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Simultaneous HPLC analysis of fat-soluble vitamins in selected animal products after small-scale extraction

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Abstract

A method for simultaneous determination of fat-soluble vitamins in animal products is presented. Milk, and two fish products containing many ingredients, were used as test materials. The vitamins determined were tocopherols, β-carotene, all-*trans*-retinol and in the case of fish, cholecalciferol. The sample preparation procedure, consisting of saponification and extraction by *n*-hexane–ethyl acetate was carried out in small-scale. To cope with the highly different composition of the test materials, some modifications were needed. Normal-phase HPLC separation using diisopropyl ether-*n*-hexane gradient elution with UV and fluorescence detections for tocopherols, β-carotene and all-*trans*-retinol was used. Cholecalciferol was determined from the same extract by reverse-phase HPLC (with UV detection) after semi-preparative HPLC purification. Recoveries of spiked samples varied from 80 to 111% for all determined fat-soluble vitamins. The day-to-day repeatability was in most cases under 7%, and the within-day variation of this method was small, under 5.5% for all vitamins. The described analytical method is effective and fast, enabling the processing of a large number of samples.

Keywords: Fat-soluble vitamins; Foods; Simultaneous HPLC determination; Small-scale extraction

1. Introduction

Vitamin A activity in food is related to the presence of both retinol and a large number of provitamin carotenoids. In milk and in meat, vitamin A occurs mainly as fatty acid retinyl esters. Typical retinoids in fish are all-*trans*-retinol and all-*trans*-3,4-dehydroretinol. All-*trans*-3,4-dehydroretinol is a typical vitamin A compound in fishes like pike (*Esox lucius*), pikepearch (*Lucioperca lucioperca*), pearch (*Perca fluviatilis*) and whitefish (*Coregonus* sp.) (Ollilainen, Heinonen, Linkola, Varo & Koivistoinen, 1989b). β-Carotene is the predominant form of carotenoids in milk and meat. Other carotenes such as α-carotene are absent or present in such low quantities that they may be ignored as a source of vitamin A (Indyk, 1988). The most common pigments in fishes are astaxanthin, tunaxanthin and canthaxanthin, which do not possess vitamin activity (Simpson, Katayama & Chichester, 1981). Astaxanthin or its esters are important carotenoids in salmon, shellfish and shrimps (Elmadfa & Majchrzak, 1998; Weedon, 1971). Naturally occuring vitamin E is also composed of a number of tocopherol analogues (α, β, γ, δ) and their corresponding unsaturated tocotrienols. The major form of vitamin E in milk fat is α-tocopherol (Syväoja, Piironen, Varo, Koivistoinen & Salminen, 1985). In marine animals α-tocopherol is the principal tocopherol but also minor amounts of β-, γ- and δ-analogues occur in some fish species (Syväoja, Salminem, Piironen, Varo, Koivistoinen & Salminen, 1985). The predominant vitamin D compound in fish is cholecalciferol (vitamin D_3), which occurs in highly varying concentrations in different species (Mattila, 1995).

Simultaneous determination of fat-soluble vitamins has been widely used for serum and plasma samples. α-Tocopherol, retinoids and carotenoids can be determined simultaneously from these samples by reverse-phase chromatography using UV detection for

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carotenoids and retinoids and fluorescence detection for tocopherols (e.g. Chuang, Trosclair & Lopez, 1994; Hess, Keller, Oberlin, Bonfanti & Schüep, 1991; Manzi, Panfili & Pizzoferrato, 1996; Yakushina & Tarakova, 1995).

Fat-soluble vitamins of foods are, however, usually determined by time-consuming separate chromatographic methods. All four tocopherols and tocotrienols can be separated and quantified by normal-phase chromatography with silica stationary phases and fluorescence detection. Furthermore, removing of nonpolar substances like triacylglycerides is not needed (Panfili, Manzi & Pizzoferrato, 1994; Piironen, 1986). The use of reverse-phase chromatography for separating tocopherols and tocotnenols is less frequent in food analysis due to its poorer separation efficiency; α- and δ-tocopherols can be separated but β- and γ-analogues coelute (Indyk, 1988; Manzi et al., 1996). In the case of vitamin D compounds, reverse-phase chromatography with UV detection is preferred because this technique allows the use of vitamin D_2 as an internal standard for vitamin D_3 (Mattila, 1995). Because of weak retention of carotenes on silica phases, reverse-phase chromatography with UV detection is mainly applied for carotenoids. On the other hand, normal-phase chromatography is required for retinoid analysis especially if *cis*- and *trans*-isomers of retinol have to be separated. Chromatographic conditions for the simultaneous measurement of tocopherols, retinols and β-carotene have been reported for few foods only, namely dairy products and cheese (Hewavitharana, van Brakel & Harnet, 1996; Indyk, 1988; Manzi et al., 1996; Panfili et al, 1994). To our knowledge, there are no studies on simultaneous determination of fat-soluble vitamins in complex animal products with varying concentration levels of vitamins.

Saponification followed by extraction is the most widely used sample preparation method for fat-soluble vitamins in food samples. Saponification removes the bulk of fat and facilitates extraction by releasing carotenoids, retinoids, tocopherols and vitamin D compounds from the matrix. Factors to be optimised in a saponification method are sample size, potassium hydroxide concentration in the solution, saponification temperature and time, composition of the saponified solution before extraction and extraction solvents. Selection of an antioxidant and removing of oxygen by nitrogen are also important. It is still quite common to use large-scale extraction methods when analysing fat-soluble vitamins in foods. These are, however, laborious and reagents-consuming (Aminullah Bhuiyan, Ratynayake & Ackman., 1993; Mattila, 1995; Ollilainen et al., 1989a, 1989b; Piironen, 1986; Rettenmaier & Schüep, 1992). There are few small-scale applications concerning simultaneous extraction of β-carotene, tocopherols and all-*trans*-retinol from dairy products and cheese (Indyk, 1988; Panfili et al, 1994) but not from more complex animal products. Furthermore, vitamin D compounds have been determined from the same extract as the other fat-soluble vitamins only after large-scale extraction (Aminullah Bhuyian et al.).

The aim of the present study was to develop a small-scale saponification and extraction procedure and HPLC method for simultaneous determination of different fat-soluble vitamins from animal based food materials. When developing the sample preparation procedure, the aim was to decrease the amount of work and consumption of reagents compared with methods traditionally used (Aminullah Bhuiyan et al., 1993; Mattila, 1995; Ollilainen et al., 1989a, 1989b; Piironen, 1986; Rettenmaier & Schüep, 1992). Tocopherols, all-*trans*-retinol and β-carotene were determined simultaneously in the same run using normal-phase chromatography. The tested samples were two fish products (fried Baltic herring fillet and fried Baltic herring burger) and standard milk. The sample types were chosen so that they varied extensively in their compositions and vitamin levels. In the case of fish, which is the most important natural source of vitamin D_3, our further aim was to determine this vitamin from the same extract. Reverse-phase chromatography was used for its quantification.

2. Materials and methods

2.1. Chemicals

HPLC-grade *n*-hexane and ethyl acetate were purchased from Rathburn (Walkerburn, UK). Diisopropyl ether (J.T. Baker, Deventer, The Netherlands), 2-propanol, methanol, tetrahydrofuran (Rathburn) and Milli Q water were of HPLC-grade. Ethanol was either spectrophotometric grade (AAS, 99.5%) or 94% (A) (Primalco Oy, Finland). Ascorbic acid, EDTA and pyrogallol were purchased from Merck (Darmstadt, Germany) and were of pro-analysis grade. KOH pellets for saponification solution were purchased from EKA Nobel (Bohus, Sweden). The saponification solutions were KOH (100) containing 100 g of KOH dissolved in 100 ml of water and KOH (50) containing 50 g of KOH dissolved in 100 ml of water.

DL-α-Tocopherol (Art. no. 8283, 98.0–102.0%) and tocopherol-isomers (α-, β-, γ-, and δ-tocopherol für biochemische Zwecke, Art no. 15496) were purchased from Merck, all-*trans*-β-carotene (C-0126, type IV from carrots), vitamins D_2 and D_3 from Sigma (St Louis, USA). Vitamin-A-alcohol (all-*trans*-retinol, isomeric purity approximately 95% all-*trans*-retinol, 5% 13-*cis*-isomer, no. 95144) was purchased from Fluka (Buchs, Switzerland). Rye and barley flour were extracted to produce a qualitative standard for tocotrienols.

The standard stock solutions of the vitamins for analysing milk samples were prepared to a concentration of approximately 5 mg/ml of α-tocopherol in ethanol (AAS), 400 μg/ml of all-*trans*-retinol in ethanol (AAS) and 20 μg/ml of β-carotene in *n*-hexane; they were stored at −20°C in the dark. These solutions were diluted in ethanol (AAS) or *n*-hexane as appropriate and the concentrations were confirmed spectrophotometrically using known absorption coefficients of each vitamin (see below). The combined working solution was prepared by pooling suitable volumes of each stock solution and diluting with *n*-hexane to obtain concentrations ranging from 0.4 to 1.4 μg/ml for each vitamin.

The standard stock solutions of the vitamins for analysing fish samples were prepared to a concentration of approximately 5 mg/ml, 500, 400 and 600 μg/ml of α-, β-, γ-, and δ-tocopherols (in ethanol, AAS), 400 μg/ml of all-*trans*-retinol (in ethanol, AAS), 20 μg/ml of β-carotene (in *n*-hexane), 200 μg/ml of vitamin D_2 (internal standard, in ethanol, AAS) and 200 μg/ml of vitamin D_3 (in ethanol, AAS). All others were stored at −20°C but vitamin D stock solutions were stored at +4°C. These solutions were diluted in ethanol (AAS) or *n*-hexane as appropriate and the concentrations were confirmed spectrophotometrically using the known absorption coefficients of each vitamin. The specific absorption coefficients ($E^{1\%}_{1\ cm}$) used were 75.8 for α-tocopherol, 89.4 for β-tocopherol, 91.4 for γ-tocopherol and 87.3 for δ-tocopherol at 292, 296, 298 and 298 nm, respectively, 1850 for all-*trans*-retinol at 325 nm (Frolik & Olson, 1984), 2592 for β-carotene at 450 nm (De Ritter & Purcell, 1981), 475 for D_2 and 480 for D_3 at 265 nm (British Pharmaceutical Codex: Anon, 1979). The combined working solution was prepared by pooling suitable volumes of each stock solution and diluting with *n*-hexane to obtain concentrations ranging from 0.2 to 75 μg/ml.

2.2. Samples

When developing the method, the samples were standard milk and more complex matrixes, a fish product (fried Baltic herring fillet) coated with rye flour and fried in rapeseed oil. Suitability of the method for analysis of a differing fish product (fried Baltic herring burger), coated with wheat flour and bread crumbs, and fried in rapeseed oil, was also tested.

2.3. Sample preparation

All work was carried out under subdued light conditions.

2.3.1. Milk samples

For saponification, 1 g of thermostated (40°C, in order to stabilize the distribution of milk fat) milk was weighed into a 10 ml Kimax tube. AAS ethanol (4 ml), a spatletip of pyrogallol and a spatletip of ascorbic acid were added to the tube and the tube was capped, vortexed and allowed to stand for 10 min. Nitrogen was led into the tube to remove oxygen and 0.5 ml of saturated EDTA and 0.5 ml of KOH (KOH50) were added to the tube. The tube was capped, shaken, and transferred to a boiling water bath for 20 min. The tube was shaken once after 10 min. After boiling, the tube was cooled in an ice-water bath for 10 min (Fig. 1).

After saponification and subsequent cooling of the milk sample, 2 ml of water and *n*-hexane–ethyl acetate (8 + 2, v/v) were added to extract the fat-soluble vitamins. The tube was shaken with 500 strokes/min (Edmund Bührer Laborgerätebau, Glastechnik, Umwelttechnik) for 10 min prior to separating the layers. The extraction was repeated with another 2 ml portion of *n*-hexane-ethyl acetate (8 + 2). The combined organic layer was evaporated with nitrogen (30°C) and the dry residue was dissolved in 1 ml of *n*-hexane and filtered (Whatman, Puradisc 25 TF, 0.45 μm) prior to HPLC analysis (Fig. 2).

2.3.2. Fish samples

For saponification of fish product, I g of homogenized sample was weighed into a Pyrex tube, 5 ml of 2% ascorbic acid (in water), 10 ml of ethanol (A) and 1 ml of internal standard (0.2 μg of vitamin D_2) were

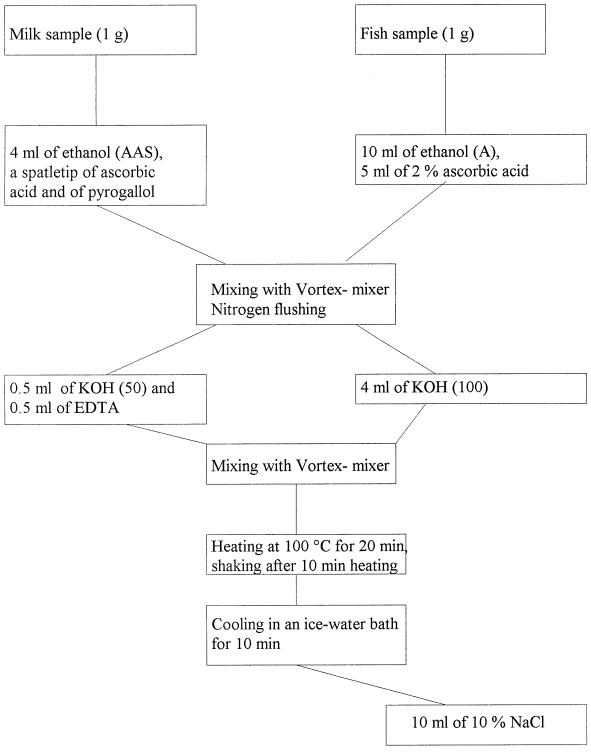

Fig. 1. Saponification procedure for standard milk and fish product samples.

added. The tube was vortexed and flushed with nitrogen, 4 ml of KOH (KOH 100) was added, the tube was capped and transferred into a boiling water bath for 20 min. The tube was vortexed after 10 min of boiling. After boiling, the tube was cooled in an ice-water bath for 10 min. Ten ml of 10% NaCl was added to the tube to avoid emulsion formation (Fig. 1).

After saponification of the fish sample, the fat-soluble vitamins were extracted using three 20 ml portions of *n*-hexane-ethyl acetate (8 + 2). The tube was shaken with a Desaga mixer for 2 min after each extraction, and the phases were allowed to separate. The organic layers were collected in a 100 ml tube where the combined extract was washed with 20 ml of 5% NaCl. After washing, the organic layer was transferred to a 100 ml bottle and evaporated with a Rotavapor (30°C). Five ml of ethanol (AAS) and 5 ml of *n*-hexane were added and the solution was evaporated. The residue was quantitatively transferred with a small amount of *n*-hexane to a Kimax tube and evaporated to dryness with nitrogen. The residue was dissolved in 1 ml of *n*-hexane and filtered (Whatman, Puradisc 25 TF, 0.45 μm) prior to HPLC analysis (Fig. 2).

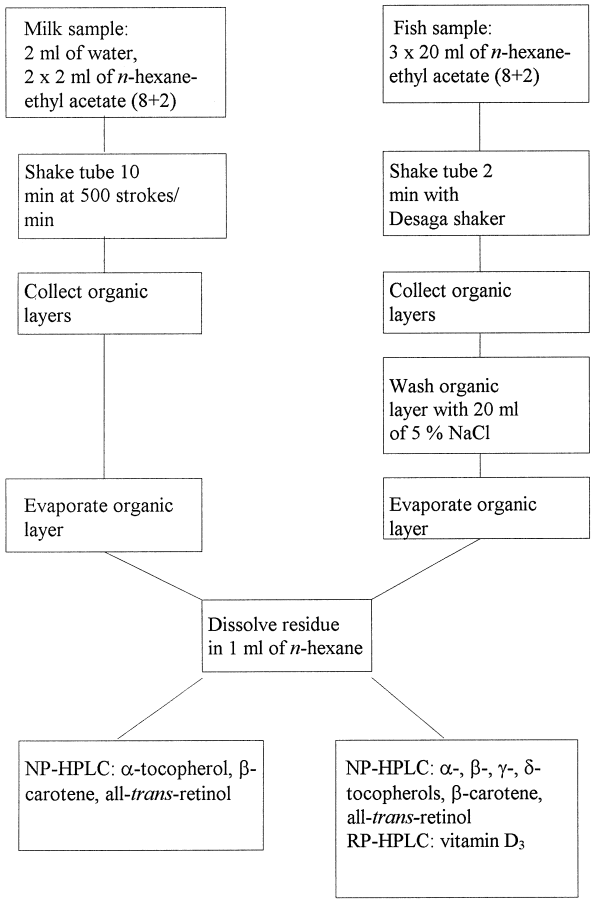

Fig. 2. Extraction and HPLC determination of fat-soluble vitamins, from standard milk and fish product samples.

2.4. Analytical and semipreparative HPLC

Normal-phase chromatography with UV (β-carotene and all-*trans*-retinol) and fluorescence detections (tocopherols) was used. When analysing vitamin D compounds, reverse-phase chromatography with UV detection was used after the semipreparative clean-up procedure.

2.4.1. Quantification of tocopherols, β-carotene, and all-trans-retinol with HPLC

The analytical HPLC system consisted of a Varian Vista 5500 liquid chromatograph equipped with a UV detector (Waters 486), a fluorescence detector (Waters 470), an autosampler with a cooling module (Waters 700 Satellite WISP), and a μPorasil column (10 μm, 30 cm×3.9 mm, Waters) with a silica guard column (Guard-Pak Silica, Waters). The temperature of the column oven was 30°C. Separation of vitamins was based on step gradient elution (Table 1). The injection volume used was 75 μl.

Tocopherols, β-carotene, and all-*trans*-retinol were quantified with an external standard method in which quantification was based on peak areas. Standard curves (four concentration levels) were obtained daily by standard injections. The variations of detector response and the retention times were obtained by standard injections, after every third sample injection.

2.4.2. Vitamin D: Clean-up with semipreparative HPLC

Fish extracts were purified by using the normal-phase HPLC method. The semipreparative cleanup system consisted of a Waters 510 HPLC pump, a Merck-Hitachi 1-4200 UV–VIS detector set at 265 nm, and a μPorasil column (10 μm, 30 cm×3.9 mm, Waters) with a silica guard column (Guard-Pak Silica, Waters). The mobile phase consisted of *n*-hexane, tetrahydrofuran, and 2-propanol (98:1:1); the flow rate was 1 ml/min, and the injection volume was 300 μml of the sample extract. A collecting time of from 1.5 min before to 1.5 min after the retention time of co-eluting vitamin D_2 and D_3 standard peak was used. The collected fraction was evaporated under nitrogen, and the residue was dissolved in 150 μl of 7% water in methanol (Mattila et al., 1992).

2.4.3. Quantification of vitamin D_3 with analytical HPLC

The analytical HPLC system consisted of a Waters 6000 pump, a UV detector set at 265 nm (Waters 486), an autosampler (Waters 712), and a Vydac TP 54 column (5μm, 25 cm×4.6 mm, The Separations group) with an ODS quard column (Nova-Pak C18, Waters). The temperature of the column oven was 25°C. The mobile phase contained 93% methanol and 7% water.

Table 1
HPLC method for the determination of tocopherols, β-carotene, and all-*trans*-retinol: step gradient and detector programme[a]

Time (min)	Pump A (%)	Pump B (%)	Pump C (%)	Flow (ml/min)	% DIPE in *n*-hexane	UV (nm)	Fluorescence (ex/em)
0	97	0	3	1.5	0.3	450	292/325
4.0	97	0	3	1.5	0.3	325	292/325
4.1	20	0	80	1.5	8.0	325	292/325
24.0	20	0	80	1.5	8.0	325	292/325
24.1	70	30	0	1.5	30.0	325	292/325
45.0	70	30	0	1.5	30.0	325	292/325
45.1	97	0	3	3.0	0.3	325	292/325

[a] Pump A, *n*-hexane; pump B, diisopropyl ether (DIPE); pump C, diisopropyl ether, 10% (v/v) in *n*-hexane.

The flow rate of the mobile phase was 1 ml/min, and the injection volume 75 μl. The vitamin D_3 was quantified with an internal standard method using vitamin D_2 as the internal standard. Quantification was based on peak areas (Mattila, Piironen, Bäckman, Asunmaa, Uusi-Rauva & Koivistoinen, 1992). Standard curves (four concentration levels) were obtained daily by standard injections. The variation of detector response and the retention times were obtained by standard injections, after every third sample injection.

2.5. Method validation

The linearity ranges of the standard curves, as well as the detection and determination limits of the vitamins, were determined. Recovery tests were run by spiking the samples with vitamins before saponification. The day-to-day repeatability and within-day variation of the results were determined by analysing the test samples (milk sample, fish product) frequently. The stability of the column was tested by calculating the variation of the retention times of each vitamin. The variation of detector response was evaluated by the variation of the slope of the standard curves.

3. Results and discussion

In the method presented in this paper, tocopherols, β-carotene and all-*trans*-retinol were quantified by normal-phase HPLC with UV (β-carotene and all-*trans*-retinol) or fluorescence detection (tocopherols) and vitamin D_3 was quantified by reverse-phase HPLC with UV detection, all from the same sample extract.

The sample preparation procedures presented here are partly based on some previous work (CEN, 1993; Panfili et al., 1994). They were developed further to enable simultaneous extraction of fat-soluble vitamins from animal products with different structures and vitamin levels. Because of the highly different compositions of the materials tested and the aim to determine vitamin D_3 from fish simultaneously with the other vitamins, two modifications of the same basic method were developed.

The saponification procedure of milk samples was based on the CEN (1993) method for α-tocopherol, but larger sample size was used which allowed quantification of β-carotene and all-*trans*-retinol from the same sample extract. The concentration of alkali was optimized to release the all-*trans*-retinol from its esteried forms but not to destroy tocopherols which are sensitive against alkaline conditions. When amounts of 0.123, 0.25 and 0.35 g of KOH/1 g of sample were tested (the total volume of saponification solution was 5 ml; Fig. 3), the α-tocopherol results were nearly the same at the two lowest concentrations of alkali but decreased markedly at the highest level. This same effect was noticed in the case of β-carotene. For all-*trans*-retinol, the highest values were obtained by using the 0.25 g concentration of alkali (Fig. 3). Because of the highest average yields for the vitamins, the 0.25 g concentration was chosen for the KOH concentration (Fig. 3). For extraction of the vitamins, a more polar solvent system (*n*-hexane-ethyl acetate instead of *n*-hexane) than in the CEN method was needed to extract vitamins of varying polarity. To improve the extractability of vitamins, the amount of water used was larger than in the CEN method.

The saponification procedure of the fish sample was based on the cold-saponification method of Ollilainen et al. (1989b), but was modified to small-scale using a hot-saponification step. Optimum sample size was found to be 1 g, and this allowed vitamin D3 determination from

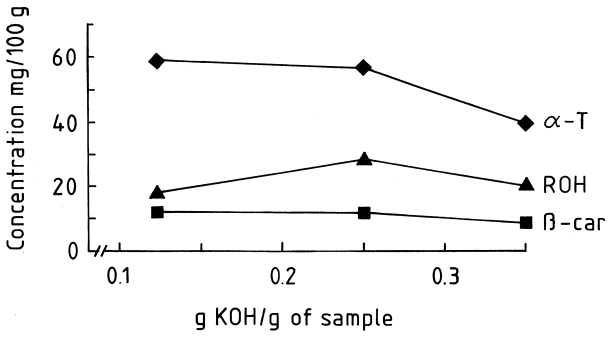

Fig. 3. The effect of KOH concentration on α-tocopherol, β-carotene and all-*trans*-retinol during saponification of a standard milk sample.

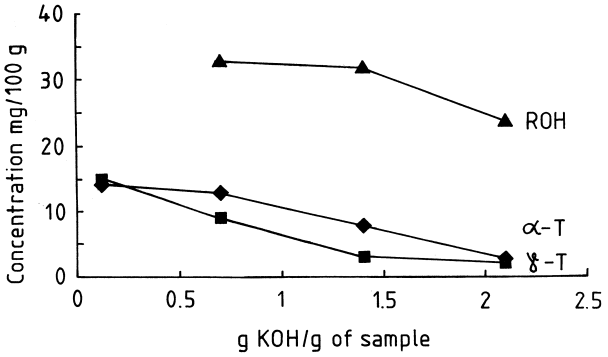

Fig. 4. The effect of KOH concentration on α- and γ-tocopherols and all-*trans*-retinol during saponification of a fish product (fried Baltic herring fillet).

the same sample extract. The tested concentration ranges of KOH for fish samples were 0.123, 0.7, 1.4 and 2.1 g of KOH/1 g of sample when the total volume of the saponification solution was 19 ml (Fig. 4). The lowest concentration of alkali did not liberate all-*trans*-retinol adequately. At the chosen concentration level, 0.7 g of KOH/ 1 g of sample, liberation of all-trans-retinol was mostly complete and the destruction of tocopherols minimal (Fig. 4). The only antioxidant used in this method was ascorbic acid because pyrogallol gave unknown peaks between γ- and δ-tocopherol which interfered with the quantification of δ-tocopherol. *n*-Hexane-ethyl acetate (8 + 2) was used as an extraction solvent instead of *n*-hexane-diethyl ether (Ollilainen et al., 1989b) or of diethyl ether (Aminullah Bhuiyan et al., 1993) because there is no risk of peroxides and possibly interfering stabilizers. The extraction step was otherwise similar to the extraction step used for milk samples but larger volumes of solvents (3×20 ml of *n*-hexane–ethyl acetate) were used.

The sample preparation procedure presented in this paper can be applied for determining tocopherols, retinol and β-carotene from a variety of animal-based foods. Only some adjustment of sample size and solvent volumes may be needed. On the other hand, vitamin D levels of animal products other than fish are so low that extraction has to be carried out in a larger scale. There are reasons to believe that after modifying the method further by adjusting sample size, the amount of KOH and solvent volumes, the method could be suitable also for determining tocopherols and β-carotene occuring in plant-based foods.

In normal-phase separations of fat-soluble vitamins, silica columns like Ultrasphere Si, Nova-Pak Si and Alltech Econosphere Si with quite small particle size (3–

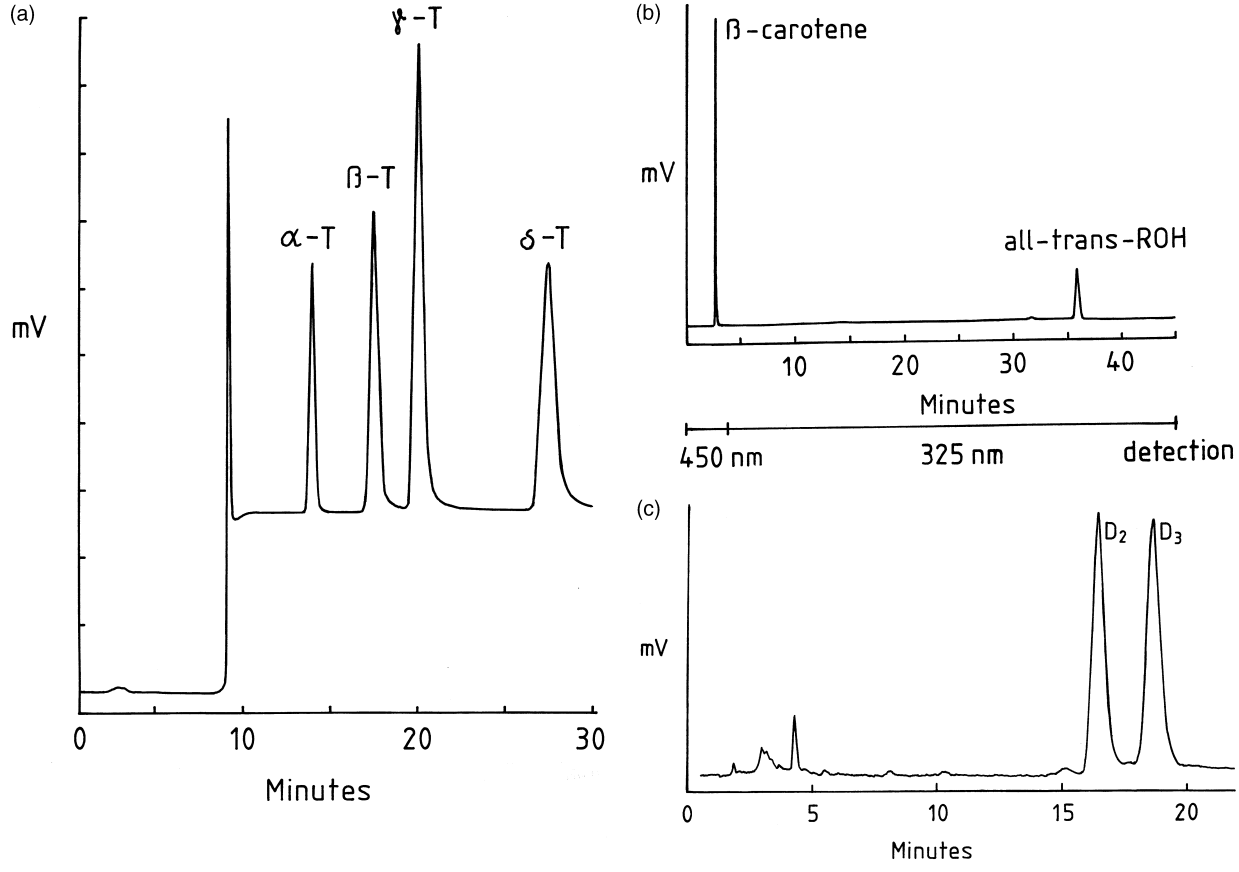

Fig. 5. HPLC chromatograms of a standard solution: (a) the separation of tocopherols (T), (b) β-carotene and all-*trans*-retinol and (c) vitamin D compounds.

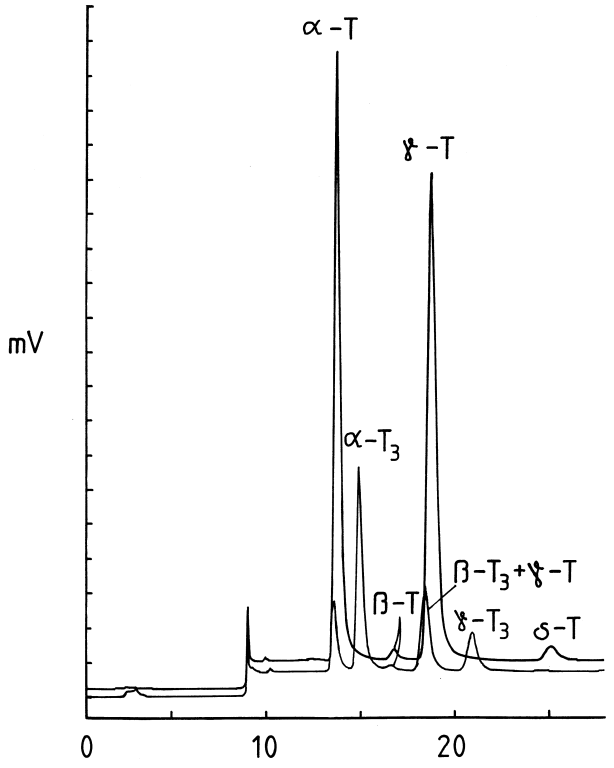

Fig. 6. HPLC chromatograms of a standard solution (T, tocopherols separation) and a barley extract (T3, tocotrienols separation).

5 μm) and varying elution conditions have been used (Hewavitharana et al., 1996; McGeachin & Bailey, 1995; Panfili et al., 1994). When choosing the analytical column and elution conditions for this study, the main problem was the weak retention of nonpolar β-carotene to silica. When comparing different silica phases, LiChrocart Supersher Si 60, Spherisorb Si with 5 μm particle size and μPorasil with 10 μm particle size, a great variation in the nature of silica packings was observed. The best retention for β-carotene was achieved by using μPorasil with 10 μm particle size. Instead of the 2-propanol-hexane gradient elution used by Panfili et al. (1994), step gradient elution with *n*-hexane and diisopropyl ether was used and this enabled proper retention to β-carotene and good separation of all the other vitamins.

The retention time of void volume (t_0) was 1.94 min (measured with dodecane) and the retention times of measured vitamins varied between 3.0 min for β-carotene and 36.8 min for all-*trans*-retinol. This shows that β-carotene did have retention in the column. Also lycopene was well separated from other non-polar carotenes, like β-carotene, with retention time of 7.0 min. The capacity factors (k') varied between 0.5 for β-carotene and 18.0 for all-*trans*-retinol. For α-tocopherol the capacity factor was in optimal range, being 6.2 All tocopherols were well separated between retention times, 13.9 for α-tocopherol and 24.7 min for δ-tocopherol (Fig. 5). The total run time was 61 min. α-Tocotrienol was well separated between α- and β-tocopherols and γ-tocotrienol was separated from γ- and δ-tocopherols but β-tocotrienol and γ-tocopherol co-eluted (Fig. 6). This good separation efficiency extends the use of this chromatography for samples with more complex tocopherol and tocotrienol compositions. If β-tocotrienol and γ-tocopherol need to be separated, it might be possible by optimising the chromatography by adjusting flow rate and column temperature (Piironen, Varo, Syväoja, Salminen & Koistoinen, 1984). The method chosen separates some carotenoids like β-carotene and lycopene, present in tomato and watermelon containing foods.

Astaxanthin, occurring in some fishes like salmon, is an alkaline-labile carotenoid which is converted to astacene during alkaline hydrolysis (Goodwin, 1980). Astacene is, however, strongly retained in silica phases and cannot be determined with this method. In this study, all-*trans*-retinol was the major retinoid to be determined. Furthermore, 13-*cis*-retinol can be separated by shortening the 8% diisopropyl ether in *n*-hexane step from 20 to 16 min (Table 1). The separation of all-*trans*-3,4-dehydroretinol from all-*trans*-retinol was verified with rainbow trout oil samples. The efficiency of saponification in the case of all-*trans*-retinol was also possible to monitor as retinol palmitate was well separated from the other compounds at the retention time 9.1 min.

The μPorasil column proved to be very stable. The within-day variation of retention times of tocopherols, β-carotene and all-*trans*-retinol was small, being under 0.6% ($n=9$). The day-to-day variation of these vitamins was also small, under 2.7% ($n=29$).

Detection limits defined as a signal three times the height of the noise level were 3 ng/100 g for vitamin D_3, <1μg/100 g for β-carotene, and 2 μg/100 g for tocopherols and all-*trans*-retinol. Determination limits (three times the detection limit) were 0.01 μg /100 g for vitamin D_3, 2 μg/ 100 g for β-carotene and 6 μg/100 g for the other vitamins (α-, β-, γ- and δ-tocopherol and all-*trans*-retinol).

The detector response was linear in the tested ranges (ng/injection) of 35–4700 for α-tocopherol ($R=0.9999$), 0.4–27 for β-tocopherol ($R=0.9994$), 27–3700 for γ-tocopherol ($R=0.9999$), 1.3–180 for δ-tocopherol ($R=0.9993$), 0.7–92 for β–carotene ($R=0.9999$), 0.8–11 for all-*trans*-retinol ($R=0.9996$) and 3–700 for vitamin D_3 ($R=0.9999$). The variation of the slope of standard curves varied from 3.7 to 6.0%, which also proves the stability of the detector response.

Recoveries ($n=8$, 4 days) of added vitamins in standard milk samples were good and variation was small: 91±1.4% (CV% 1.5) for α-tocopherol (mean value in standard milk was 57 μg/100 g, Fig. 7a), 86±1.7% (CV% 2.0) for β-carotene (13 μg/100 g in standard milk, Fig. 7c) and 82±5.6% (CV% 5.6) for all-*trans*-retinol (28 μg/100 g in standard milk, Fig. 7c). The day-to-day repeatabilities (CV%, $n=13$) for these vitamins were

5.4%, 3.9% and 7.0%, respectively. The within-day variation ($n=13$) was small: 1.0% for α-tocopherol, 1.3% for β-carotene and 3.2% for all-*trans*-retinol.

The major tocopherols in fish products are α- and γ-tocopherols: mean value for α-tocopherol content in fried Baltic herring fillet was 6220 μg/100 g and for γ-tocopherol 2830 μg/100 g, and β- and δ-analogues occur in some cases at concentrations under the determination limit (mean value for β-tocopherol was 8 μg and for δ-tocopherol was 75 μg/100 g in fried Baltic herring fillet, Fig. 7b). The content of other fat-soluble vitamins in fried Baltic herring were: all-*trans*-retinol 8 μg, β-carotene 107 μg and vitamin D_3 7 μg/100 g, (Fig. 7d,e). Recoveries ($n=6$, 3 days) of added vitamins in the fish product tested (fried Baltic herring fillet) were 90±5.0% (CV% 5.6) for α-tocopherol, 90±12% (CV% 13) for γ-tocopherol, 93±6.2% (CV% 6.7) for δ-tocopherol, 80±4.5% (CV% 4.6) for β-carotene, 111±9.1% (CV% 8.2) for vitamin D_3 and 92±14% (CV% 15) for all-*trans*-retinol. The day-to-day repeatabilities (CV%, $n=6$) for these vitamins varied, being 2.1% (α-tocopherol), 5.3% (γ-tocopherol), 16.4% (δ-tocopherol),

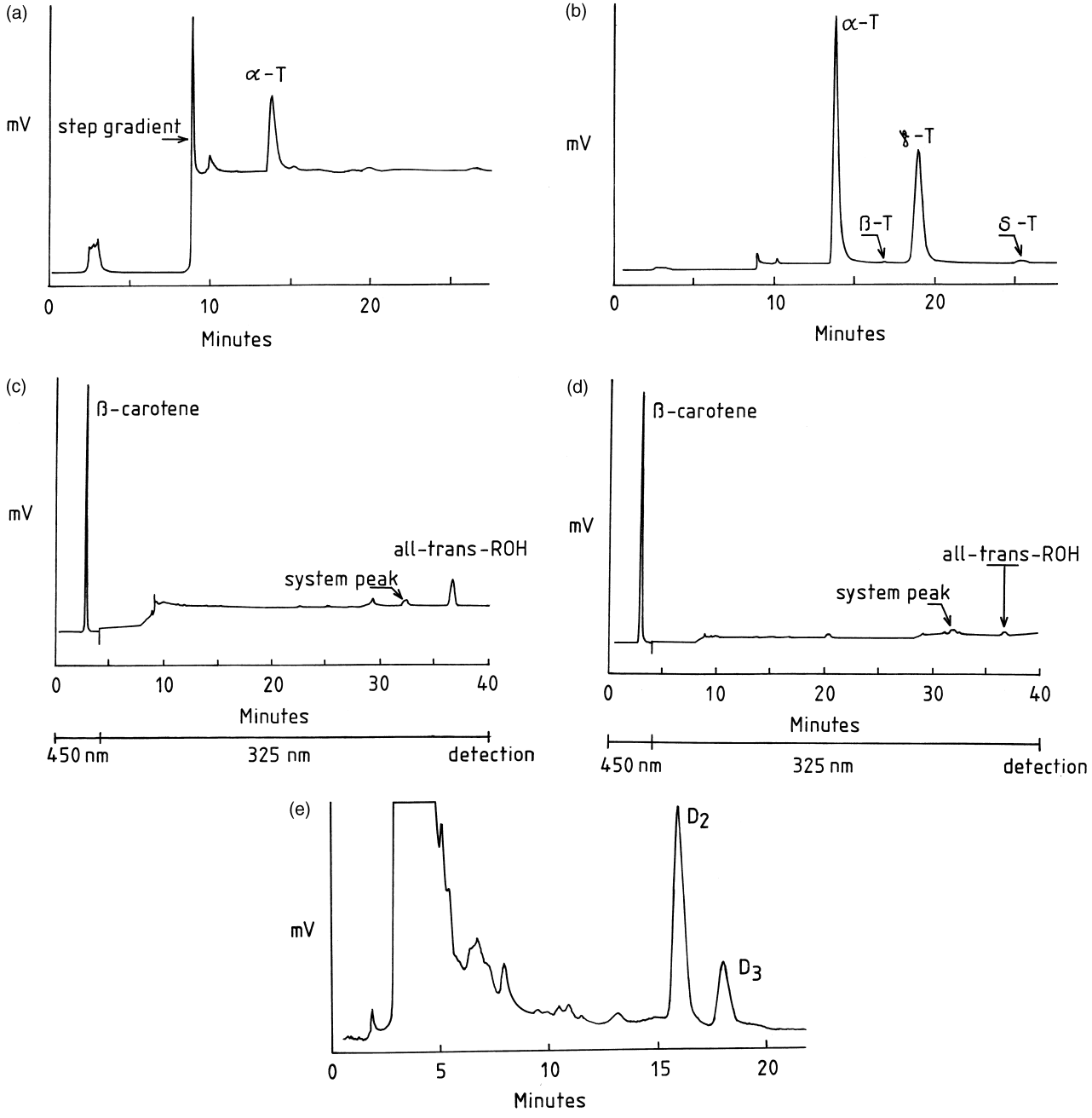

Fig. 7. Analytical HPLC chromatograms of tocopherols [a (milk) and b(fish)], β-carotene [c (milk) and d (fish)], all-*trans*-retinol [c (milk) and d (fish)] and vitamin D compounds [e (fish)]; milk = standard milk and fish = fried Baltic herring fillet.

7.3% (β-carotene), 2.4% (vitamin D_3) and 20.6% (all-*trans*-retinol). The relatively high variation in the values for all-*trans*-retinol and δ-tocopherol was due to their low concentrations in fish products. The within-day variation ($n=6$) was small for all vitamins: 1.7% for α-tocopherol, 2.0% for γ-tocopherol, 1.9% for δ-tocopherol, 2.0% for β-carotene, 1.5% for vitamin D_3 and 5.5% for all-*trans*-retinol. When this method was tested for the other fish sample (fried Baltic herring burger), the day-to-day repeatabilities for α-, β-, γ-, and δ-tocopherols were 1.3, 5.8, 4.2 and 5.4%, respectively, and for β-carotene 5.0% ($n=5$). The concentration of all-trans-retinol was in this case under the determination limit.

The present small-scale extraction method with its two modifications was found to perform well for analysis of fat-soluble vitamins from samples of animal origin with differing composition and varying levels of vitamins. This method also saves time, solvent, waste and cost, which allows high sample capacity compared to the traditional large-scale methods. Repeatability and reproducibility of the present method was good. Although this method is not suitable for separating position isomers of carotenoids, the separation efficiency and repeatability of the chromatography makes it suitable as it is, or with slight modifications, for a wide range of food materials when analysing tocopherols, β-carotene and retinols.

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