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# On-Line Supercritical Fluid Extraction/Enzymatic Hydrolysis of Vitamin A Esters: A New Simplified Approach for the Determination of Vitamins A and E in Food

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An on-line supercritical fluid extraction (SFE)/enzymatic hydrolysis procedure using immobilized lipase has been developed for the determination of vitamin A in dairy and meat products. Several lipases were tried, of which Novozyme 435 (*Candida antarctica* type B) showed the highest activity toward retinyl palmitate. There was no observed activity with  $\alpha$ -tocopheryl acetate. When pressure, temperature, modifiers, flow rate, extraction time, and water content were varied, high vitamin A recovery was obtained in milk powder. Collected extracts were analyzed by reversed-phase high-performance liquid chromatography with ultraviolet and fluorescence detection without additional sample cleanup. The procedure gave reliable values of vitamin A as well as of vitamin E in other food items such as infant formula, minced pork and beef meat, and low- and high-fat liver paste. The described method is faster and more automated than conventional methods based on liquid–liquid extraction, or SFE using off-line saponification, for vitamin A and E determination. Results obtained with the new method did not differ significantly from those obtained with the other two methods mentioned above.

**Keywords:** *Enzymatic hydrolysis; fat-soluble vitamins; lipase; milk powder; supercritical fluid extraction*

## INTRODUCTION

Fat-soluble vitamins are important to determine because they have key roles in several functions of the human body, such as vision (vitamin A), calcium absorption (vitamin D), antioxidative protection in cell membranes (vitamin E), and blood coagulation (vitamin K) (1). Their determination is conventionally based on a hot saponification procedure followed by liquid–liquid extraction with hexane and/or ether and HPLC (2, 3). Increasing environmental concern with minimizing the use of organic solvents has directed the interest toward supercritical fluid extraction (SFE) with carbon dioxide (4, 5). Applications of analytical SFE for fat-soluble vitamin determination in food products include carotenoids from vegetables (6), vitamin K<sub>1</sub> from powdered infant formulas (7), vitamin A palmitate from breakfast cereals (8), and vitamin A palmitate and  $\beta$ -carotene from calf liver (9).

The first SFE method for the determination of the total concentration of vitamins A and E in milk and meat products as their retinol and tocopherol analogues via the introduction of a subsequent off-line saponification step was recently presented (10). The advantage of the saponification procedure after SFE is the same as when using saponification in conventional vitamin analysis, that is, facilitated determination, because the vitamin esters are hydrolyzed to their mother compounds and the triacylglycerols are converted to free

fatty acids. These elute close to the solvent front, well separated from the vitamins in the HPLC system.

Another way of hydrolyzing triacylglycerols and vitamin esters is to use enzymes. If this step is performed at supercritical conditions, the enzyme must be able to withstand high pressure and elevated temperature. One class of enzymes that is interesting in this respect is lipases. They have already been used in immobilized form in analytical SFE for hydrolysis of glycerol esters in a work describing total fat determination (11) and for the determination of fatty acid composition after transformation of fatty acids to their corresponding methyl esters (12). No attempts have as far as we know been made up to now to use enzymes for the hydrolysis of vitamins at SFE conditions. However, it has been concluded by Harrison (13) that lipases can be used for the hydrolysis of retinyl esters in aqueous solutions.

In this paper we show the possibility of performing a lipase-catalyzed hydrolysis of retinyl esters and fat components in the SFE extraction cell while simultaneously extracting the formed retinol compound and fatty acids. Because naturally occurring vitamin E in most food products already is present as nonesterified tocopherols (14), it should be possible to modify a determination procedure for vitamin A in food formulas to include vitamin E. This possibility has also been investigated.

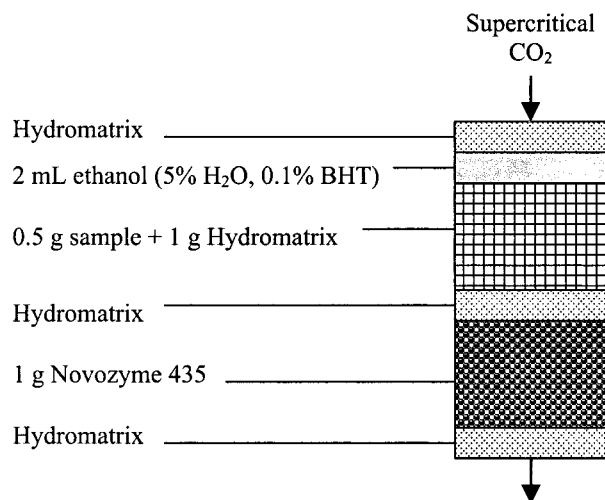
## EXPERIMENTAL PROCEDURES

**Apparatus.** The analytical HPLC system consisted of a Waters 600E HPLC pump (Millipore Corp., Milford, MA), a Waters 490E variable UV detector, a Thermo Separation FL2000 fluorescence detector (ThermoQuest, San Jose, CA),

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**Figure 1.** Illustration of the different layers in the extraction cell.

a Thermo Separation SP8780 autosampler, and a ChromQuest chromatography data system (ThermoQuest). The SFE instrument used in this study was an Isco SFX 3560 (Isco Inc., Lincoln, NE), equipped with two 100DX pumps. A Pierce Reacti-Therm heating module (Rockford, IL) was used for solvent evaporations.

**Reagents.** Carrier fixed enzymes Chirazyme L-1 (EC 3.1.1.3, *Pseudomonas cepacia*, 10,000 tributyrin units/g), Chirazyme L-5 (EC 3.1.1.3, *Candida antarctica*, type A, 1000 tributyrin units/g), and Chirazyme L-9 (EC 3.1.1.3, *Rhizomucor miehei*, 8000 tributyrin units/g) were purchased from Boehringer Mannheim Corp. (Indianapolis, IN) and Lipozyme IM (EC 3.1.1.3, *Rhizomucor miehei*, 5–6 batch acidolysis units Novo/g) and Novozyme 435 (EC 3.1.1.3, *Candida antarctica*, type B, 7,000 propyl laurate units/g) from Novo Nordisk Inc. (Franklinton, NC). Analytical grade butylated hydroxytoluene (BHT) was obtained from Sigma (St. Louis, MO), methanol (HPLC grade) from Fisher Scientific (Fair Lawn, NJ), 99% ethanol from AAPER Alcohol and Chemical Co. (Shelbyville, KY), and water adsorbing, silica-based Hydromatrix from Varian (Harbor City, CA). SFE grade carbon dioxide was purchased from Air Products (Allentown, PA), and industrial grade carbon dioxide and nitrogen ( $\geq 99.9995\%$ ) were obtained from BOC Gases (Murray Hill, NJ). *all-trans*-Retinol,  $\alpha$ -tocopheryl acetate, and *dl*- $\alpha$ -tocopherol were acquired from Fluka Chemie AG (Buchs, Switzerland). Vitamin A palmitate,  $\gamma$ -tocopherol, and  $\delta$ -tocopherol were obtained from Sigma. All of the above standards were of analytical grade, 98% purity or higher.

**Analytical Procedure.** One gram of Novozyme 435 or Lipozyme IM or 0.75 g of Chirazyme L-1, L-5, or L-9 was loaded into the extraction cell at the outlet side, as shown in Figure 1.

The fresh immobilized lipase filled up one-third to half of the total volume of the extraction cell (10 mL). A small layer of Hydromatrix was added to separate the immobilized enzyme from the sample mixture. One-half gram of sample was weighed accurately and mixed with 1 g of Hydromatrix and then added on top of the Hydromatrix layer in the extraction cell. Two milliliters of ethanol containing 5% water (v/v) and 0.1% BHT (w/v) was thereafter added on top of the sample mixture to facilitate better extraction (15). Water was added to speed the enzymatic reaction; BHT was added to protect the vitamins from degradation. Finally, the remaining cell volume was filled with Hydromatrix. During the entire analytical procedure, care was taken to avoid sample degradation caused by light, oxygen, or excessive heating. The samples were extracted with supercritical carbon dioxide modified with 5 vol % ethanol, and collection was performed in 10 mL of ethanol containing 0.1% BHT (w/v). After extraction, the sample extracts were evaporated under a stream of nitrogen to dryness and then redissolved in 1.0 mL of ethanol.

The fat-soluble vitamins were determined by RP-HPLC, using a Merck column (LiChrospher RP-18, 5  $\mu$ m, 250 mm length  $\times$  4 mm i.d.), with one UV and one fluorescence detector connected in series in this order from the column outlet. A 20  $\mu$ L ethanol injection was made into a mobile phase consisting of methanol/water (98:2 v/v) at a flow rate of 1.0 mL/min. Vitamin A (*all-trans*-retinol) and retinyl palmitate were detected by employing UV detection at 325 nm, and  $\alpha$ -tocopheryl acetate was detected at 284 nm. The nonesterified tocopherols were determined by fluorescence detection using 294 nm for excitation and 330 nm for emission.

The vitamin concentrations in the samples were calculated by comparison with peak areas obtained for vitamin standard solutions in the range of 0.1–10  $\mu$ g/mL. Recoveries were calculated on the basis of the average of results obtained for samples from the same batch at seven laboratories using either SFE methodology combined with alkaline saponification (five of the laboratories) or conventional methodology (two laboratories). These values were obtained in an intercomparison study (16) within an EU project (SMT4-CT96-2089) and were as follows (mg/100 g): milk powder, 0.12 (vitamin A), 0.29 ( $\alpha$ -tocopherol); infant formula, 0.52 (vitamin A), 5.68 ( $\alpha$ -tocopherol), 3.20 ( $\beta/\gamma$ -tocopherol); liver paste (12%), 5.78 (vitamin A), 0.77 ( $\alpha$ -tocopherol), liver paste (23%), 1.55 (vitamin A), 0.29 ( $\alpha$ -tocopherol); minced pork meat, 0.007 (vitamin A), 0.32 ( $\alpha$ -tocopherol); and minced beef meat, 0.006 (vitamin A), 0.42 ( $\alpha$ -tocopherol).

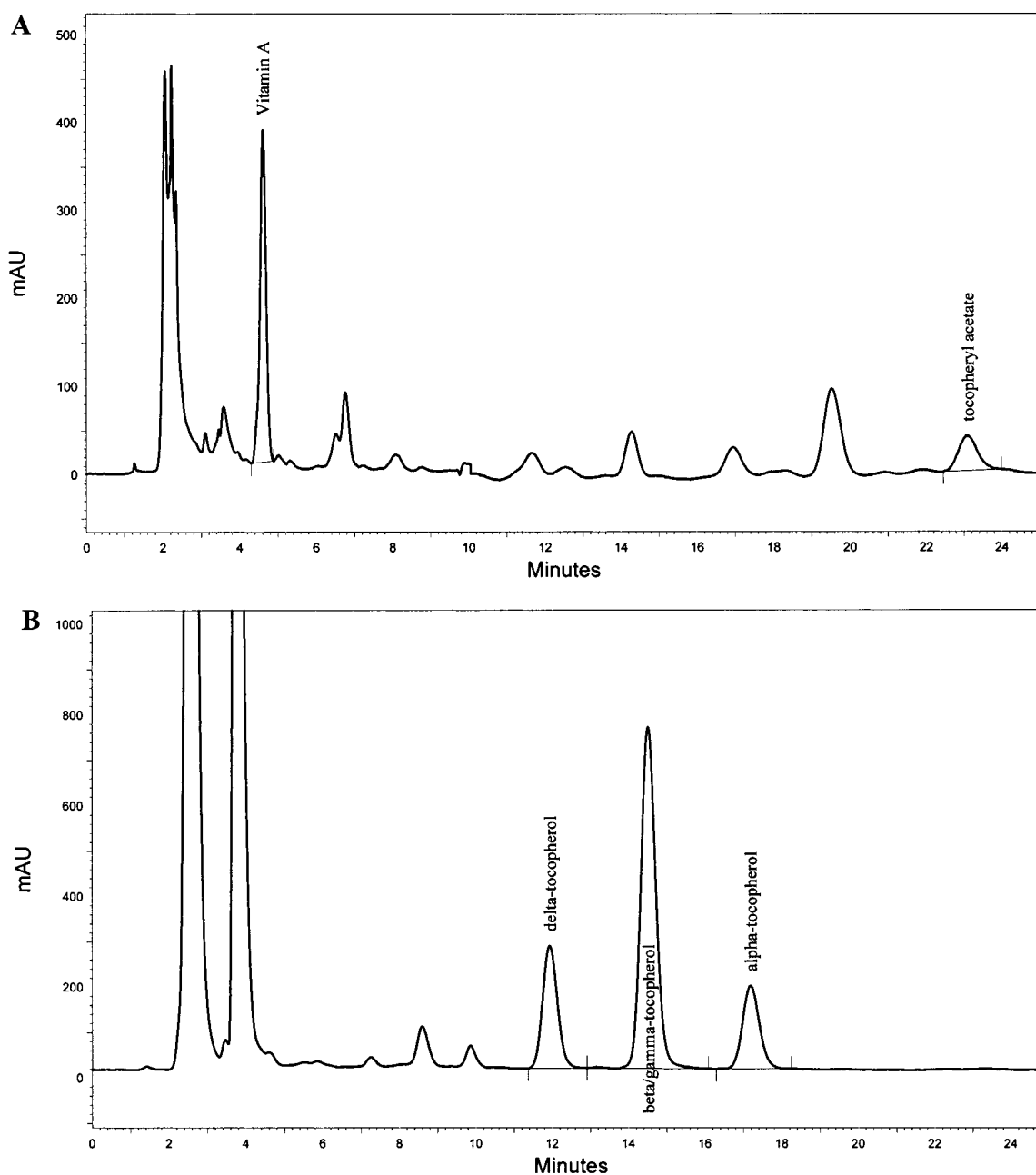
## RESULTS AND DISCUSSION

**Chromatography.** With serially connected variable UV and fluorescence detectors it was possible to determine all analytes of interest in one run. Figure 2 shows the results of an analysis of an infant formula sample.

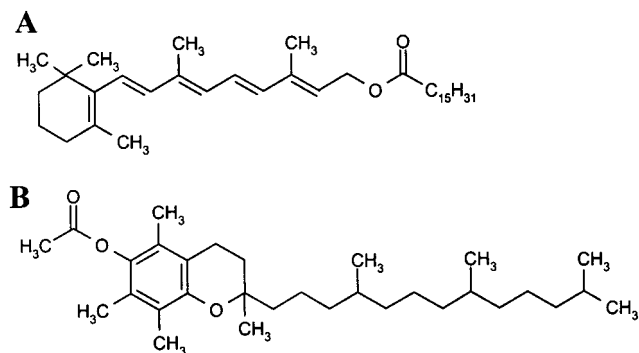
It is clear that a determination of vitamins A and E in all of the considered food samples could be achieved within 30 min. The sensitivity using fluorescence detection for tocopherols is  $\sim 10$  times higher than that using UV detection. Tocopheryl acetate is practically nonfluorescing (17) and was determined with UV detection at 284 nm. Of the considered food formulas (milk powder, meat, liver paste, and infant formula), only infant formula had detectable amounts of tocopheryl esters ( $\alpha$ -tocopheryl-acetate). The analysis time for the other food formulas can thus be reduced to 20 min.

**Choice of Enzyme.** Several commercially available immobilized lipases were tried to find an enzyme with proper catalytic activity for fat-soluble vitamin esters. Initial SFE experiments, where 1 mL of ethanolic solution containing retinyl palmitate and  $\alpha$ -tocopheryl acetate was used as sample, showed that Novozyme 435 and Chirazyme L-5 gave highest transformation of the retinyl ester. There was no observed enzymatic reaction for  $\alpha$ -tocopheryl acetate with any of the investigated lipases. The active sites of these enzymes are probably too small to accommodate the bulky aromatic group of  $\alpha$ -tocopheryl acetate (Figure 3B). The ester group of retinyl palmitate is bound to a long carbon chain, which obviously does not prevent reaction at the active site of the enzymes (Figure 3A).

The implication that  $\alpha$ -tocopheryl acetate, and probably other tocopheryl esters as well, cannot be cleaved by any of the lipases investigated leads to a slightly more complex final analysis when also vitamin E is to be determined. In this case all tocopherols and tocopheryl esters must be determined. The vitamin E concentration then has to be calculated using the concentration values for the different compounds plus their vitamin E activities. Fortunately, in most natural food products only tocopherol itself (and in a few cases  $\alpha$ -tocopheryl



**Figure 2.** HPLC chromatogram after SFE/enzymatic hydrolysis of an infant formula sample using serially connected detectors: (A) variable UV (0–10 min, 325 nm; 10–25 min, 284 nm); (B) fluorescence (294 nm excitation/330 nm emission). Analytical procedure was as under Experimental Procedures.



**Figure 3.** Chemical structures of (A) retinyl palmitate and (B)  $\alpha$ -tocopheryl acetate.

acetate) gives significant contribution to the vitamin E content (14). It is notable that a saponification step has

so far normally been included in the determination of total vitamin E content in food, although there is a considerable risk of vitamin degradation (18). An enzymatic hydrolysis of vitamin A esters (and triacylglycerols) at gentle conditions may thus be a better way of determining vitamin E.

Novozyme 435 was chosen for all further experiments because it worked well for vitamin A and had previously been found to be efficient for quantitative hydrolysis, alcoholysis, and interesterification of lipids in supercritical carbon dioxide (11, 19–20).

**Optimization of Parameters for Vitamin A Determination in Milk Powder.** Optimization of the analytical procedure was performed using 0.5 g milk powder samples (obtained from AB Västgöta Mjölkrädling, Falköping, Sweden) mixed with 1 g of Hydro-matrix. Preliminary experiments with the addition of



**Table 1. Investigation of Pressure Dependency on the Recovery of Vitamin A in Milk Powder ( $n = 2$ ) after Enzymatic Hydrolysis<sup>a</sup>**

pressure (psi)	vitamin A recovery (%)
2500	51
4000	81
5500	106
7000	17

<sup>a</sup> The SFE parameters were as follows: CO<sub>2</sub> with 1 vol % ethanol as modifier, 70 °C, 15 min static and 30 min dynamic extraction at a flow rate of 0.5 mL/min. The pressures 2500, 4000, 5500, and 7000 psi correspond to densities of 0.59, 0.77, 0.85, and 0.90 g/mL, respectively, at the given temperature.

**Table 2. Effects of Different Extraction Temperatures on Vitamin A Recoveries in Milk Powder ( $n = 3$ )<sup>a</sup>**

temperature (°C)	vitamin A recovery (%)
40	57 (4)
50	79 (9)
60	102 (2)
70	97 (11)
80	101 (12)

<sup>a</sup> The SFE parameters were as follows: CO<sub>2</sub> with 1 vol % ethanol as modifier, 0.8 g/mL [corresponding to 2313–5308 psi (157–361 atm) in the temperature range 40–80 °C], 15 min static and 45 min dynamic extraction at 0.5 mL/min. RSD values (%) are given in parentheses.

different amounts of ethanol solution (containing 0.1% BHT w/w) and using 1 g of Novozyme 435 with the sample showed that 2 mL of ethanol gave full vitamin A recovery (102%), whereas 1 mL of ethanol gave only 85% recovery. The relative standard deviations (RSDs) of the recoveries (three replicates) were considerably lower when using the larger amount of entrainer (2% compared to 12%). Accordingly 2 mL of ethanol was used in further experiments.

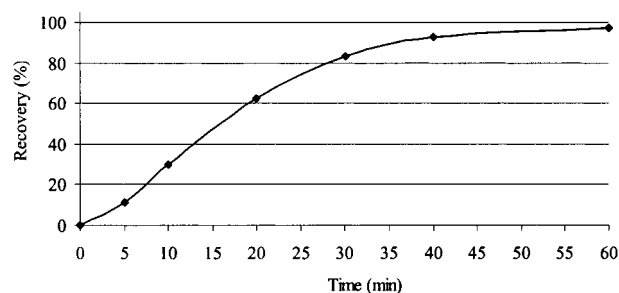
**Effect of Pressure.** The optimal pressure in the range of 2500–7000 psi (170–476 atm) at 70 °C was investigated, giving the results shown in Table 1.

The results in Table 1 show that vitamin A recovery increases with higher extraction/reaction pressure and density to >100% at 5500 psi (374 atm) and drops dramatically at 7000 psi (476 atm). This decrease is most likely caused by pressure-induced inactivation of the enzyme and not by reduced extraction capability, because increased pressure, which gives higher density of the supercritical fluid, normally should lead to higher solubility of the analyte and hence higher recoveries.

**Extraction/Reaction Temperature.** The effect of different extraction/reaction temperatures was investigated by applying 40, 50, 60, 70, and 80 °C while keeping the density constant at 0.8 g/mL. These results are shown in Table 2.

Table 2 implies that quantitative vitamin A recovery is obtained in the temperature range of 60–80 °C. Our findings confirm earlier observations of the high thermal stability exhibited by *Candida antarctica* in supercritical CO<sub>2</sub> (21). With respect to the thermolability of other fat-soluble vitamins as well as the long-term activity of the enzyme, the lowest temperature (60 °C), which gave 100% recovery, was chosen as the extraction temperature in further experiments. With a density of 0.8 g/mL, the extraction pressure becomes 3757 psi (256 atm), which is still below pressures leading to decreased enzyme activity.

**Kinetics.** To investigate the kinetics of the extraction of vitamin A from milk powder at an extraction tem-

**Figure 4.** Extraction profile of vitamin A in milk powder. The points are average values of two extractions. The SFE parameters were as follows: CO<sub>2</sub> with 1 vol % ethanol as modifier, 60 °C, 0.8 g/mL (3757 psi, 256 atm), and 0.5 mL/min.

perature of 60 °C, the dynamic extraction time was divided into several steps with subsequent collection in separate vials. The process was initiated by a 1-min static extraction step to ensure a constant flow rate from the beginning, followed by dynamic extraction with collection intervals of 5, 5, 10, 10, 10, and 20 min, giving a total dynamic extraction time of 60 min. The extraction profile in Figure 4 shows that 45 min of dynamic extraction should be sufficient, especially because 15 min instead of 1 min of static extraction was used in further experiments.

The kinetics of the enzymatic hydrolysis/alcoholysis was also examined because fast reaction kinetics might allow higher flow rates, resulting in shorter analysis times. Samples were extracted using the same total volume of supercritical fluid by applying 45 min of extraction at 0.5 mL/min, 23 min at 1.0 mL/min, and 12 min at 2.0 mL/min. The dynamic step was in all cases initiated by 15 min of static extraction. Higher vitamin A recovery (89%) was obtained at 0.5 mL/min, whereas 1.0 and 2.0 mL/min gave 79 and 81% recoveries, respectively. The lowest flow rate was chosen in further experiments.

**Influence of a Delay Time on the Recovery.** The SFE instrument (Isco SFX 3560) used is a fully automated instrument, which permits overnight extractions. In the experiments described until now the instrument carousel was loaded with samples in the evening, and the method was started automatically at midnight, making the samples ready for analysis in the morning. This minimizes the risk of analyte degradation due to long waiting times. Occasionally the same experiments were repeated during the daytime, with the extraction starting immediately after the samples had been prepared. It then turned out that the recoveries were much lower. This was further investigated by applying 1, 3, and 7 h delay times. The relative recoveries increased with almost 40% by increasing the delay time to 3 h and were not significantly improved using a delay time of 7 h. Hence, the sample disruption process, which is obtained by modifier addition to the sample prior to extraction, seems to be rather slow. Accordingly, 3 h of delay time was applied in further experiments.

**Influence of Modifier on the Recovery.** In a previous work 5% of methanol as modifier was used to get quantitative vitamin extraction from different food formulas (15). The addition of methanol makes the supercritical fluid more polar, which facilitates its ability to break interactions between the vitamins and the sample matrix. This results in a faster transport of the vitamins through the sample, which can be considered as a chromatographic column. Thus, to achieve a

**Table 3. SFE Parameters for the Developed Method**

SFE parameter	outcome
entrainer (added to the sample in the extraction cell)	2.0 mL of ethanol (0.1% BHT, 5% H <sub>2</sub> O)
delay time	3 h
static extraction time	15 min
dynamic extraction time	45 min
extraction temperature	60 °C
pressure	3757 psi (256 atm)
density	0.80 g/mL
modifier	5% ethanol
flow rate	0.5 mL/min
collection solvent	10 mL of ethanol (0.1% BHT)
collection temperature	10 °C

good recovery, the modifier concentration as well as the extraction time is of importance. Because a high modifier concentration might lead to decreased enzyme activity (22), the optimal condition of using ethanol as modifier was investigated with an extraction time of 60 min. Ethanol concentrations of 1, 2, and 5% gave recoveries of 78, 81, and 92%, respectively. Because no retinyl esters could be found in the extracts, the increased recoveries at 5% modifier concentration most likely depend on most of the vitamin being passed through the extraction cell after 60 min of extraction time, corresponding to a volume of extraction fluid of 22.5 mL. Accordingly, CO<sub>2</sub>/ethanol (95:5/v:v) was chosen for further experiments.

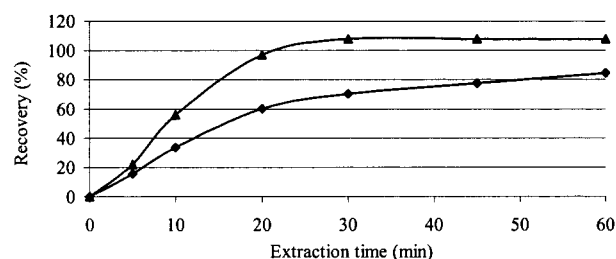
**Water Concentration.** The water concentration of the extraction/reaction system affects the activity and the stability of the enzyme (23). It is therefore important to control the total amount of water entering the system. For a sample of 0.5 g it turned out that 100  $\mu$ L of water was sufficient to give high recoveries and good precision (RSD of 4%,  $n = 3$ ). This amount was achieved by adding 2 mL of 95% ethanol to the sample before the extraction procedure was begun. Moreover, the 5% of ethanol continuously added during the extraction procedure contained ~1% of water. Hence, the total water concentration of the supercritical fluid was ~0.05%, which prevents it from stripping water from the enzyme.

**Determination of Vitamins A and E in Different Food Samples.** The optimal parameters obtained for vitamin A in milk powder are given in Table 3.

The conditions for SFE given in Table 3 for vitamin A are similar to those used previously for the determination of vitamins A and E in different food formulas using a subsequent saponification step (15). Accordingly, the conditions in Table 3 were used in the investigation of other food formulas as well.

**Kinetics of the Extraction Process.** This was investigated for infant formula as well as liver paste. The kinetics for the extraction of vitamin A (as retinol) and vitamin E (as  $\alpha$ -tocopherol and  $\alpha$ -tocopheryl acetate) in infant formula according to the conditions in Table 3 is illustrated in Figure 5. The points on the curve are obtained using a fractionated extraction-collection procedure, starting with a 15 min static step. The time axis represents the dynamic extraction.

It seems that an extraction time of even >60 min might be beneficial for the determination of vitamin A as retinol in infant formula. The extraction of vitamin E, however, is quite fast and complete within ~30 min of dynamic extraction. The longer time needed for the extraction of vitamin A compared to vitamin E is in contrast to our previous experiences, when extractions were performed without including enzyme in the extraction cell. The faster extraction of vitamin A found



**Figure 5.** Extraction profile of vitamins A (●) and E (▲) in infant formula. The points are average values of two extractions. The SFE parameters were as follows: CO<sub>2</sub> (5% ethanol), 60 °C, 0.8 g/mL (3757 psi, 256 atm), and 0.5 mL/min. The  $\alpha$ -tocopherol recoveries are based on the sum of extracted  $\alpha$ -tocopherol and  $\alpha$ -tocopheryl acetate.

**Table 4. Recoveries of Vitamins A and E in Different Food Formulas ( $n = 3$ )<sup>a</sup>**

sample	recoveries (%)		
	retinol	$\alpha$ -tocopherol	$\beta/\gamma$ -tocopherol
milk powder	102 (2)	85 (3)	
infant formula	79 (13)	92 <sup>b</sup> (7)	104 (6)
liver paste (12%)	119 <sup>c</sup> (12)	152 (71)	
liver paste (23%)	104 <sup>d</sup> (12)	91 (59)	
minced pork meat	103 (19)	117 (5)	
minced beef meat	98 (21)	121 (28)	

<sup>a</sup> The SFE parameters were as in Table 3. RSD values (%) are given in parentheses. <sup>b</sup> The value includes 75%  $\alpha$ -tocopheryl acetate. <sup>c</sup> The value includes 32% retinyl palmitate. <sup>d</sup> The value includes 20% retinyl palmitate.

previously could depend on the fact that it was mainly extracted as retinyl palmitate, whereas vitamin E also here was extracted mainly as free tocopherols. The slower transport of retinol compared to the vitamin E compounds through the extraction cell probably depends on differences in hydrogen bonding to the matrix.

The kinetics for liver paste samples containing 12 or 23% of fat was investigated as above. In this case it was found that the recoveries for vitamin A as retinol were somewhat lower than 100%, whereas tocopherol could be quantitatively extracted. For vitamin A the recoveries were 87 and 84% for low-fat and high-fat liver paste, respectively. However, when the unhydrolyzed vitamin A palmitate present in liver paste samples was also determined, the recoveries increased to 119 and 104%, respectively. The difficulties in obtaining 100% recoveries based on solely retinol for vitamin A in liver paste with its high fat content may depend on the competing procedure of triacylglycerol hydrolysis slowing the enzymatic hydrolysis of the vitamin A esters.

**Vitamin Concentrations in Different Food Formulas.** Finally the procedures used for infant formula and liver paste were applied to some other food formulas. Results concerning all food formulas considered are collected in Table 4.

For all samples except liver paste the recoveries of vitamin A were based on retinol alone. Because the value in some cases even exceeds 100%, it can be anticipated that the used enzymatic hydrolysis/alcoholysis step is a milder treatment than alkaline saponification. This obviously results in somewhat higher and probably truer values than those obtained with methods based on a saponification step. Concerning vitamin E, all of the values except for infant formula are based on tocopherol alone, which implies that the concentrations of tocopheryl esters are so low that they are not

normally expected to give significant contribution to the values calculated.

The recovery values in Table 4 are based on averages of results from seven laboratories that had participated in an intercomparison study, where food formulas from the same batches were used. The deviations among different results within this study were in some cases rather large, which could be one explanation for the varying recovery values in this work. For instance, the vitamin E concentration in milk powder was determined in this work as 0.24 mg/100 g, which gave 85% recovery when compared to the average value from the intercomparison of 0.29 mg/100 g. This average value is based on seven results ranging from 0.13 to 0.42 mg/100 g, of which one result (the lowest) was an outlier. In fact, none of the determined vitamin concentrations in this investigation is outside the range of values obtained for the same food formulas in the intercomparison study.

**Concluding Remarks.** In summary, the developed method is faster and more automated than the method using SFE with off-line saponification (10, 15) or conventional extraction techniques (2, 3) because no additional saponification or cleanup steps are needed. As a comparison, the extraction of six samples requires ~9 h of manual work using conventional techniques, whereas ~24 samples can be run and handled using SFE/enzymatic hydrolysis with about the same demand on manual work. Moreover, the new method consumes several times fewer (20–25) volumes of organic solvents. The use of the integrated enzymatic hydrolysis/alcoholysis with SFE combined with HPLC gives an analytical procedure that is gentler toward the easily degradable vitamins. The optimized parameters should be applicable to other food formulas as well. However, one parameter that should be especially considered is the dynamic extraction time, which might be necessary to prolong if the sample matrix has strong adsorbing properties.

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