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Determination of vitamin A palmitate in cereal products using supercritical fluid extraction and liquid chromatography with electrochemical detection

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Abstract

Vitamin A palmitate (retinol hexadecanoate, retinol palmitate) was extracted from cereal products using supercritical CO_2 at 55 MPa and 60°C. Quantitative extraction required only 20 min. Retinol palmitate in the extract was determined by reversed-phase liquid chromatography (LC) using an oxidative mode electrochemical detector with a glassy carbon electrode polarized at +1.2 V vs. saturated calomel electrode. The LC run time was 12 min. The detection limit was 0.17 ng for a 20 μl injection, and response was linear over at least three orders of magnitude. For corn, wheat and oat cereals fortified with retinol palmitate at four levels in the 10–125 μg/g range, the overall average recovery was 95% with an overall R.S.D. of 5%. For a wheat sample spiked at three levels in the 25–100 μg/g level, the within-day average recovery was 101% with 3% R.S.D., and the between-day recovery was 100% with 6% R.S.D. Sample lipid in the form of added soybean oil had no adverse effect on the extraction or the LC. The method was applied to commercial breakfast cereals. Co-extractants from the samples elute well before the retinol palmitate peak.

Keywords: Food analysis; Vitamins; Vitamin A palmitate

1. Introduction

The status of official methods for the determination of vitamin A in foods and feeds was evaluated in depth by Thompson [1], who emphasized the need to introduce liquid chromatographic (LC) methods and to improve sample preparation techniques. Numerous citations were given to works on both established procedures and LC methods for vitamin A. We describe here a method using quantitative supercritical fluid extraction (SFE) for isolation of the unsaponified retinol palmitate form of the vitamin from commercial cereal products followed by LC with a selective electrochemical detector in the oxidative mode.

Ready-to-eat cereals are most commonly fortified with vitamin A in the retinyl palmitate form, at the 25–125 μg/g level. Compendial procedures for the isolation from foods and feeds of retinol palmitate, which is sensitive to heat, light and oxygen, often

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involve harsh techniques for the removal of lipids and other matrix components. Usually the first step is an alkaline hydrolysis [1–5], which converts the vitamin A ester to all-*trans*-retinol [6]. This is generally followed by liquid–liquid extraction and open-column chromatographic purification prior to a spectrophotometric determination of the retinol such as the Carr–Price procedure [5]. Enzymatic hydrolysis [7] is gentler but time-consuming. Solid-phase extraction of the saponified retinol onto diatomaceous earth has also been applied [8]. Size-exclusion chromatography has also been used as a fractionation and clean-up method [9]. LC methods for the determination of the isolated retinol are based on conventional reversed-phase (RP) [10,11], nonaqueous RP [12,13] and normal-phase [14] modes, using UV or fluorescence detectors.

SFE was shown previously by us [15] to be a fast, gentle method conducted in a dark, oxygen-free environment, for the extraction of vitamin K1 from infant formulas. Reversed-phase LC with electrochemical detection was used for the determination. The electrochemical detector has the advantages of selectivity and sensitivity. Direct voltammetry has been applied to the determination of vitamin A in static systems [16] and as a detector for low pressure LC of vitamin A in multivitamin preparations [17], for reversed-phase LC of retinol and its isomers in human serum extracts [18], and for normal-phase LC of retinoids [19]. SFE was recently applied by Scalia et al. to the isolation of vitamins A and E and their esters from tablets [20] and cosmetic creams [21] followed by nonaqueous reversed-phase LC with a UV detector.

2. Experimental

Note: To prevent photochemical decomposition of vitamin A, samples, standard solutions and extract solutions must be protected from light by storing in actinic glassware or in Al foil-wrapped vials and volumetric glassware.

2.1. Apparatus

The laboratory-built static supercritical fluid extractor was described in detail previously [22]. It is based on a Newport Scientific (Silver Spring, MD, USA) 70 MPa diaphragm compressor with a Circle Seal (Anaheim, CA, USA) dome-loaded pressure regulator. The extraction chamber was a 15 cm×0.95 cm I.D. stainless steel tube housed in a Lab-Line thermostat (Fisher Scientific, Springfield, NJ, USA) connected through an Autoclave Engineers (Erie, PA, USA) medium pressure metering valve to a 15 cm× 0.64 cm O.D. stainless steel extract trap packed with Chromosorb W, 177–250 μm (Alltech Associates, Deerfield, IL, USA).

The HPLC was a laboratory-constructed instrument [22] based on a Varian 8500 syringe pump, the pulseless output of which is ideal for the electrochemical detector. The injector was a Rheodyne 7125 valve (Cotati, CA, USA) modified to provide facile degassing of the sample and pneumatic loading of the 20 μl sample loop. The column was 15 cm×3.9 mm I.D., 5 μm Altex C_8 (Beckman Instruments, Fullerton, CA, USA) used at ambient temperature. The electrochemical detector was laboratory-constructed [22] based on standard potentiostat and electrometer circuits. The stainless steel auxiliary electrode (2.5 cm×2.5 cm, 1.25 cm thick) was separated by a PTFE spacer from a Kel-F block of the same size containing a 3.2 mm diameter glassy carbon rod working electrode (Type GC-30, Tokai Carbon, Tokyo, Japan). The cell volume was 9.6 μl. The working potential was set at +1.2 V vs. the saturated calomel reference electrode which was located downstream in a nylon housing.

2.2. Chemicals

CO_2 was Linde bone-dry grade (Prest-O-Sales and Service, Long Island City, NY, USA).

Acetonitrile and 2-propanol were Baker HPLC grade, obtained from J and H Berge (Plainfield, NJ, USA). Water was glass-distilled. Reagent grade $NaClO_4$ was purchased from GFS Chemicals (Columbus, OH, USA). Retinol palmitate (Sigma, Cat. no. R-3375, St. Louis, MO, USA) was all-*trans* oil equivalent to $1.67 \cdot 10^6$ retinol units/g, stabilized with 0.9% (w/w) each butylated hydroxyanisole and butylated hydroxytoluene.

The LC eluent was acetonitrile–2-propanol–aqueous 25 m*M* $NaClO_4$ (45:45:10, v/v), filtered and

degassed by helium sparging. The flow-rate was 2.0 ml/min.

Retinol palmitate stock solution, 350 μg/ml in 2-propanol was diluted daily with eluent to prepare working solutions in the range of 0.35 μg/ml to 3.5 μg/ml.

2.3. Samples

Packaged samples of ready-to-eat corn, wheat, rice and bran breakfast cereals were purchased locally. Samples from two different lots of each brand were mixed to form composite samples prior to analysis. Dried corn kernels, milled oats and wheat porridge were purchased locally in health food stores. Fortification of these samples with retinol palmitate was carried out by the addition of stock solution to ground, weighed samples.

2.4. Sample extraction procedure and assay

An accurately weighed 2 g sample was packed into the extraction tube, held in place with glass wool plugs, and connected to the high pressure manifold. The sample was equilibrated at the required oven temperature (45–65°C) with a low flow of CO_2 to remove air. The exit valve was closed, and the system was pressurized to 55 MPa and allowed to equilibrate for 15 min. The exit valve was carefully cracked open to allow the CO_2 plus extract to depressurize across the valve and deposit the extract onto the Chromosorb W trap. The trap was removed and the extract eluted with 30 ml 2-propanol. The solvent was evaporated at 45°C in rotary evaporator in the dark, the extract reconstituted in 2.0 ml of eluent, and 20 μl injected into the HPLC. The retention time of retinol palmitate was 10 min ($k'=9.0$) and the total run time, 12 min. Quantitation was accomplished by comparison of peak heights of samples and standards.

3. Results and discussion

3.1. HPLC and electrochemical detector conditions

Reversed-phase LC with an aqueous eluent was used here mainly because of the greater compatibility of this type of mobile phase with the electrochemical detector. The addition of 2-propanol to the acetonitrile–aq. 25 m*M* $NaClO_4$ eluent produced better peak shapes, shorter retention times, and lower background currents. The 2-propanol may improve peak shape by better wetting of the bonded alkyl phase by the eluent [18]. The retention time of retinol palmitate decreased in a sigmoidal fashion from 40 min to 5 min as the 2-propanol concentration was increased from 22% to 58%, while maintaining the aqueous component at 10%. Background current, which is typically large with an electrochemical detector, decreased linearly with (% 2-propanol)$^{-1}$, from 1200 nA at 10% 2-propanol to 120 nA at 90% 2-propanol, which we presume reflects the decreased dielectric constant of the eluent.

For the electrochemical detector to function properly, some electrolyte is needed in the eluent. With the acetonitrile–2-propanol–aqueous $NaClO_4$ (45:45:10, v/v) eluent, the height of the retinol palmitate peak initially increased smoothly with increasing concentration of $NaClO_4$ and then passed through a broad maximum at 25 m*M*.

The dependence of peak current on eluent flow-rate was found, as in our earlier work [15], to reflect a convective mass transfer-limited mechanism [23]; peak current increased with the one-third power of the flow-rate up to flows of about 100 ml/h, and became independent of flow-rate above this. Thus 2 ml/min of acetonitrile–2-propanol–aqueous $NaClO_4$ (45:45:10, v/v) eluent was selected to provide good selectivity and sensitivity, and a reasonable retention time for retinol palmitate.

The electrochemistry of retinols was discussed by MacCrehan and Schönberger [18], who presented a hydrodynamic voltammogram for retinol in a MeOH–aq. pH 3.2 buffer (69:31, v/v) which was quite similar in form to the one we determined in our eluent. The retinol palmitate peak height and the background increase with electrode potential; to compromise, the working electrode potential was set at +1.2 V vs. saturated calomel electrode.

The peak-to-peak noise level of the detector was 90 pA. The detection limit, corresponding to a retinol palmitate peak height twice the noise, was 8.6 ng/ml, or 0.17 ng absolute for a 20 μl injection. This is about double that reported by MacCrehan and

Schönberger [18], whose more sophisticated potentiostat may be presumed to have had a lower noise level. Response of the detector was linear to at least 40 μg/ml; higher concentrations were avoided to minimize the possibility of electrode fouling. A typical working calibration curve for concentrations up to about 3 μg/ml was linear (r^2=0.997) and passed close to the origin (y-intercept=0.28 nA).

3.2. Supercritical fluid extraction conditions

Optimal supercritical fluid extraction conditions were established by extracting 1 g samples of Chromosorb W spiked with 50 μg of retinol palmitate, at 45°C and 60°C, at pressures in the range of 34–55 MPa, and for extraction times from 5 to 90 min. As shown in Fig. 1, recovery increased sharply with pressure, because of the increase in density of the CO_2. Recovery was essentially quantitative (≥90%) at pressures greater than about 55 MPa at the higher temperature. Temperature plays a role because of its effects on both the vapor pressure of the retinol palmitate and the density of the CO_2. At these pressures, the increase in solute vapor pressure with temperature exceeds the decrease in supercritical fluid density with temperature, leading to higher recovery at the higher temperature. Because the extraction is a diffusion-controlled process, sufficient time must be allowed. Triplicate extractions of 50 μg of retinol palmitate adsorbed on Chromosorb W were carried out at 55 MPa and 60°C for different equilibration times; for 5 min equilibration the average recovery was 74%; for 10 min, 78%; for 15, 20, or 30 min, 93%. The relative standard deviations (R.S.D.s) of the recoveries averaged 4%. The extraction conditions used in all further work were 55 MPa CO_2, 60°C, and 20 min equilibration time. Samples were ground by mortar and pestle; in our experience with porous materials such as these, particle size is not a factor limiting recovery, as it is in the case of nonporous materials such as plastics [24].

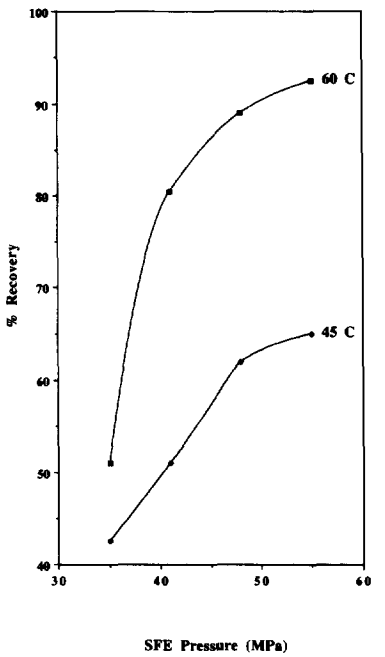

Fig. 1. Effect of CO_2 pressure on SFE efficiency at 60°C and 45°C. Equilibration time, 20 min. Samples, 1 g Chromosorb W spiked with 50 μg retinol palmitate. HPLC conditions: column, 15 cm×4.6 mm I.D. Altex C_8, 5 μm; eluent, acetonitrile–2-propanol–aqueous 25 m*M* $NaClO_4$ (45:45:10, v/v), 2.0 ml/min; electrochemical detector with glassy carbon electrode at +1.2 V vs. SCE.

3.3. Application to spiked and fortified cereal samples

Fig. 2 shows a chromatogram of an unfortified

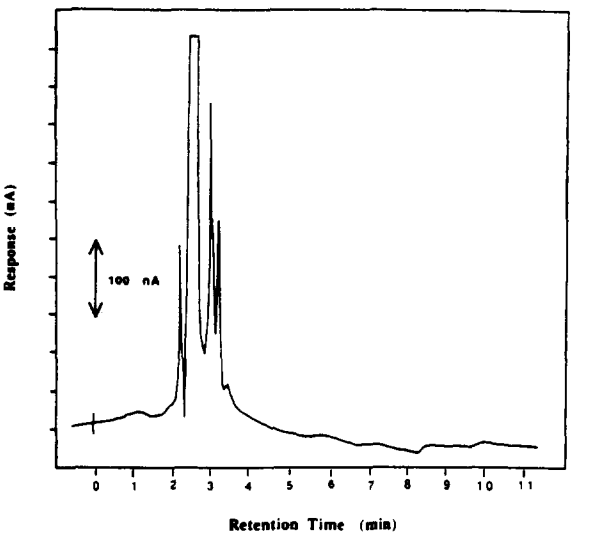

Fig. 2. Chromatogram of supercritical fluid extract of an unfortified wheat sample. SFE conditions, 55 MPa CO_2, 60°C, 20 min. Chromatographic conditions, same as Fig. 1.

wheat sample taken through the entire procedure. It is clear there are few oxidative-electrochemically-detectable materials extracted from the wheat, and nothing detectable elutes near the retention time (10 min) of retinol palmitate. Similar chromatograms were obtained for the unfortified corn and oat samples, with no interfering peaks near 10 min.

A precision study was performed on the wheat sample fortified as above, using the SFE and LC conditions established above. The results are given in Table 1. The within-day precision (R.S.D.) varied from 2% to 4%. The day-to-day precision ranged from 4% to 8%.

As a test of the recovery of the procedure, extractions were carried out in triplicate on cereal products spiked by addition of stock solution of retinol palmitate at four levels ranging from 10–125 μg/g. The average recoveries were 95% and average R.S.D. values were 5%. The type of cereal had no significant effect on the SFE recoveries or R.S.D. values.

To address the concern of the effect of sample lipids on the efficiency and ruggedness of the procedure, retinol palmitate dissolved in soybean oil was added to fortified corn samples which were well mixed and subjected to the analytical method. To 1 g samples of corn previously fortified with 125 μg/g retinol palmitate was added 250 mg of soy oil containing 10, 20, 30 and 40 μg of the vitamin. The recoveries ranged from 93% to 103% with a R.S.D. of 4%. The recovery of retinol palmitate is not significantly affected by the additional oil or the existing vitamin level. During this work, over 50 cereal extracts were chromatographed on the same HPLC column without any change in the retention time or peak shape of the retinol palmitate. Column deterioration resulting from lipid carryover is consequently not a problem.

The procedure was also applied to the quantitation of retinol palmitate in ready-to-eat commercial breakfast cereals. Five fortified cereal products with label declarations of 25% and 100% of the US RDA were analyzed. The results are given in Table 2. For

Table 1
Precision study of retinol palmitate assay procedure

Fortification level (μg/g)	Mean found (μg/g)	n^a	% R.S.D.
Within-day			
25.0	25.8	3	4
50.0	48.8	3	3
100	102	3	2
Between-day			
25.0	24.6	5	8
50.0	51.2	4	6
100	98.6	4	4

Supercritical fluid extraction and chromatographic conditions: same as Fig. 2.
[a] n=Number of samples analyzed on one day for within-day results, and the number of days for the between-day results.

Table 2
Retinol palmitate in commercial breakfast cereals using the SFE–LC–electrochemical detector procedure

Cereal	Base	Retinol palmitate		
		% US RDA[1]	Found (μg/g)	% R.S.D. (n=3)
A	Rice	25	33	2
B	Rice	25	39	3
C	Corn	25	30	3
D	Bran	25	41	5
E	Bran	100	110	2

Supercritical fluid extraction and chromatographic conditions: same as Fig. 2.
[a] Manufacturers' label declaration of the % of the US Department of Agriculture Recommended Daily Allowance of the vitamin in the product; 25% of the US RDA for retinol palmitate in breakfast cereals corresponds to 25 μg/g.

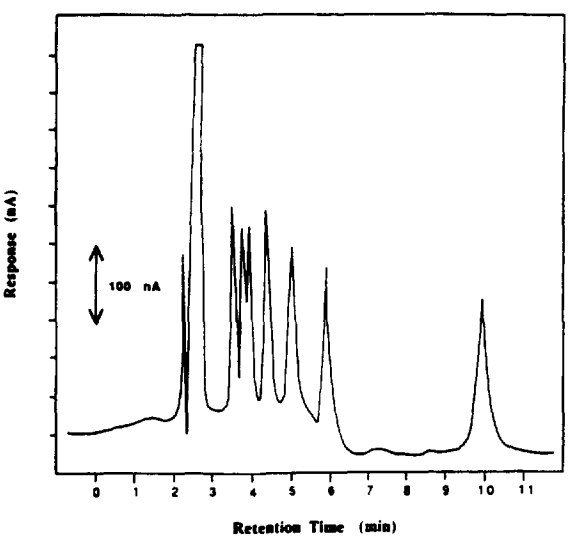

Fig. 3. Chromatogram of supercritical fluid extract of a bran-based ready-to-eat breakfast cereal. Conditions, same as Fig. 2. The peak at 10 min is retinol palmitate.

all samples, the level of retinol palmitate found exceeded the label declaration. A typical chromatogram of a supercritical fluid extract of a bran-based cereal is shown in Fig. 3. The retinol palmitate is well resolved from other extracted materials. Confirmation of the identity of the retinol palmitate peak was achieved by co-injection of a standard. Retinol palmitate was the only form of the vitamin detected in these cereals.

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