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Simultaneous determination of fat-soluble vitamins and provitamins in dairy products by liquid chromatography with a narrow-bore column

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A reversed-phase high-performance liquid chromatographic method is described for the simultaneous determination of vitamins A, D_2, D_3, E and K_1, retinyl acetate, retinyl palmitate, tocopherol acetate, ergosterol and 7-dehydrocholesterol in milk and butter. Narrow-bore columns are recommended because this alternative provides a good separation and efficiency, plus greater economy and sensitivity. Detection limits for individual vitamins range from 0.14 to 6.9 ng. All vitamins are separated in less than 33 min. For the simultaneous determination of these vitamins and provitamins we use two sample pre-treatment methods, a liquid–liquid extraction with hexane or a solid-phase extraction with a C_{18} cartridge. Recovery studies show good results for all solutes (84–108% and 85-108% for milk and butter, respectively) and the intra-day coefficients of variations range from 1.6 to 4.5%. These methods permit the simple determination of fat-soluble vitamins using a small sample volume.

Introduction

Vitamins are a large group of indispensable compounds for the development and normal growth of living organisms, and their absence is the cause of serious physiological problems. These compounds differ in their chemical composition, physiological action and nutritional importance. In food technology, the analysis of vitamins is of great interest in the evaluation of several biochemical and nutritional disorders. In recent years a great deal of research has been devoted to developing adequate methods for the analysis of fat-soluble vitamins in foods,[1–3] because clinical studies exist which relate fat-soluble vitamin deficiency states with certain pathologies and, at the same time, cases have been described which were triggered by high doses of fat-soluble vitamins.[4,5] Therefore, the analysis of vitamins in milk is of major importance because dairy food plays a vital role in human nutrition.

Traditional methods of vitamin assays have required that each vitamin be determined individually using widely differing physical, chemical and biological methods. However, fluorimetric and spectrophotometric methods may not provide accurate and precise results for the food matrices tested. Several excellent HPLC separations of fat-soluble vitamins have been published[6–12] but very few simultaneous and complete characterizations of fat-soluble vitamins in foods are available in the literature.[13]

The determination of fat-soluble vitamins of real samples includes a sample preparation step that is usually laborious and time consuming. The aim of sample preparations is to extract the analytes from the matrix to be analysed and to concentrate them, if necessary, so as to obtain a measurable response in a chromatographic or any other type of system. Whereas the actual analytical procedure may only take a few minutes, the sample preparation step may take several hours.

Some authors[3] recommend alkaline hydrolysis followed by extraction with an organic solvent (hexane, petroleum ether, *etc.*) of the vitamins from the non-saponifiable matter. While saponification is generally desirable, it is not always necessary. The presence of large amounts of lipids that yield no signal at the preselected detection wavelength is not necessarily detrimental, although column efficiency may be affected. For example, this approach is useful for the determination of vitamins A, D and E but it is not satisfactory for vitamin K compounds, which are rapidly decomposed in alkaline media, and also provides no information on the amount or composition of retinyl derivatives in the sample. The liberation of the unstable retinol and tocopherols demands that protective measures with respect to light and oxygen be implemented throughout the analysis.

Other authors, in order to allow vitamin K determination, propose performing an enzymatic digestion.[14,15] A third alternative is the direct extraction of these vitamins. A non-hydrolytic extraction procedure using a suitable solvent system can be applied to the determination of naturally occurring vitamin A esters and supplemental α-tocopheryl acetate. Among the many published procedures, the Rose–Gottlieb[3] method is particularly suitable for extracting the total fat from milk products but the use of ammonia solution makes the method unsuitable for vitamins E and K, which are labile under alkaline conditions.

At the same time, antioxidants (such as butylated hydroxytoluene, pyrogallol, *etc.*) may be added at any stage of sample preparation to protect against oxidation, since it has been confirmed that the antioxidant will not interfere with subsequent analysis. Moreover, clean-up procedures have been used in fat-soluble vitamins assays. In this aspect, the interest aroused by solid-phase extraction has increased, not only because of the use of disposable prepacked cartridges, but also because concentration of the sample extract can be achieved if the elution volume is less than the volume of sample extract applied to the cartridge.

The aim of the present work was to design a sample treatment method without hydrolysis for the simultaneous determination of vitamins A, D_2, D_3, E, K_1, retinyl acetate, retinyl palmitate, provitamin D_2 (ergosterol) and provitamin D_3 (7-dehydrocholesterol) by liquid chromatography using a narrow-bore (2.1 mm id) octadecylsilane column. The proposed method was applied to the determination of the vitamin content in milk and butter samples.

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Experimental

Equipment

The experiments were performed using an analytical HPLC system equipped with two Kontron (Milan, Italy) Model 422 pumps and a Rheodyne Model 8125 injection valve with a 5 μl loop. A Kontron Model 430 UV-vis detector with a 3 μl flow cell and a data station with Data System 450 software were used.

Before use, the mobile phases were vacuum filtered through a 0.45 μm nylon filter and de-gassed with helium. The column used was Extrasil ODS2, 150 × 2.1 mm id, 3 μm (Teknokroma, Barcelona, Spain).

The chromatographic experiments were performed at room temperature (20 ± 2 °C).

Reagents

Methanol was of HPLC grade and was used as received. Ultrapure water was obtained through a Milli-Q System (Millipore, Milford, MA, USA). Analytical-reagent HPLC grade (purity 98–99%) retinyl acetate, retinyl palmitate, retinol, tocopherol acetate and vitamin K_1 (Sigma, St. Louis, MO, USA), DL-α-tocopherol, cholecalciferol and ergocalciferol (Merck, Darmstadt, Germany), ergosterol and 7-dehydrocholesterol (Fluka, Buchs, Switzerland) were used. Butylated hydroxytoluene (BHT) was purchased from Sigma. Absolute ethanol was purchased from Romil (Loughborough, Leicestershire, UK). Hexane was purchased from Merck.

Preparation of standards

Individual stock standard solutions of each vitamin were prepared by mass in ethanol containing 0.025% (m/v) of BHT, to provide a concentration of 1 mg ml^{-1} for all fat-soluble vitamins (retinol, retinyl acetate, retinyl palmitate, ergocalciferol, cholecalciferol, tocopherol, tocopherol acetate and vitamin K_1) and provitamin D_2 (ergosterol) and D_3 (7-dehydrocholesterol).

Working standard solutions were prepared by appropriate dilution of the stock standard solution to obtain final concentrations ranging from 1 to 4 μg ml^{-1} for retinol, retinyl acetate, retinyl palmitate and vitamin D_3 and from 2 to 8 μg ml^{-1} for vitamin D_2, tocopherol, tocopherol acetate, vitamin K_1 and provitamins D_2 and D_3. These solutions were de-gassed with helium and stored in dark glass flasks at −20 °C. All solutions were filtered through a 0.45 μm membrane (Millex-HV_{13}, Millipore) before being injected into the system.

Sample preparation: clean-up and pre-concentration

Milk samples were drawn directly into glass tubes. All tubes were protected from light. The tubes were then immediately frozen at −20 °C. Butter samples were protected from the light and were then kept at 4 °C. Both samples were submitted to one of these procedures for clean-up and pre-concentration.

Solid-phase extraction. The milk sample, 1 ml for fresh milk and 2.5 ml for fortified milk (larger volumes plug the cartridge), was transferred into a glass tube and 500 μl of ethanol with 0.025% (m/v) of butylated hydroxytoluene (BHT, to protect the vitamins against oxidation) were added to initiate protein precipitation; the sample was subsequently treated for 2 min in an ultrasonic bath to disrupt the fat globule membranes (proteins and phospholipids) that encapsulate fat-soluble vitamins; it was then diluted with Milli-Q water to 4 ml. After this, solid-phase extraction using Mega Bond Elut C_{18} cartridges (Varian, Mulgrave, Victoria, Australia), was used to remove potential interfering compounds in the sample and to pre-concentrate and extract the fat-soluble vitamins.

The cartridge had previously been prepared with 25 ml of methanol and 10 ml of Milli-Q water. The sample was passed through the Mega Bond Elut C_{18} cartridge at a controlled flow rate, which was adequate to ensure complete retention of vitamins. The cartridge was then washed with 5 ml of methanol–water (1 + 9, v/v) followed by elution of the fat-soluble vitamins with 6 ml of methanol. The eluate was passed through a 0.45 μm filter. This was then evaporated to dryness under nitrogen. The residue was reconstituted in 20 or 40 μl ethanol with 0.025% (m/v) of BHT for fresh milk and fortified milk samples, respectively. A 5 μl aliquot of these solutions was injected into the HPLC system.

Liquid–liquid extraction. The sample (5 ml for fresh milk and fortified milk, 1 g for butter) was transferred into a 10 ml glass tube, 5 ml of ethanol with 0.025% (m/v) of BHT were added and the mixture was treated for 2 min in an ultrasonic bath.

The mixture was then transferred to a separating funnel and 15 ml of hexane were added, followed by mechanical shaking for 5 min. The organic layer was transferred to another separating funnel and the extraction process was repeated with a further 15 ml of hexane. The two organic layers were combined, and washed with two portions (5 ml each) of methanol–water (9 + 1, v/v). The organic layer was separated, passed through a 0.45 μm filter, and the larger part of the hexane extract evaporated (final volume 1.5 ml approximately) under nitrogen stream. Then, this solution was transferred to a conical vial and evaporated until dryness. The residue was re-dissolved quantitatively by vortex-mixing in 20 μl of methanol for milk samples and 1 ml for butter samples. A 5 μl aliquot of each solution was injected into the HPLC system.

Alkaline hydrolysis. Samples (5 ml of fortified milk) were saponified overnight at room temperature with 3 ml of potassium hydroxide solution (KOH 60%, m/v) in water and 10 ml of ethanol with 0.025% (m/v) of BHT to avoid oxidation. The vitamins were extracted by liquid–liquid extraction.

Chromatographic method

Reversed-phase chromatography was used with two solvents as mobile phases, methanol–water (99 + 1, v/v) (A) and methanol–tetrahydrofuran (70 + 30, v/v) (B) in gradient mode, the mobile phase being pumped at a flow rate of 0.15 ml min^{-1} to 5.5 min and then 0.2 ml min^{-1} after to 6.3 min, also in gradient mode.

As a result of optimisation, the gradient run conditions were programmed as follows: 0–20 min 0% B; 20–22 min 85% B; 22–29 min 85% B; 29–33 min 0% B.

The wavelength of the detector was set at 325 nm for retinol, retinyl acetate and retinyl palmitate, 264 nm for vitamin D_2 and vitamin D_3, 280 nm for vitamin K_1, tocopherol, tocopherol acetate, ergosterol and 7-dehydrocholesterol, and was programmed in gradient run as follows: 0–7 min, 325 nm; 7–9.5 min, 264 nm; 9.5–24 min 280 nm; 24–33 min 325 nm.

Results and discussion

Using earlier studies in our laboratory[16,17] as a basis, we investigated the effect of elution conditions, particularly flow rate and mobile phase composition, on the resolution of the fat-soluble vitamins which are present in milk and butter. This was achieved with a narrow-bore column because, in comparison with ordinary columns, it is clear that less mobile phase solvent

is consumed (flow-rate < 0.2 ml min^{-1}) and that the analytical cost is therefore less. Besides, lower amounts of analyte can be determined because a substantial increase in peak concentration is obtained. The maximum peak concentration is inversely proportional to the square of the column internal diameter.

Taking into account the high retention capacity of the octadecylsilane packing chosen as the stationary phase, mobile phases with a high elution capacity, such as methanol or methanol–tetrahydrofuran mixtures, were employed. The use of methanol–water mobile phases in the isocratic mode provides retention times which are very long and the addition of tetrahydrofuran decreases the retention time but increases problems of overlapping.

In order to decrease retention times, another approach is to use the flexibility offered by simultaneous flow and solvent programming.

It is important to emphasize that the object of these studies was to optimize the simultaneous separation of ten analytes. Therefore, the gradient mode for optimizing sample on-column residence time with mobile phase polarity and flow-rate was used.

As can be seen in Fig. 1, which shows the chromatogram obtained under these conditions, an adequate resolution in an acceptable analysis time was obtained.

Another problem, relating to the simultaneous determination of several structurally different analytes, depends on the detection mode. Unfortunately, not all of the fat-soluble vitamins can be determined directly by a sensitive detection system, such as a fluorescence or electrochemical detector. Consequently, because of its capability of simultaneous determination, spectrophotometric detection was chosen.

Using a UV detector, and in spite of the fact that a selected wavelength and a narrow-bore column were used, a sample preconcentration step was necessary, as the detection limits obtained (based on a signal-to-noise ratio of 3) and shown in the order of elution in the Table 1, suggest. In fact, this sensitivity appears to be inadequate for the determination of the vitamin levels usually encountered in unprocessed milk and butter, although for several kinds of fortified milk it does appears to be suitable.

In order to demonstrate the applicability of reversed phase chromatography using narrow-bore columns for the separation of fat-soluble vitamins in real samples, different milk and butter samples were analysed.

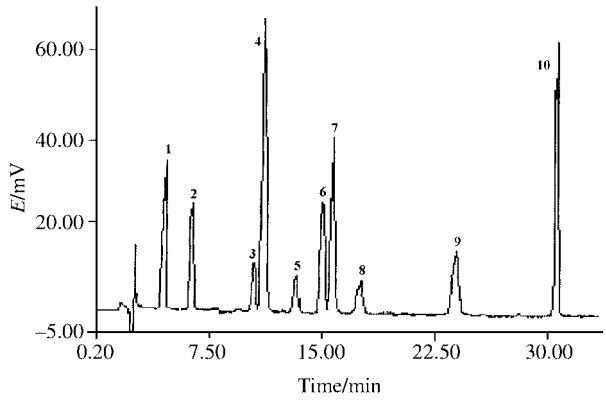

Fig. 1 Chromatogram obtained in the gradient mode from a standard solution of the fat-soluble vitamins. Column, Extrasil ODS-2, 150 × 2.1 mm id, 3 μm. Flow rate of 0.15 ml min^{-1} to 5.5 min and 0.2 ml min^{-1} after to 6.3 min. Injection volume, 5 μl. Mobile phase, solvent A methanol–water (99 + 1), solvent B methanol–tetrahydrofuran (70 + 30). Gradient conditions, 0–20 min 0% B, 20–22 min 85%, 22–29 min 85% B, 29–33 min 0% B. Detection at 325 nm for retinol, retinyl acetate and retinyl palmitate, 264 nm for vitamin D_2 and D_3 and 280 nm for vitamin E and K_1, tocopherol acetate, ergosterol and 7-dehydrocholesterol: 1, retinol; 2, retinyl acetate; 3, vitamin D_2; 4, vitamin D_3; 5, vitamin E; 6, provitamin D_2; 7, provitamin D_3; 8, tocopherol acetate; 9, vitamin K_1; 10, retinyl palmitate.

An analytical problem as complex as the aforementioned simultaneous determination of vitamins and provitamins required a sample pre-treatment step designed to protect the compound of interest, present in the original material, during liberation from the sample matrix. So, the application of classical methodology that generally requires a hydrolysis process of the sample is undesirable with regard to vitamin stability. It was necessary to take into account alternative approaches for sample preparation, such as those indicated above.

In fact, as can be appreciated in the chromatograms in Fig. 2, which correspond to a fortified milk sample which was subjected to a liquid–liquid extraction, the peaks corresponding to retinyl acetate and retinyl palmitate disappeared while the peak due to tocopherol acetate decreased notably when the sample was subjected to an alkaline digestion, increasing simultaneously the retinol and tocopherol peaks as a consequence of the saponification process.

Figs. 3 and 4 show the chromatograms corresponding to fresh milk and butter samples obtained under similar conditions to those of Fig. 2B.

Identification was achieved by comparing the retention times with those of the standards, and obtaining UV spectra to confirm

Table 1 Detection limits of fat-soluble vitamins determined by using an Extrasil ODS-2 column (150 × 2.1 mm id, 3 μm)

Vitamin	Detection limit/ng
Retinol	0.14
Retinyl acetate	0.15
Vitamin D_2	4.10
Vitamin D_3	0.30
Vitamin E	4.80
Ergosterol	0.80
7-Dehydrocholesterol	1.25
Tocopherol acetate	6.90
Vitamin K_1	4.65
Retinyl palmitate	0.29

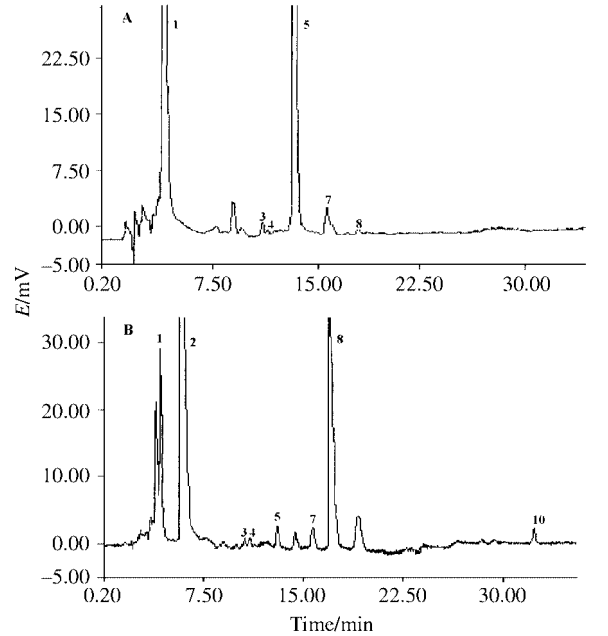

Fig. 2 Typical chromatogram of the fat-soluble vitamins in a fortified milk sample submitted to liquid–liquid extraction: (A) with alkaline hydrolysis and (B) without alkaline digestion. Column, Extrasil ODS-2, 150 × 2.1 mm id, 3 μm. Flow rate of 0.15 ml min^{-1} to 5.5 min and 0.2 ml min^{-1} after to 6.3 min. Injection volume, 5 μl. Other conditions as in Fig. 1: 1, retinol; 2, retinyl acetate; 3, vitamin D_2; 4, vitamin D_3; 5, vitamin E; 7, provitamin D_3; 8, tocopherol acetate; 10, retinyl palmitate.

peak identity. Ten vitamins and provitamins were present in all samples and these were all determined.

Alternative studies using solid–liquid extraction were carried out in milk and butter samples.

As an example, Fig. 5 shows the chromatogram obtained for a fortified milk sample submitted to a stage of clean-up and pre-concentration by solid–liquid extraction.

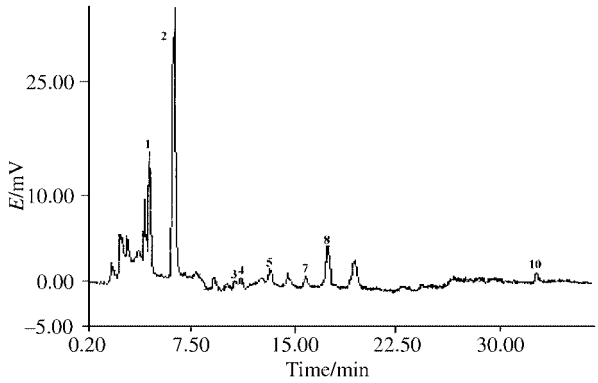

Fig. 3 Chromatogram of the fat-soluble vitamins from a fresh milk sample submitted to a liquid–liquid extraction. Column, Extrasil ODS-2, 150 × 2.1 mm id, 3 μm. Flow rate of 0.15 ml min^{-1} to 5.5 min and 0.2 ml min^{-1} after to 6.3 min. Injection volume, 5 μl. Other conditions in Fig. 1: 1, retinol; 2, retinyl acetate; 3, vitamin D_2; 4, vitamin D_3; 5, vitamin E; 7, provitamin D_3; 8, tocopherol acetate; 10, retinyl palmitate.

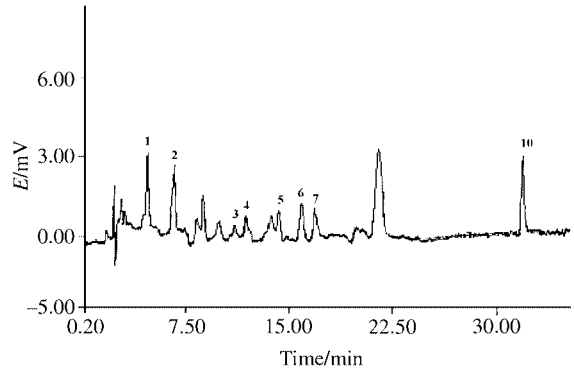

Fig. 4 Typical chromatogram of the fat-soluble vitamins in a butter sample submitted to a liquid–liquid extraction. Column, Extrasil ODS-2, 150 × 2.1 mm id, 3 μm. Flow rate of 0.15 ml min^{-1} to 5.5 min and 0.2 ml min^{-1} on to 6.3 min. Injection volume, 5 μl. Other conditions in Fig. 1: 1, retinol; 2, retinyl acetate; 3, vitamin D_2; 4, vitamin D_3; 5, vitamin E; 6, provitamin D_2; 7, provitamin D_3; 10, retinyl palmitate.

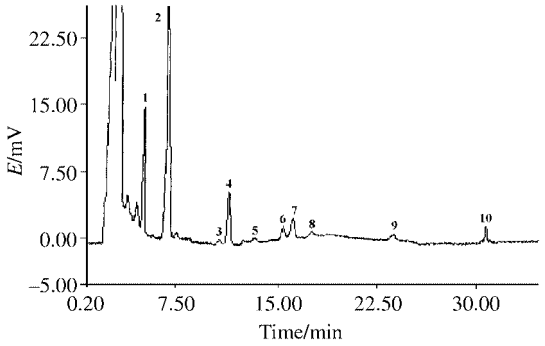

Fig. 5 Chromatogram of the fat-soluble vitamins in a fortified milk sample submitted to a solid phase extraction. Column, Extrasil ODS-2, 150 × 2.1 mm id, 3 μm. Flow rate of 0.15 ml min^{-1} to 5.5 min and 0.2 ml min^{-1} after to 6.3 min. Injection volume, 5 μl. Other conditions as in Fig. 1: 1, retinol; 2, retinyl acetate; 3, vitamin D_2; 4, vitamin D_3; 5, vitamin E; 6, provitamin D_2; 7, provitamin D_3; 8, tocopherol acetate; 9, vitamin K_1; 10, retinyl palmitate.

As can be seen, practically all the studied vitamins are present in the sample and an adequate cleaning of the same is obtained although notable advantages in the clean-up are not observed utilizing the solid-phase extraction method.

The solid-phase extraction of fat-soluble vitamins from butter samples was not satisfactory because the solvents used to dissolve the butter compete with the functional sorbent groups of the cartridge. Consequently, the fat-soluble vitamins are not quantitatively retained.

The determination of vitamins was achieved by using the external standard method owing to the difficulty encountered in selecting an adequate internal standard for all vitamins and because it was necessary to switch the detection wavelengths in order to select the absorption maximum for each vitamin.

The calibration graphs constructed daily from peak areas *versus* vitamin concentrations were linear ($r > 0.998$) from the determination limit up to at least 4 ppm for vitamin A, D_3, retinyl acetate and retinyl palmitate and up to at least 8 ppm for vitamin D_2, tocopherol, vitamin K_1, tocopherol acetate, ergosterol and 7-dehydrocholesterol.

A precision study was performed on five fresh milk, fortified milk and butter samples using liquid–liquid extraction and the LC conditions described above and on five fresh and fortified milk samples using solid-phase extraction and the LC conditions established above. The samples submitted to a liquid–liquid extraction procedure showed an inter-day precision (standard deviation) lower than 5.7%; similarly, the samples pre-treated with SPE showed a precision lower than 6.8%. All analyses were carried out in triplicate and the intra-day coefficients of variations ranged from 1.6 to 4.5%.

As a test of the recovery of the procedure, extraction was carried out at three fortification levels on milk and butter samples spiked by means of the addition of stock solutions. Table 2 reports the recovery obtained for the fortified milk using liquid–liquid extraction, which ranged between 84 and 108%, testifying to the accuracy of the proposed method. Similar

Table 2 Recovery studies on fat-soluble vitamins added to a fortified milk sample submitted to a liquid–liquid extraction

Vitamin	Concentration in milk/μg l^{-1}	Concentration added/ μg l^{-1}	Concentration found/ μg l^{-1}	Recovery (%)
Retinol	23.9	3.2	26.7	85.2 ± 4.1
		7.2	31.5	104.4 ± 4.5
		10.9	35.4	105.2 ± 3.8
Retinyl acetate	599.5	126.7	725.4	99.4 ± 2.6
		252.0	842.0	96.2 ± 2.7
		410.7	1025.7	103.8 ± 3.2
Vitamin D_2	10.0	7.5	16.7	89.4 ± 4.1
		16.2	25.0	92.4 ± 2.7
		23.8	31.9	92.3 ± 3.5
Vitamin D_3	7.7	4.7	11.9	90.2 ± 3.2
		9.4	17.3	102.1 ± 2.6
		14.8	22.0	96.5 ± 2.4
Vitamin E	102.9	17.9	120.2	96.2 ± 4.3
		21.8	122.0	87.0 ± 3.7
		25.2	126.3	92.6 ± 2.4
Engosterol	163.3	54.2	215.3	96.0 ± 3.6
		65.9	223.2	90.8 ± 2.8
		73.8	235.5	97.8 ± 2.3
7-Dehydrocholesterol	—	2.9	2.7	92.8 ± 3.8
		4.5	4.6	103.1 ± 2.5
		13.8	13.5	98.0 ± 3.2
Tocophenol acetate	5589.6	1618.3	7070.4	91.5 ± 1.6
		2346.1	7897.4	98.4 ± 2.0
		3252.2	9098.5	107.9 ± 2.3
Vitamin K_1	—	10.8	10.5	97.0 ± 1.9
		19.1	17.6	92.2 ± 2.2
		32.7	29.3	89.6 ± 1.8
Retinyl palmitate	66.7	18.3	82.0	83.8 ± 3.4
		55.7	115.0	86.7 ± 2.7
		70.4	129.6	89.4 ± 2.4

results were obtained for the other samples analysed: 79–105% for fresh milk and 85–110% for butter using liquid–liquid extraction; and 74–116% in fresh milk and 80–104% in fortified milk using solid-phase extraction.

Conclusion

Data supporting our work suggest that the combined use of the pre-concentration technique with conventional UV detection allows the simultaneous determination of fat-soluble vitamins and provitamins in milk and dairy foods.

With regard to the possibility of using SPE for sample pre-concentration and clean-up, it seems to be clear that this deserves further study.

This research confirms the usefulness of combining both liquid–liquid extraction and reversed-phase HPLC with narrow-bore columns packed with 3 μm particles to establish fat-soluble vitamins profiles for milk and butter.

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