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Journal of Chromatography A, 874 (2000) 275–283

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Determination of vitamins A and E in milk powder using supercritical fluid extraction for sample clean-up

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Received 15 July 1999; received in revised form 5 November 1999; accepted 17 January 2000

Abstract

A method for the analysis of the natural contents of vitamins A and E in milk powder has been developed. The method utilises supercritical fluid extraction, a miniaturised alkaline saponification procedure and reversed-phase HPLC with UV detection. Modifications of the sample matrix, combinations of static and dynamic modes of extraction and effects of changes in extraction parameters such as temperature, flow-rate, time, collection solvent and collection temperature were studied to optimise the extraction efficiency and selectivity. Supercritical CO_2 at 80°C and 37 MPa, modified with 5% methanol and pumped at a flow-rate of 1.0 ml/min, gave recoveries of 99 and 96% for vitamins A and E, respectively, using a 15 min static followed by a 15 min dynamic extraction. The measurements gave a within-day RSD of 4% for both vitamin A and E, and between-day RSDs of 4 and 8% for vitamins A and E, respectively.

Keywords: Milk powder; Vitamins

1. Introduction

Fat-soluble vitamins are compounds essential for the human body, as they regulate various processes. For example, deficiency of vitamin A may give rise to serious disorders like night blindness and xerophthalmia [1], and lack of vitamin E may lead to breakage of cell membranes, possibly leading to heart diseases and certain cancers [2]. Vitamin A is a group of vitamins containing retinoids (all-*trans*-retinol, 13-*cis*-retinol and others), and carotenoids (such as β-carotene) [3]. Normally (and also here), vitamin A refers to all-*trans*-retinol, which is the most active form of the vitamin. Vitamin E is a family including tocols (tocopherols) and trienols, of which α-tocopherol has the highest vitamin E activity [4].

Vitamins A and E are not synthesised within the body, and must be supplied in the diet [5]. Since fat-soluble vitamins are sensitive towards oxygen, light, heat and extreme pH values the fortification of food is commonly achieved with the more stable vitamin esters, such as retinol acetate, retinol palmitate and tocopherol acetate. To simplify the determination of the vitamins, the sample is normally saponified to transform the vitamin esters into their corresponding parent vitamin. This facilitates separation as well as quantitation, since the number of peaks to be determined is reduced. The hydrolysis of lipids facilitates the further sample work-up.

Traditionally, fat-soluble vitamin analysis is performed by hot saponification followed by liquid–

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0021-9673/00/$ – see front matter
PII: S0021-9673(00)00096-0

liquid extraction with organic solvents like petroleum ether or hexane [6,7]. This procedure consumes large volumes of solvents, and requires a large amount of manual work leading to a low sample throughput. For example, the American standard method of analysis for nutrition labelling concerning vitamin A in milk powder consumes ~85 ml of organic solvents per sample including 25 ml of hexane [6,7].

A faster, less labour intensive and more work environmentally friendly extraction technique would be of great value. Modern supercritical fluid extraction (SFE) could be an interesting alternative in this respect. Some papers on analytical SFE of fat-soluble vitamins have been published which illustrates the potential of the SFE technique. These papers include determination of carotenoids in vegetables [8–13] and plants [14], and of vitamin A palmitate in meat [15], cereal products [16] hydrophobic ointment [17] and cosmetic creams [18]. The only application so far for fat-soluble vitamin extraction of dairy products seems to be the extraction of vitamin K_1 spiked to infant formulas with supercritical carbon dioxide as the extraction fluid [19]. To the best of our knowledge, there is no published work that describes an analytical application of SFE for the determination fat-soluble vitamins, where a saponification step has been included.

In this work we have investigated the possibility of using SFE as a work-up procedure in the determination of the fat-soluble vitamins A and E in milk powder. This study has been performed within an EU development project concerning the determination of vitamins A, D, E and β-carotene in different food formulas.

2. Experimental

2.1. Chemicals

Analytical grade L-(+)-ascorbic acid, palmitoyl ascorbic acid, methanol, 2-propanol and diisopropyl ether were purchased from Merck (Darmstadt, Germany), potassium hydroxide from Eka Nobel (Bohus, Sweden), ethanol (99.5%) from Primalco Oy (Rajamäki, Finland), Hydromatrix (SFE Grade) from Sorbent (Frölunda, Sweden) and petroleum ether (60–80°C, reagent grade) from Lab-scan (Dublin, Ireland). Carbon dioxide (≥99.998%) used as extraction medium and carbon dioxide (industrial grade) used as cryo gas, required for cooling different zones in the SFE apparatus, were obtained from AGA Gas (Sundbyberg, Sweden). Argon (≥99.996%) and nitrogen (≥99.9995%) were also purchased from AGA Gas. All-*trans*-retinol (≥99%) and D,L-α-tocopherol (≥98%) standard samples were obtained from Fluka (Buchs, Switzerland). Standard solutions were prepared by diluting with ethanol. The purity of retinol and the concentration of respective vitamin were determined spectrophotometrically [20,21].

2.2. Equipment

The SFE instrument used was an Isco SFX 3560, Isco (Lincoln, USA), equipped with two pumps (model 260D and 100DX), of 7500 and 10000 PSI capacity, respectively. The HPLC system consisted of an HPLC pump, Isco (Lincoln, USA), a Uvis 204 UV detector, Linear Instruments (Reno, USA), an SP8875 autosampler, Spectra-Physics (Fremont, USA) and a reversed-phase column (Merck LiChrospher® RP-18, 5 μm, 250×4 mm) with a protective guard column (Merck LiChrospher RP-18, 5 μm, 4×4 mm). A Uvikon 930 spectrophotometer, Kontron Instruments (Montigny-le-Bretonneux, France) was used for the concentration and purity determinations of vitamin standard solutions.

2.3. Sample preparation

Milk powder purchased from Västgöta Mjölkförädling (Falköping, Sweden) was carefully homogenised and vacuum packed in aluminium covered plastic bags in 50-g portions. The samples were stored at −18°C to maintain the stability of the vitamins. The inhomogeneity was checked by conventional methodology, and the total variation, also including analytical errors, was below 11% for both vitamins.

2.4. Analytical procedure

The analytical procedure included SFE of the vitamins and their esters followed by a saponification step and final determination by HPLC.

2.4.1. Supercritical fluid extraction

A 0.5-g amount of milk powder was carefully mixed with 1.0 g of Hydromatrix and 0.2 g of ascorbic acid in a mortar. Thereafter, the extraction thimble was filled with three layers of: (1) Hydromatrix (~0.3 g, 1 cm), (2) the above described sample mixture and (3) more Hydromatrix so that the thimble was filled up. Finally 2.0 ml of ethanol was added on top. The samples were then extracted with supercritical carbon dioxide (density 0.80 g/ml) containing 5% of methanol. The extracted substances were collected into a glass tube filled with 16 ml of solvent containing palmitoyl ascorbic acid (≈0.5 mg/ml), to suppress oxidation of the extracted fat-soluble vitamins. After extraction, the sample solvent was evaporated to dryness under a stream of nitrogen. During the optimisation of the SFE method, milk powder samples were taken from the same batch, to ensure that the sample inhomogeneity was kept as low as possible. The SFE conditions, which were kept constant in all optimisation experiments, included a modifier concentration of 5% of methanol, a density of carbon dioxide of 0.8 g/ml and a temperature of the automatic restrictor always 5°C above the extraction temperature. From previous measurements of spiked vitamin solutions added to Hydromatrix it was known that a density of carbon dioxide of 0.8 g/ml and 5% methanol was sufficient to move the vitamins through the extractor, once the vitamins had been liberated from the sample matrix. A relatively low density is advantageous in that the coelution of fat, present in most food samples, is minimised.

2.4.2. Saponification

The extracts were saponified both to degrade the fat and to transform the fat-soluble vitamin esters to their corresponding vitamins. A miniaturised saponification procedure was developed, where 4 ml of ethanol, 10 mg of ascorbic acid and 1 ml of 50% aqueous KOH solution were added to the sample tubes. This reaction mixture was protected from oxygen with argon, and incubated in a water bath at 40°C for 30 min. The samples were shaken rigorously every 10 min during the saponification process. When it was finished, the samples were cooled under tap water, and the unsaponificable fraction was extracted by shaking the sample rigorously for at least 1 min with 6 ml of Milli-Q water and 5 ml of petroleum ether. The two phases were allowed to separate in cold tap water at room temperature. The organic phase was then washed twice with cold Milli-Q water and 4 ml of the organic phase containing the vitamins were finally evaporated to dryness under a gentle stream of argon. The fat-soluble vitamins were reconstituted in 500 μl of ethanol and analysed by HPLC soon afterwards. The reason for choosing ethanol instead of methanol, used in the mobile phase for HPLC, was that co-extracted compounds had higher solubility in ethanol, which assured a one-phase system.

2.4.3. Final analysis

Vitamins A and E were analysed by injecting 20 μl sample into a reversed-phase HPLC system, using a mobile phase of methanol–water (96:4, v/v) and a flow-rate of 1.0 ml/min. Detection was carried out at 325 and 295 nm for vitamins A and E, respectively. The data were evaluated with a BORWIN chromatography software system, JMBS Developements (Le Fontanil, France). A typical chromatogram of a milk powder sample is shown in Fig. 1. The SFE followed by saponification gives a high degree of selectivity as illustrated in Fig. 1.

The vitamins were determined using retention times and peak areas for vitamins in standard solutions. Recoveries were calculated on the basis of results obtained at the Department of Dairy Research and Bacteriology, Agricultural University, Vienna. They used a standard method [20,21], starting with hot alkaline saponification of the sample followed by conventional liquid–liquid extraction and finally HPLC. The average contents from their measurements were 0.11 mg/100 g for vitamin A and 0.34 mg/100 g for vitamin E, with a total relative standard deviation (RSD) of 11% for both (n=6).

3. Results and discussion

The SFE of fat-soluble vitamins from milk powder includes processes such as desorption of the analyte from the matrix, diffusion through and out from the sample pores, partition into the supercritical fluid and finally transportation to the collection device, where trapping is achieved in a solvent. Also a disruption

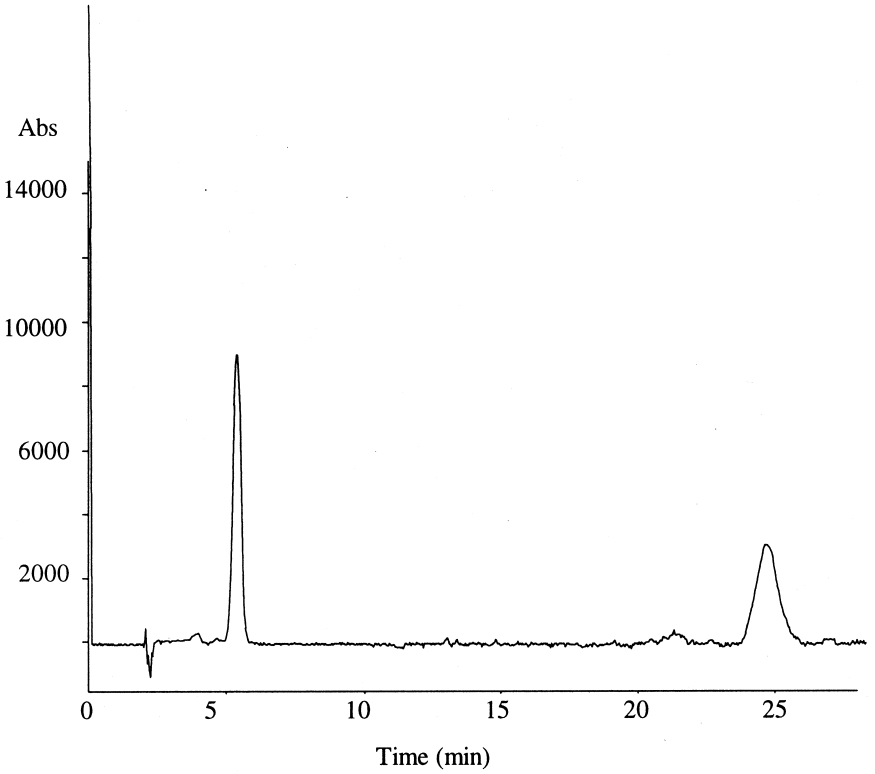

Fig. 1. HPLC chromatogram of vitamin A and α-tocopherol in a milk powder extract, with a mobile phase of methanol–water (96:4). Other HPLC conditions were as follows: flow-rate 1.0 ml/min, injection volume 20 μl, and variable UV detection at 325 and 295 nm for vitamins A and E, respectively.

of lactose shells and fat globules to give the supercritical fluid access to the interior of the sample needs to be considered during the method development. The hard shells of lactose are formed during the short crystallisation time when the milk is spray-dried [22].

Parameters affecting all these processes have been investigated in this work, including modifier addition, extraction temperature, flow-rate, static respectively dynamic extraction mode, extraction time, collection solvent and collection temperature. Various amounts of ethanol were added to study the possibility of breaking the lactose shells.

3.1. Collection

For good recoveries, it is necessary that the collection procedure works properly. Generally a solvent which can provide sufficient strength and has a quite high viscosity should be chosen, to allow the formation of smaller CO_2 bubbles and a longer rising time [23]. Preliminary measurements on standard solutions spiked on Hydromatrix showed that a flow-rate of 1.0 ml/min, a solvent volume of 16 ml (corresponding to a height of 7 cm in the collection device), a restrictor temperature of 85°C, a collection temperature of 10°C and 2-propanol with 0.5 mg/ml of palmitoyl ascorbic acid as solvent gave close to 100% recovery. However, when analysing complex samples like food, it is important that not only the target analytes, but also that coextracted major components (in this case fat), are trapped. Otherwise analytes may be lost due to aerosol formation [24]. Accordingly, the trapping procedure was further investigated using authentic milk powder samples run with the same set of parameters as in the preliminary experiments. The solvent composition and the collection temperatures were further investi-

Table 1
Collection performances when extracting milk powder; a study of two different solvents and three collection temperatures[a]

Collection solvent	Collection temperature (°C)	Recovery (%)		*n*
		Vitamin A	Vitamin E	
2-Propanol	5	98 (7)	87 (14)	3
EtOH–IPE (1:1)	5	98 (4)	101 (5)	6
EtOH–IPE (1:1)	10	103 (6)	95 (2)	3
EtOH–IPE (1:1)	15	101 (4)	89 (4)	3

[a] RSD for each experiment is given in parentheses. SFE conditions were: CO_2 at 0.80 g/ml with 5% MeOH as modifier, 80°C, 37 MPa, 15 min static and 15 min dynamic extraction, 1.0 ml/min; 2.0 ml of ethanol was added to the sample prior to extraction. EtOH–IPE (1:1) is an ethanol–diisopropyl ether mixture at a volume ratio of 1:1 in a test tube. Solvent volume 16 ml and height 7 cm.

gated by including ethanol–diisopropyl ether (1:1) with 0.5 mg/ml of palmitoyl ascorbic acid and by varying the temperature between 5 and 15°C.

Results are given in Table 1. The recovery values are calculated from the vitamin concentrations determined for the same batch of milk powder with a reference method [20,21].

According to the results shown in Table 1 the vitamin E recovery is higher using ethanol–diisopropyl ether (1:1, v/v) than with pure 2-propanol, while vitamin A recoveries are unaffected. This mixture was therefore chosen as collection medium in all further experiments. Collection temperature is expected to influence analyte trapping by changing the solvent viscosity [25]. In these experiments it was found that 5°C was significantly better for vitamin E at 95% confidence level while vitamin A was not affected (Table 1). In addition, a low temperature also increases the stability of the fat-soluble vitamins. Taking all results into consideration, the ethanol–diisopropyl ether mixture at a collection temperature of 5°C was chosen for all further experiments.

3.2. Matrix modifying

In preliminary experiments on milk powder, low recoveries were obtained even when using alcoholic modifiers mixed with supercritical carbon dioxide. This was believed to result from the lactose shell surrounding the milk powder particles. In a work by Roy et al. [26] it was shown that the extraction rate of oil from tomato seeds almost totally determined the overall extraction process. The oil recovery was also significantly improved by milling the seeds to smaller particles. In this work, experiments were performed, where relatively large volumes (2 ml) of methanol or ethanol were added manually to the milk powder prior to extraction in an attempt to make the vitamins more accessible to the supercritical fluid, which was carbon dioxide containing 5% alcohol. Results are given in Fig. 2.

The results in Fig. 2 indicate that both methanol and ethanol enable quantitative extractions, in contrast to samples without modifier, which gave recoveries of only 57 and 30% for vitamins A and E,

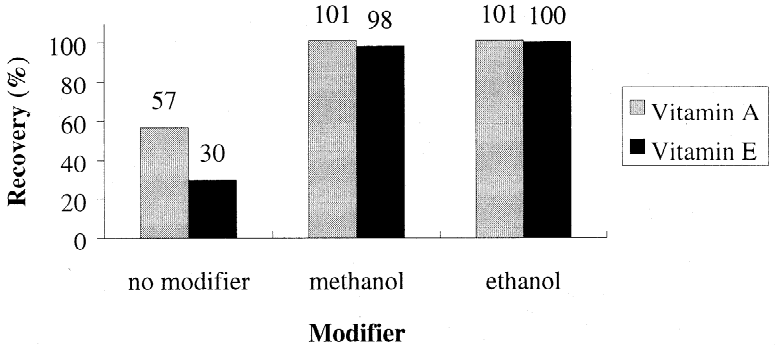

Fig. 2. Effects of manually adding modifier to milk powder samples before SFE. Samples were wetted with 2.0 ml of methanol or ethanol and a reference sample with no modifier added were extracted with CO_2 containing 5% of methanol. For each type of sample three extractions were performed. Other SFE conditions: 37 MPa, 80°C, 15 min static and 15 min dynamic extraction, 1.0 ml/min, collection into 16 ml of ethanol–diisopropyl ether (1:1) at 5°C.

respectively. With the modifier the RSDs of the recoveries were below 5% for both vitamins A and E, compared to 48 and 115%, respectively, without modifier. The most probable explanation for the improvement in recovery and precision is that the high concentration of alcohol in the extractor (proportions around 1:1 (w/w) between the alcohol and the rest of the sample), disrupts the lactose shells covering the milk powder particles, so that the supercritical fluid gets access to the vitamins. In all subsequent experiments, 2.0 ml of ethanol was added to the sample prior to extraction.

3.3. Extraction temperature

Higher extraction temperature should facilitate both the disruption of hard structures and the desorption process of analytes bound to the matrix, and increase the diffusion rate of the analyte out from the matrix pores to the supercritical bulk flow [27]. The influence of temperature on the recovery of vitamins A and E from milk powder was investigated by extracting samples at 40, 60 and 80°C with three experiments at each temperature. The other SFE conditions were 0.80 g/ml, 5% MeOH as modifier, 1.0 ml/min, 15 min static and 15 min dynamic extraction, collection into 16 ml of ethanol–diisopropyl ether (1:1) at 5°C and 2.0 ml of ethanol added to the sample prior to extraction.

The obtained recoveries were, for both vitamins at all temperatures, above 90%, with an average RSD of 4.5% with the highest recoveries (101 and 100%) at the highest temperature (80°C) for vitamins A and E, respectively. Accordingly, an extraction temperature of 80°C was chosen for the subsequent experiments. Higher temperatures should be avoided since they carry an increased risk of vitamin degradation was found in extractions of standard solutions.

3.4. Flow-rate

Milk powder samples were extracted with 15 ml CO_2–methanol (95:5), at flow-rates of 0.5, 1.0 and 2.0 ml/min with an initial static extraction step of 15 min and other SFE parameters as above. Results from three replicate experiments at each flow-rate showed that the flow-rate had virtually no effect on the extraction efficiency, with vitamin A recoveries of 105, 101 and 110% for flow-rates of 0.5, 1.0 and 2.0 ml/min, respectively.

During the static step, most of the fat-soluble vitamins have probably already been extracted from the milk powder matrix. The dynamic extraction step is mainly needed to transport the dissolved vitamins through the extraction thimble to the collection device. The flow-rate of 1.0 ml/min was chosen in subsequent experiments to make the method more robust. Lower flow-rates require longer extraction times, and higher flow-rates may cause reduced trapping efficiency, because of the formation of larger CO_2 bubbles, creating a longer diffusion paths for the analytes in the bubbles out to the solvent [28].

3.5. Extraction time and extraction mode

The extraction mode (static or dynamic) as well as the time used for these two different extraction steps is expected to have a large influence on the recovery. In this work, one set of milk powder samples was subjected to 0, 5, 10 and 15 min static extraction followed by 15 min dynamic extraction. Another set of samples was exposed to 15 min static extraction followed by 5, 10, 20, 30, 45, 60 and 90 min dynamic extraction. The other SFE conditions were as outlined above. The outcome of these experiments, performed in triplicate for each set of conditions was that vitamin A and E were readily extracted from milk powder. Using 5 min static and 15 min dynamic extraction, alternatively 15 min static and 10 min dynamic extraction, recoveries above 90% for both vitamins were obtained at an average RSD of 5%. For further experiments 15 min was chosen for the static as well as for the dynamic extraction step.

3.6. Quantitation

From the optimisation experiments discussed in 3.1–3.5 final conditions were chosen for the extraction of vitamins A and E from milk powder. These conditions are given in Table 2.

Based on these conditions a number of milk powder samples were run to obtain accuracy and precision values. The recovery values were as previ-

Table 2
Optimised SFE conditions for the extraction of vitamins A and E from milk powder

SFE parameters	Outcome
Extraction temperature	80°C
Pressure	37 MPa
Density	0.80 g/ml
Modifier in the CO_2	5% methanol
Modifier added to the extraction cell	2.0 ml ethanol
Flow-rate	1.0 ml/min
Static extraction time	15 min
Dynamic extraction time	15 min
Restrictor temperature	85°C
Collection solvent	Ethanol–diisopropyl ether (1:1) containing 0.5 mg/ml of palmitoyl ascorbic acid
Collection temperature	5°C
Solvent volume	16 ml

ously obtained by comparing with values obtained from the reference method for vitamins used on the same batch of milk powder. Results are shown in Table 3.

The accuracy is good at the low concentrations of vitamins A and E occurring in milk powder. The within-day RSD is below 4% for both vitamins. For vitamin E the between-day RSD of 8% is significantly larger than the variation within-day, while for vitamin A it is 4% for both. For these oxidation, light and temperature sensitive compounds, the accuracy as well as the precision is considered to be sufficient. One source of uncertainty in the determination of the recoveries is that the values obtained by the reference method also may have some errors and that there is always an inhomogeneity between solid samples of the same batch with respect to the analyte. The sample inhomogeneity of the milk powder, determined by the reference method, is $<11\%$, including analytical errors.

3.7. Remarks

The advised method reduces the amount of organic solvent needed for the entire analysis, which is favorable with respect to the work environment and also increases the sample throughput. One important difference in the advised method is that sample work-up starts with SFE followed by saponification in contrast to conventional vitamin analysis, where the starting point is a saponification followed by a solvent extraction. This new approach results in similar and simpler matrices for different food extracts before the saponification procedure, which should be advantageous with respect to interferents. For food formulas the automated SFE procedure,

Table 3
Recoveries for milk powder samples, utilising the developed SFE method[a]

Fat-soluble vitamin	Vitamin content (mg/100 g sample)	Average recovery (%) ($n=9$)	RSD (%)		
			Within-day ($n=9$)	Between-day ($n=3$)	Total ($n=9$)
Vitamin A	0.11	99	4	4	5
Vitamin E	0.34	96	4	8	8

[a] Recovery values are based on results obtained by conventional methodology. The within-day and between-day variations are calculated using single factor Anova [29]. The results are obtained by measuring three replicate samples on 3 different days.

which can be run overnight, combined with the micro saponification procedure, where only minute amounts of solvent needs to be evaporated, results in a considerably higher sample throughput than conventional vitamin determinations based on solvent extraction. The two rate determining steps, the SFE extraction and the saponification procedure both take ~35 min, which should allow a sample throughput of at least 24 samples per day including all other sample preparation steps and the final HPLC determination. This is at least a factor of 3 higher than using manual procedures.

Internal standards are mainly used to reduce volumetric errors or to account for analyte losses during sample work-up. Due to difficulties to find appropriate internal vitamin standards all transfers of volumes were performed with volume calibrated pipettes. With recoveries above 99% for vitamin A and 96% for vitamin E the need for internal standards to account for losses during sample work-up was not judged to be necessary.

4. Conclusions

The results obtained here show that SFE is a promising candidate for replacing traditional liquid–liquid extraction techniques in the determination of vitamins A and E in food formulas. It is faster, more automated and uses smaller amounts of organic solvents than the traditional method. This work also points out that matrix modifications are needed prior to extraction to get high recoveries. The developed SFE methodology has the potential to be used for other food formulas and other fat-soluble vitamins as well. Such work is in progress within a current EU project.

Acknowledgements

Mr. F. Ulberth and Mr. R. Gabernig, of the Department of Dairy Research and Bacteriology, Agricultural University, Vienna, are gratefully acknowledged for performing the vitamin analysis with conventional methodologies. The financial support from the European Union (EU project SMT4-CT96-2089) is also acknowledged.

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