gregory and Sartain [232] evaluated the efficacy of both enzymatic and acid-catalysed hydrolyses of glucosylated forms of vitamin B_6. The enzymatic treatments were selective and included incubation with β-glucosidase, to measure PN-glycoside and other forms of vitamin B_6, and acid phosphatase treatment for the selective dephosphorylation of PLP and PMP. They found that the combination of these two enzymatic treatments would be useful in reducing the problem of interference in the measurement of PLP when the SSA extraction procedure is used.

TABLE 19

CONDITIONS FOR HPLC DETERMINATION OF B_6 VITAMERS

Sample preparation	Stationary phase	Mobile phase	Detection	Food	Ref.
Extraction with MPA	Biosil ODS-5S	0.066 M KH_2PO_4 buffer, (pH 3); flow, 1 ml/min	Fluorescence, 290/395 nm (ex./em.)	Meats	229
Bound vitamers: autoclave (0.44 M HCl, 2 h), add SSA, stir, remove SSA with AG 1-X8 anion-exchange column. Nonbound vitamers: homogenize in 0.01 M CH_3COONa (pH 6.8), filter through paper and adjust pH to 4.7	RCM-100, 4 μm	(A) 0.033 M H_3PO_4, octane- and heptanesulphonic acid in water–2-propanol (97.5:2.5), pH 2.2. (B) 0.33 M H_3PO_4 in water–2-propanol (82.5:17.5), pH 2.2; flow, 1 ml/min	Fluorescence, 338/425 nm (ex./em.). Postcolumn reaction with $NaHSO_3$	Potatoes	228
Autoclave in 0.1 M H_2SO_4 at 121°C for 30 min, centrifuge	Novapak C_{18}, 5 μm, 35°C	Methanol–5 mM hexanesulphonic acid in 1% CH_3COOH (8:92); flow, 1.5 ml/min	Fluorescence, 290/390 nm (ex./em.)	Various foods	230
(1) All vitamers: homogenize in 0.1–0.5 M $HClO_4$, centrifuge, adjust pH to 7.5 with KOH, filter, adjust pH to 4 with HCl, filter through 0.45-μm membrane. (2) Esters: adjust pH to 4, enzymatic treatment (alkaline phosphatase, 30 min, 25°C), filter through 0.45 μm membrane	Lichrospher RP-18, 5 μm	(A) Methanol (B) 0.03 M phosphate buffer (pH 2.7) plus 4 mM octanesulphonic acid. Gradient of B from 90% to 69%, then to 90%	Fluorescence, 330/400 nm (ex./em.). Postcolumn reaction with $NaHSO_3$	Pork, liver, milk	234
Mix with 5% SSA, add I.S. solution (600 nmol/l 4-deoxypyridoxine), homogenize, add CH_2Cl_2, centrifuge, purify by anion-exchange chromatography, then either enzymatic hydrolysis (β-glucosidase, pH 5, 37°C, 2.5 h) or incubation in 6 M KOH (in the dark, 20°C, 3 h)	Ultrasphere ODS	(A) 0.033 M H_3PO_4–8 mM octanesulphonic acid (pH 2.2). (B) 0.033 M H_3PO_4–2-propanol (83:17). Gradient: 0 min, 17% B; 11 min, 31% B; 15 min, 100% B; held for 15 min; flow, 1 ml/min	Fluorescence, 295/405 nm (ex./em.)	Various foods	232
Add 0.5 M HCl, sonicate (10 min, 40°C), then autoclave (105°C, 10 min), add methanol, filter through paper, add TCA, (50°C, 10 min), centrifuge, adjust pH to 7, filter through 0.45-μm membrane	Novapak C_{18}	Methanol–5 mM hexanesulphonic acid in CH_3COOH (8:92); flow, 1.5 ml/min	Fluorescence, 290/395 nm (ex./em.)	Various foods	234

4.4.2. High-performance liquid chromatography

RP methods are of general use with various kinds of columns, eluting with isocratic mobile phases [19,183,236], while IP-RPC is used both isocratically [230,231] and with gradients [228,232,234] (Table 19).

Bitsch and Möller [234], employing IP-RPC with a binary gradient, achieved very good separations of all vitamers and PA in a relatively short time (about 30 min); the method was also highly reproducible.

PLP, the first B_6 vitamer eluted, can be separated from the solvent front more effectively using an Ultrasphere IP column than any other commercially available column [232]. However, to alleviate the problems encountered in the measurement of PLP in direct analysis, such as the presence of peaks eluted in the vicinity of PLP which render peak integration difficult or impossible, Gregory and Sartain [232] proposed an acid phosphatase treatment to hydrolyse PLP to PL.

Detection was mainly fluorimetric [229–232] and to enhance the fluorescence intensity some workers [228,234,235] carried out the $NaHSO_3$ postcolumn reaction of Coburn and Mahuren [237,238]. Bitsch and Möller [234] observed that the interferences due to matrix substances are minimized with this postcolumn reaction, because of the simultaneous shift from acidic to weakly alkaline pH.

Gregory and Sartain [232] and Bitsch and Möller [234] improved the accuracy and precision of their HPLC methods by using 4-deoxypyridoxine as a routine I.S., which is added at the time of extraction and behaves similarly to the naturally occurring B_6 vitamers during sample preparation. Under their analytical conditions this compound elutes between PN and PM, but Gregory and Sartain's [232] conditions provide longer retention times than those reported by Bitsch and Möller [234] with more resolved peaks.

Ang et al. [229] found with FLD of an MPA extract DLs of about 0.19, 0.1, 1.97 and 0.2 nmol for PMP, PM, PLP and PL, respectively, while Bitsch and Möller [234] claimed a DL ranging from 0.4 to 0.7 pmol for the seven vitamers, using postcolumn reaction with $NaHSO_3$.

Hou et al. [236] proposed ED for the determination of vitamin B_6 in multivitamin tablets, using a μBondapack C_{18} column eluted at 20°C with meth-anol–0.02 M phosphate buffer (pH 5) (10:90). They compared the use of UV detection at 254 nm, a glassy carbon electrode (GCE) and a carbon fibre microelectrode (CFE) with respect to linear range and DL. They pointed out that with the detector based on CFE, the working electrode can be reactivated only by chemical or electrochemical methods, and that after electrochemical pretreatment its sensitivity increases considerably. Generally, they found that the linear dynamic range for ED is wider than that for UV detection, with DLs (at a signal-to-noise ratio of 2) varying according to the vitamer and the detector. The DLs claimed were for PM, 1 ng, whatever the detection mode; for PL, 0.3, 1 and 0.5 ng with UV, CFE (1.2 V) and GCE (1.0 V), respectively; and for PN, 2.5 ng (UV and CFE) and 2 ng (GCE).

4.4.3. Micellar electrokinetic capillary chromatography

Swaile et al. [239] evaluated MECC for separating PLP, PA, PMP, PL, PM and PN and compared the results with those obtained by IP-RP. For MECC they used a 1 m × 75 μm I.D. fused-silica capillary column filled with 0.05 M SDS, 0.01 M Na_2HPO_4 and 0.006 M $Na_2B_4O_7$ in deionized water as mobile phase and an on-column laser-based FLD system equipped with a helium–cadmium laser ($\lambda_{ex.}$325 nm, $\lambda_{em.}$ 430 nm). For IP-RP the conditions were an Alltech C_{18} 10-μm column and a stepwise solvent gradient with 0.01 M sodium heptane-sulphonate in 15% methanol in water (pH 2.75) as initial mobile phase, which after 12 min was changed to 45% methanol in water (pH 2.75) without an IP reagent.

They reported that the stepwise gradient separation of IP-RPC led to tailed peaks and baseline deviations when the mobile phase composition is changed. In addition, comparing IP-RPC and MECC, they found that, apart from the elution order being different for the two separations, the efficiency is far superior with MECC. They also pointed out that the composition of the mobile phase in MECC is critical and, to reduce the band-spreading effect, the surfactant concentration must be kept relatively high. They stated that capillaries of ≤50 μm I.D. should be employed when laser-based FLD is possible; otherwise, larger diameters (50–100 μm I.D.) are necessary when absorbance detection is used.

Yik et al. [240] studied a system for interfacing ED with MECC which couples the separation capillary column to the detection capillary, with a section of porous graphite tubing which forms an electrically conductive joint. They pointed out that the graphite joint has to be kept immersed in a buffer reservoir, to prevent it from drying out, together with the ground electrode of the high-power source. The reservoir is electrically insulated from the electrochemical cell containing the carbon fibre electrode. They claimed that their ED system allows better sensitivity than laser-excited FLD, with a DL two orders of magnitude lower than those reported for laser FLD (0.4 fmol), while the linear dynamic range of the calibration plot was slightly over two orders of magnitude (from ca. 1 to 200 ppm).

4.5. Folacin

The folacin complex consists of a group of related compounds derived from pteroylglutamic acid, exhibiting varying physiological activity, bioavailability and stability. It includes folic acid (FA), 7,8-dihydrofolic acid (DHF), 5,6,7,8-tetrahydrofolic acid (H_4folic acid), 5-methyltetrahydrofolic acid (5-CH_3-H_4folic acid), 5-formyltetrahydrofolic acid (5-CHO-H_4folic acid) and 10-formyltetrahydrofolic acid (10-CHO-H_4folic acid) plus p-aminobenzoylglutamic acid (PBGA) and pterine-6-carboxylic acid (PT-6-COOH). In most plant and animal materials, they exist in long-chain polyglutamyl forms, with chain lengths ranging from five to seven γ-linked glutamyl residues [24].

4.5.1. Sample preparation

The major problems during extraction and cleanup are the lability of folacin both to oxidation and thermal treatment [19] and the conjugation of polyglutamyl forms of the vitamin. An additional complication is the extremely low level present in most foods, less than 100 ng/g [242] (Table 20).

The thermal degradation kinetics of 5-CH_3-H_4folic acid in both the presence and the absence of oxygen were studied by Barrett and Lund [243]. To protect 5-CH_3-H_4folic acid from degradation during the thermal extraction procedure, Schulz et al. [244] developed a method for removing oxygen from food samples by ultrasonication followed by flushing with nitrogen and by the addition of sodi-

um ascorbate as an antioxidant. This technique of extraction was also applied by Bitsch et al. [245] to determine folates from cabbage. They achieved the autolysis of polyglutamates using a raw cabbage extract as a source of endogenous conjugase activity.

Engelhardt and Gregory [246] evaluated the properties of pteroylpolyglutamate hydrolase (conjugase) from hog kidney and the efficacy of this enzyme in deconjugating sample extracts for folate analysis. They stated that the appropriate combination of enzyme concentration and incubation time should be determined for each type of sample, as extracts of a variety of foods caused detectable inhibition of the enzyme.

The addition of antioxidants (ascorbate and 2-mercaptoethanol) to sodium phosphate buffer (pH 4.5) was adopted by Holt et al. [247] for milk and dairy products, whereas lyophilization followed by incubation in trifluoroacetic acid was used by Andondonskaja-Renz and Zeitler [248] for royal jellies and caviar.

For citrus juices, White [249] carried out a solid-phase extraction procedure using a phenyl-bonded phase conditioned with tetrabutylammonium phosphate, which permitted the separation of 5-CH_3-H_4folic acid from sample interferences. The folate was then easily eluted from the cartridge with a buffer–methanol mixture without IP reagent. He found that ascorbic acid was the main interferent, and hindered the selective detection of 5-CH_3-H_4folic acid when amperometric detection was used. After solid-phase extraction the ascorbate concentration was considerably reduced, facilitating the detection of the analyte. This purification procedure was automated by White et al. [250], using a switching ten-port valve that allowed direct injection of filtered juice into the HPLC system. Sample clean-up was effected on a C_{18} precolumn followed, after the elution of ascorbic acid (monitored by UV detection), by the backflushing of 5-CH_3-H_4folic acid to the analytical column. This procedure was claimed to be effective in reducing oxidative losses of folate during sample preparation.

4.5.2. High-performance liquid chromatography

RP separation methods with buffer–organic mobile phases is still preferred [248–251], although IP methods are also used [19,247]. Hahn et al. [251]

tested several chromatographic systems based on IP-RPC and conventional RPC. They found that the best resolution of folacins is achieved by using a 3-μm ODS Hypersil column in a gradient of 5 mM KH_2PO_4 (pH 2.3) and ACN.

UV detection is considered sufficient for normal contents of folates [245], whereas for small amounts FLD is necessary [251]. Because of the low specificity of UV detection, Bitsch et al. [245] used LC–MS to identify the various forms of folate in cabbage. Hahn et al. [251] studied the postcolumn derivatization of folacins, paying particular attention to the influence of the flow-rate of the derivatization reagent (1% $K_2S_2O_8$) on the fluorescence of the resulting products, finding that higher flow-rates resulted in smaller peak areas.

They also compared their DLs with literature data, finding that, with UV detection at 295 nm, the values were lower than those in the literature (DHF 1.77, H_4folic acid 2.67, 5-CH_3-H_4folic acid 1.15, 5-CHO-H_4folic acid 2.37 ng per injection). The same was found for fluorimetry (365/450 nm for DHF and H_4folic acid and 295/356 nm for 5-CH_3-H_4folic acid and 5-CHO-H_4folic acid), the DLs being 0.89, 0.04, 0.007 and 0.28 ng per injection for DHF, H_4folic acid, 5-CH_3-H_4folic acid and 5-CHO-H_4folic acid, respectively. However, fluorimetry does not achieve the sensitivity of ED [251].

4.6. Vitamin C

Vitamin C occurs in two biologically active forms: *l*-ascorbic acid (AA) and *l*-dehydroascorbic acid (DHAA), its oxidized form. Nowadays the most common mode of analysis is HPLC, which overcomes the main drawbacks of chemical methods in the presence of interfering compounds in the matrix of foods, as it is more selective and sensitive.

4.6.1. High-performance liquid chromatography

There is a wide range of HPLC methods [19,183]; the most common modes of separation are RP, IP-RP and weak anion-exchange with an NH_2-bonded phase, with various columns, elution conditions, detection systems and extraction techniques (Table 21). Most methods, however, suffer from an AA retention time that is too close to the void volume, which may lead to errors in quantitative work.

The type of extraction media and stabilizing solutions in order to prevent AA oxidation are very important. A well established extractant and stabilizer is metaphosphoric acid (MPA) [252–260]. Gennaro and Bertolo [253] stressed that the addition of MPA prevents the decay of ascorbic acid during the first few hours, losing its efficacy after about 12 h. In fact, according to Bushway et al. [257], vitamin C begins to degrade within 15 min after homogeniza-

TABLE 20

CONDITIONS FOR HPLC DETERMINATION OF FOLACIN VITAMERS

Sample preparation	Stationary phase	Mobile phase	Detection	Food	Ref.
Homogenize, adjust pH to 4.5 with CH_3COOH, centrifuge, add sodium phosphate buffer (pH 4.5) (10% ascorbate, 1 M 2-mercaptoethanol), incubate with conjugase, centrifuge, filter through 0.45-μm membrane	Microsorb C_{18}, 3 μm	Methanol–phosphate buffer (pH 6.8) and Bu_4NBr; flow, 1 ml/min	Fluorescence, 238/340 nm (ex./em.). Postcolumn oxidation with hypochlorite	Milk dairy products	247
Lyophilize, suspend in 0.1 M trifluoroacetic acid, stir at 37°C for 20 min, centrifuge, adjust pH to 4 with 2 M K_3PO_4	Spherisorb ODS, 5 μm	3.5 mM K_2HPO_4 (pH 6.5); flow, 1 ml/min	Fluorescence, 360/460 nm (ex./em.)	Royal jelly, caviar	248
Centrifuge (2°C, 15 min), adjust pH to 5 with NaOH, (a) direct solid-phase extraction, (b) conjugase hydrolysis (37°C, 1 h) and solid-phase extraction	Zorbax ODS	Methanol–acetate buffer (pH 5.5) (25:75); flow, 1 ml/min	ED, +200 mV vs. Ag/AgCl, 3 M NaCl DAD	Citrus juices	249

TABLE 21

CONDITIONS FOR HPLC DETERMINATION OF ASCORBIC ACID

Sample preparation	Stationary phase	Mobile phase	Detection	Food	Ref.
Degas by filtration through paper, dilute 1:20 with pH 9 buffer prior to injection	HPICE-AS1	Acetonitrile–10 mM H$_2$SO$_4$ (4:96); flow, 0.8 ml/min	PAD, +0.70 V	Beer	278
Add 62.5 mM MPA, blend, centrifuge, filter through paper	Aminex HPX-87H, pore size 9 μm	4.5 mM H$_2$SO$_4$; flow, 0.5 ml/min	DAD, 245 nm	Potatoes, strawberry	252
Extract in 0.5% oxalic acid, add perchloric acid, centrifuge, two-step derivatization with 4-ethoxy- and 4-methoxy-phenylenediamine, purify through C$_{18}$ and Aminex 50W-X2 (Na$^+$) columns	PRP-1	16% ACN in 50 mM H$_3$PO$_4$ and 5 mM propanesulphonate, adjust pH to 9 with 0.15 M H$_3$PO$_4$; flow, 1 ml/min	Fluorescence, 375/475 (ex./em.)	Dairy products	273
Filter through Nucleopore Syrfil, 25 mm, 0.45-μm filter and dilute 1:5	LiChrospher RP-8, 5 μm	Octylammonium-salicylate, 5 mM; flow, 1.0 ml/min	UV, 254 nm	Milks	262
Homogenize in MPA, ultracentrifuge and filter through Nucleophore Syrfil, 25 mm, 0.45-μm filter	LiChrospher RP-18,5 μm	Octylammonium-salicylate, 5 mM; flow, 1.0 ml/min	UV, 254 nm	Fruit juices	253
Blend in 2.5% MPA–mobile phase (1:2), filter through paper, clean-up through Sep-Pak C$_{18}$ and 0.45-μm nylon 66 membrane. To detect total AA: reduce DHAA with DTT	Rainin NH$_2$, 8 μm	ACN–0.05 M KH$_2$PO$_4$ (75:25); flow, 2 ml/min	UV, 254 nm	Apple, potatoes	254
Homogenize, add 0.1 M citric acid containing 5 mM EDTA together with hexane, centrifuge, filter through 0.45-μm membrane.	Three Ultrasphere ODS C$_{18}$ in series, 4°C	Buffer (pH 5)–0.1 M NaH$_2$PO$_4$–5 mM EDTA–5 mM tetrabutylammonium phosphate	Fluorescence, 350/430 nm (ex./em.) after postcolumn derivatization	Luncheon meats	261
Homogenize in 5% MPA, centrifuge, filter through paper, dilute, clean-up through Sep-Pak C$_{18}$ and filter through 0.45-μm membrane	μBondapak NH$_2$	5 mM KH$_2$PO$_4$ (pH 4.6)–ACN (30:70); flow, 1 ml/min	UV, 254 nm	Vegetables	256
Mix with ethanol and MPA, centrifuge. For total AA: add 0.3 M Na$_3$PO$_4$ and NaSH (20 min, 35°C), dilute with MPA, filter through 0.45-μm membrane. For AA: dilute with MPA and filter through 0.45-μm membrane	Cosmosil 5 C$_{18}$, 40°C	MPA (2 g/l); flow, 1 ml/min	UV, 243 nm	Citrus juices	266
Homogenize in 0.2 M phosphate buffer (pH 2), extract with 3% MPA, filter through 0.45 μm nylon filter	PLRP-S, 5 μm 100 Å	1.8% H$_4$folic acid and 0.3% MPA in water; flow, 0.5 ml/min	DAD, 244 nm	Fruit, vegetables, juices	257
Add 12.5% TCA solution to precipitate proteins, centrifuge, filter. For total AA add homocysteine to reduce DHAA to AA, adjust pH to 7.0 (15 min, room temp.)	Nucleosil 7 C$_{18}$	2 mM Bu$_4$NOH in water (pH 2.92); flow, 1.5 ml/min	UV, 254 nm	Milk	264

tion and by 30 min 4–5% is oxidized. When large subsamples are used, they suggested replacing the expensive MPA with a phosphate buffer (pH 2) in the initial homogenization step. However, the extraction medium can cause problems during the chromatographic separation. For example, Graham and Annette [252], investigating the use of amino-bonded and RP-18 columns with and without IP to determine AA extracted using MPA, found that amino-bonded phases were unsatisfactory in analysing cooked samples because of a poor resolution and a co-eluting component contaminating the AA peak; likewise, RPC with or without the use of IPC leads to column poisoning. Vanderslice and Higgs [255], studying a large variety of fresh and cooked vegetables, fruits, fruit juices and cereals, also found that the MPA–acetic acid extraction was more efficient than the citric acid extraction used previously [261]. However, MPA extraction led to irreproducible separations on ODS and this was replaced with a microporous polystyrene–divinylbenzene polymer (PLPR-S) column, which gave clean and stable traces even when MPA extraction was used.

Gennaro et al. [262] compared the performances of various types of RP packings (C_{18} and C_8, spherical and irregular, with 5- and 10-μm particles and different degrees of end-capping), with salicylates of heptylamine, octylamine and decylamine as the ion interaction reagent. They found that a longer alkyl chain of the amine causes higher retention but, at the same time, the longest alkyl chain shows the lowest sensitivity. Further, greater retentions corresponded to stationary phases characterized by spherical packings and by the highest carbon loading. In fact, these parameters affect the degree of functionalization induced on the column by the interaction reagent [262]. They concluded that the most suitable reagent was octylammonium salicylate, which with UV detection permits a DL of the order of 113 pmol injected [253]. Gennaro et al. [263] proposed an RP ion interaction reagent method which makes use of chiral compounds as the interaction reagents, to separate D-(−)- and L-(+)-AA. After having employed the optical isomeric forms of malic, tartaric and mandelic acids as the interaction reagents, they achieved a good separation of AA using a Spherisorb ODS-2 column with 0.005 M octylamine D-(−)-tartrate.

Sapers et al. [254] developed a method to deter-

mine ascorbic acid 2-phosphate (AAP), without phosphatase treatment, simultaneously with AA using an aminopropyl column with an isocratic separation which ensures no interfering peaks. The retention times of AAP (isocratic, 30 min) could be reduced by gradient elution, increasing the proportion of buffer to 50%. DHAA was determined after reduction to AA with dithiothreitol (DTT). To determine AA, DHAA, isoascorbic acid (IAA) and dehydroisoascorbic acid (DHIAA) simultaneously, Vanderslice and Higgs [261] used three Ultrasphere ODS columns at 4°C in series and an eluting buffer (pH 5) of 0.1 M NaH$_2$PO$_4$, 5 mM EDTA and 5 mM tetrabutylammonium phosphate. After separation on the column, the compounds were converted into fluorescent derivatives by a method involving oxidation of AA and IAA with mercury(II) chloride to DHAA and DHIAA and subsequent reaction with o-phenylenediamine. This procedure allows determination at the 3-ng level.

Most methods determine DHAA as the difference between total AA after DHAA reduction and the AA content of the original sample [252] in order to determine total AA with UV detection. The sensitivity of DHAA is insufficient to detect the amounts usually found in foods even when it is monitored at its maximum wavelength (210 nm). The reduction of DHAA to AA can be accomplished using homocysteine [252,259,264] or dithiothreitol solution [254,265], and usually TCA or MPA is added to the sample prior to the reduction treatment to prevent AA and DHAA from being oxidized to diketogulonic acid (DKG) and other degradation products. Sawamura et al. [266] reduced DHAA to AA with sodium hydrosulphide and claimed that NaSH has advantages over DTT and homocysteine.

On the other hand, Graham and Annette [252] claimed that using DAD at 230 nm, AA and DHAA could be detected simultaneously if the concentration of DHAA is sufficiently high, as its DL is 100 ng/μl. Otherwise, DHAA must be determined only after reduction to AA. With their procedure (a modification of the homocysteine method), they found almost complete reduction of DHAA to AA (96%) and a DL for AA of 1 ng/μl.

However, UV detection at a single wavelength is the most often used method [253,254,256,259,260, 262,265,267–270].

Some workers detected AA with fluorescence detection after precolumn [271] or postcolumn derivatization of DHAA. Huang and Kissinger [272] proposed a highly selective method to determine AA and DHAA simultaneously using UV and ED in series. DHAA was derivatized postcolumn with o-phenylenediamine and the product was detected at 348 nm, while AA was detected at a glassy carbon electrode maintained at +600 mV vs. Ag/AgCl. Bilic (273] set up a procedure for the simultaneous determination of AA and DHAA in dairy products using a two-step precolumn derivatization with 4-methoxy- and 4-ethoxy-1,2-phenylenediamine; the derivatives formed, presumably methoxy- and ethoxyquinoxalines, exhibit a tenfold increase in fluorescence compared with the quinoxaline derived from 1,2-phenylenediamine. First, the sample is derivatized with 4-ethoxy-1,2-phenylenediamine, then the sample is derivatized with 4-ethoxy-1,2-phenylenediamine, then the derivatives are isolated and retained by a RP adsorbent. The AA is oxidized to DHAA with saturated aqueous bromide and then derivatized with 4-methoxy-1,2-phenylenediamine and retained on the same RP adsorbent. The recovery of derivatives from the RP adsorbent is accomplished by elution with 15% ACN and 0.5% trifluoroacetic acid in water. It was claimed that, at a signal-to-noise ratio of 3, the DLs were about 50 and 70 fmol of AA and DHAA, respectively, per 5-μl injection.

ED has been used either alone [274,275] or in series with UV detection [272,276,277]. To detect AA and sulphite in beers, Wagner and McGarrity [278] found that the use of a pulsed amperometric detector with a single applied voltage was unsuccessful, as the loss of detector sensitivity was extremely rapid, probably owing to the contamination of the surface area of the platinum electrode used. As an alternative, they obtained good results utilizing a standard amperometric cell, like Kitada et al. [258], and superior results were achieved when it was operated in a pulsed mode with cleaning cycles continuously applied during the analysis. Kitada et al. [258] found a DL of 5 μg/g using an amperometric detector set at +500 mV vs. Ag/AgCl.

Bode and Rose [279] studied the detection of AA based on coulometric ED, evaluating the time necessary for the complete reduction of DHAA to AA by β-mercaptoethanol, the optimum pH of the re-

duction reaction and the necessary ratio of reductant to DHAA. A high degree of sensitivity was achieved with detection of less than 1 pmol of AA.

4.6.2. Other techniques

Usually, AA is evaluated by TLC after reaction with dinitrophenylhydrazine [280]. The ascorbic acid osazone derivative can be purified by column chromatography [280] or measured by TLC and densitometry [281], achieving a DL of 5 ng, or detected in situ at 494 nm [282] with a DL of 1 ppm. To determine total AA, Touchstone et al. [281] oxidized AA to DHAA with copper(II) salts prior to reaction with dinitrophenylhydrazine. To separate AA from its oxidation products (DHAA and DKG), Tsuda and Fukuba [283] used Avicel SF as adsorbent, ethyl acetate–pyridine–water (10:4:3) as eluent and 2,6-dichlorophenolindophenol sodium salt in ethanol as spray reagent.

Application of GC to the determination of vitamin C has been on a limited scale, and only a few papers have dealt with food applications [190]. As AA is a non-volatile compound, it can be determined after derivatization as the trimethylsilyl ether on non-polar methyl polysiloxanes as stationary phase with FID. The same derivatization procedure could be suitable for obtaining volatile trimethylsilyl derivatives from DHAA and DKG, which therefore could be simultaneously determined with AA. During the derivatization, basic solvents such as pyridine have to be avoided, owing to the instability in alkaline media of AA and its oxidation products.

AA may be extracted from food using MPA, and after clean-up on a cellulose column the AA in the dried eluate is derivatized and determined [284]. An interesting approach could be the use of isotachophoresis to identify AA and its oxidized products in foods. As only acidic media have to be selected for the leading electrolyte, β-alanine is more suitable as a counter ion, as it has a wide buffer action at pH 3–4; n-caproic acid, having the lowest solubility, is better as the terminal solution [283]. To detect AA in wheat dough, Kvasnicka et al. [285] carried out a capillary isotachophoresis with a 200 × 0.8 mm I.D. preliminary column and a 200 × 0.3 mm I.D. separation column. As initial electrolyte they used 5–10 mM HCl, 12–24 mM β-alanine and 0.1% hydroxyethylcellulose, while the final electrolyte contained 5 mM caproic acid and 5 mM Tris buffer.

Similarly, Tsuda and Fukuba [283] found that the optimum conditions for the isotachophoretic determination of AA were HCl–β-alanine at pH 3.6 and n-caproic acid at pH 3.4 as the leading and terminal solutions, respectively.

With UV detection of AA at 254 nm, Kvasnicka et al. [285] found a DL of AA of 0.1 mg/kg. Using the same system, they detected DHAA after its oxidation to AA with 2-mercaptoethanol. In fact, DHAA does not appear in the isotachopherogram, even if it is present in the sample. To confirm this, Tsuda and Fukuba [283] determined the oxidation products of AA by both TLC and isotachophoresis. TLC confirmed the presence of DHAA, although in the isotachopherogram only AA and DKG were found.

5. CONCLUSIONS

This survey of the recent literature confirms that there is a trend towards the use of HPLC methods as standard techniques for the qualitative and quantitative determination of both water- and fat-soluble vitamins. To date, the single chromatographic determinations of some vitamins, such as cyanocobalamin, pantothenic acid, biotin and some pseudovitamins, have not been reported for food products, probably because nutritional scientists are not particularly interested in these vitamins; hence there has not been further research on the assay methods [19,183].

Most HPLC methods use bonded phases, particularly RP packing materials; smaller particles (3 μm), shorter columns and microbore columns are increasingly being used to improve speed and sensitivity. Although UV and FLD are widely employed, ED is becoming increasingly important for the detection of very small amounts of vitamins, as it shows enhanced sensitivity and selectivity.

The use of HPLC column-switching techniques makes the sample preparation less laborious and, at the same time, prevents some oxidizable vitamins from being oxidized. These automatic procedures are becoming more important for routine work in food control laboratories, where speed of analysis and simple sample preparation together with a reliable and reproducible chromatographic assay are needed.

Chromatographic techniques other than HPLC are seldom employed in food analysis and they are reserved for research purposes and not used as routine analytical methods.

For identification purposes, DAD, MS and FT-IR could be advantageous detection techniques, especially for the development of reference methods to validate HPLC determinations of vitamins.

6. ABBREVIATIONS

AA	Ascorbid acid
AAP	Ascorbic acid 2-phosphate
ACN	Acetonitrile
AOAC	Association of Official Analytical Chemists
CE	Capillary electrophoresis
CFE	Carbon fibre electrode
cGC	Capillary gas chromatography
CN	Cyano, nitrile
cZE	Capillary zone electrophoresis
DAD	Diode-array detection
DHAA	1-Dehydroascorbic acid
DHF	7,8-dihydrofolic acid
DHIAA	Dehydroisoascorbic acid
DKG	Diketogulonic acid
DL	Detection limit
DMF	N,N-Dimethylformamide
DMSO	Dimethyl sulphoxide
DTT	Dithiothreitol
ECD	Electron-capture detection
ECN	Effective carbon number
ED	Electrochemical detection
FA	Folic acid
FAB	Fast atom bombardment
FAD	Flavin adenin dinucleotide
FID	Flame ionization detection
FLD	Fluorescence detection
FMN	Flavin mononucleotide
FPD	Flame photometric detection
FT-IR	Fourier transform infrared spectroscopy
GC	Gas chromatography
GCE	Glassy carbon electrode
GPC	Gel permeation chromatography
H₄folid acid	5,6,7,8 Tetrahydrofolic acid
HPLC	High-performance liquid chromatography
IAA	Isoascorbic acid

I.D.	Inside diameter
IMF	Intermediate moisture food
IP-RPC	Ion-pair reversed-phase chromatography
I.S.	Internal standard
LC	Liquid chromatography
LP	Light petroleum
MD	Menadione
MECC	Micellar electrokinetic capillary chromatography
MeOH	Methanol
MK	Menaquinone
MPA	Metaphosphoric acid
MS	Mass spectrometry
MSB	Menadione hydrogensulphite
MSD	Mass spectrometric detector
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NARP	Non-aqueous reversed-phase
NP	Normal phase
NPC	Normal-phase chromatography
NPD	Nitrogen–phosphorus detection
ODS	Octadecylsilica
PA	Pyridoxic acid
PAD	Pulsed amperometric detection
PBGA	p-aminobenzoyl glutamic acid
PK	Phylloquinone
PL	Pyridoxal
PLP	Pyridoxal-5′-phosphate
PM	Pyridoxamine
PMP	Pyridoxamine-5′-phosphate
PN	Pyridoxine
PNP	Pyridoxine-5′-phosphate
Pt-6-COOH	Pterine-6-carboxylic acid
RF	Riboflavin
RP	Reversed-phase
RPC	Reversed-phase chromatography
RP-FC	Reversed-phase flash column
SCE	Saturated calomel electrode
SDS	Sodium dodecyl sulphate
SFC	Supercritical fluid chromatography
SFE	Supercritical fluid extraction
SSA	Sulphosalicylic acid
TCA	Trichloroacetic acid
THF	Tetrahydrofuran
TLC	Thin-layer chromatography
TOC	Tocopherol

UV–VIS   Ultraviolet–visible
UV   Ultraviolet

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