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Microwave-Assisted Rapid Determination of Vitamins A and E in Beverages

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A new rapid procedure for the determination of vitamins A and E in beverages has been developed and validated. Key steps include a microwave-assisted saponification of the sample and a single-step extraction of the vitamins prior to HPLC analysis. All sample preparation steps are carried out consecutively in the same vial. The vitamins are determined using normal-phase (Si-60) HPLC with fluorescence detection. The method is applicable to beverages with a content of *all-trans*-retinol >0.14 mg/L and/or a content of α-tocopherol >1 mg/L. Recoveries determined by spiking experiments ranged from 91.3 to 106.3%. The precision of the method is characterized by relative standard deviations of <2% for α-tocopherol and <5% for *all-trans*-retinol.

KEYWORDS: Microwave-assisted extraction; saponification; vitamin A; vitamin E; normal-phase HPLC; fluorescence detection; beverages; vitamin fortification

INTRODUCTION

Vitamins A and E are essential food ingredients that are usually supplied with the diet. The names refer to two groups of naturally occurring compounds, each consisting of several structurally related members exhibiting a common biological activity. *all-trans*-Retinol and *R,R,R*-α-tocopherol are among the most important natural representatives of the two groups occurring in food. The current work focused on their determination in beverages. Some beverages, for example, milk, can possess a considerable natural content of vitamins A and E, whereas genuine contents in fruit juices are usually low (*1*). Thus, beverages are often fortified with these vitamins, usually in the form of *all-trans*-retinol and *all-rac*-α-tocopherol or their esters, predominately acetates or palmitates. The esters are less sensitive to oxidation than the corresponding free retinols and tocopherols, enhancing the stability of the vitamins in the product. The ester bond is readily hydrolyzed during digestion (*2*, *3*). As these compounds are only sparsely soluble in water, they are added in special formulations, for example, in beadlets. The matrix of the beadlets consists of gelatin or similar materials and helps to form a microemulsion in aqueous medium. Fortified products include multivitamin fruit juices, energy drinks, and isotonic sport beverages. Fortification is often adjusted to the Recommended Dietary Allowance (RDA) for healthy adults. For example, 100 mL of a typical multivitamin fruit juice can contain 80 μg of *all-trans*-retinol and 3.3 mg of α-tocopherol. To satisfy the great demand for both vitamins, they are synthesized industrially on a large scale. In contrast to natural α-tocopherol, which consists only of the *R,R,R*-stereoisomer, synthetic *all-rac*-α-tocopherol usually contains equal amounts of all eight stereoisomers (*3*). These stereoisomers are difficult to separate chromatographically. Therefore, and due to the usually predictable isomeric composition in natural and synthetic material, analytical methods for food usually describe only the determination of the total α-tocopherol content.

Due to their many similar physical properties, vitamins A and E can generally be determined using an identical sample workup procedure. Typically, this includes a saponification step, extraction of the vitamins into an organic solvent, and separation by normal- or reversed-phase HPLC coupled to UV or fluorescence detection (see, e.g., refs *3*−*6*). During saponification, vitamins are freed from beadlets and natural occurring complexes, ester bonds are cleaved, and occurring fat is hydrolyzed. This step is usually carried out under reflux with alcoholic sodium hydroxide solution. The vitamins are then extracted with a lipophilic organic solvent, for example, hexane, diethyl ether, ethyl acetate, toluene, or mixtures thereof. This extract can then be used for analysis by normal-phase HPLC, either directly or after additional concentration and/or cleanup steps. Analysis by reversed-phase HPLC requires evaporation of the solvent and redissolution into a solvent compatible with the polar mobile phase.

Most published sample preparation procedures are rather time-consuming and labor intensive and use large amounts of organic solvents for extraction (*6*). The goal of the current investigation was therefore to develop a new, fast, and reliable method with reduced solvent consumption, suitable for routine analysis. The method focused on the determination of the two most important additives, α-tocopherol (sum of all stereoisomers) and *all-trans*-retinol. Esters are hydrolyzed during sample workup and determined as the corresponding phenol/alcohol. Development of the method was based on procedures routinely used in our department (*7*, *8*). Key improvements included a microwave-assisted sample saponification instead of saponification under

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Table 1. Linearity of the Chromatographic Systems and Detection Limits

	all-trans-retinol	*all-rac*-α-tocopherol
linear range, μg/mL	0.02–3.42	0.09–10.38
R^2	0.9997	0.9997
intercept	−1275	524
slope	299863	617993
detection limit, ng/mL	0.6	0.03

reflux and an optimized and miniaturized single-step extraction procedure. The new method is applicable to most food and feed samples with only minor modifications. Development and validation of a procedure suitable for the analysis of beverages is described in the present work.

MATERIALS AND METHODS

Instrumentation. An MLS Ethos Plus II microwave system (MLS GmbH, Leutkirch, Germany), maximum energy of 1000 W, equipped with easyWave software version 3.3, multiPREP-P 36 rotor, and GRV-28 glass reaction tubes (50 mL), has been used. The reaction tubes possess an external plastic coating and a high-pressure security valve and are suitable for operation at up to 15 bar. Samples were extracted on an HS 500 horizontal mechanical shaker (Janke Kunkel, Staufen, Germany) and centrifuged using a megafuge 1.0 (Heraeus, Zürich, Switzerland) at ∼1000*g*.

The HPLC system consisted of a PU 980 pump (Jasco, Tokyo, Japan), a 717Plus autosampler (Waters, Milford, MA), an FP-1520 fluorescence detector (Jasco), and a VAX Multichrom V2.20h chromatography data system (LabSystems, Chesire, U.K.).

Chemicals and Reagents. Certified reference material of *all-trans*-retinol, *all-trans*-retinyl acetate, *all-rac*-α-tocopherol, and *all-rac*-α-tocopheryl acetate was supplied by Dr. Ehrenstorfer GmbH (Augsburg, Germany). *all-trans*-Retinol often contains traces of its 13-*cis*-isomer, which can be detected using the chromatographic system described below (*7*). Fluorescence-free *n*-hexane was supplied by Roche Vitamins Ltd. (Basel, Switzerland); all other chemicals were of analytical grade and supplied by Merck (Darmstadt, Germany). For the ascorbic acid solution, 15 g of ascorbic acid was dissolved in 40 mL of water and made up to 1 L with EtOH.

Samples. Sample A was homogenized milk with 3.5% fat, sample B was a banana-flavored milk energy drink, sample C was a multivitamin multifruit juice, and sample D was orange juice from concentrate. All samples were purchased in a local grocery store.

Sample Preparation. Sample (2.0 mL) was pipetted into a reaction tube. Ascorbic acid solution (5 mL), potassium hydroxide solution 50% (1 mL), and a few boiling stones were added. The sample was saponified in the microwave system using the following timed temperature program: starting at room temperature, heat to 100 °C over 1 min and keep at 100 °C for 1 min. After saponification, the tube was removed from the microwave system and rapidly cooled to room temperature. Acetic acid (1 mL), saturated sodium chloride solution (10 mL), and cyclohexane (20.0 mL) containing BHT (500 mg/L) were added, and the mixture was mechanically shaken for 10 min at ∼250 cycles/min. The tube was centrifuged at ∼1000*g* for 5 min, and the resulting supernatant organic layer was subjected to HPLC analysis.

Chromatographic Conditions. For determination of *all-trans*-retinol a normal-phase column (Hibar LiChrosorb Si 60, 5 μm, 125 × 4 mm) was used. The mobile phase consisted of *n*-hexane/2-propanol (98:2; v/v) at a flow rate of 1.2 mL/min. Injected volume was 50 μL. The detector was set to excitation at 325 nm; emission was measured at 480 nm; gain, 10; attenuation, 1; response, 3 s. Retention times were 3.75 min for 13-*cis*-retinol and 4.75 min for *all-trans*-retinol.

For determination of α-tocopherol a normal-phase column (Hibar LiChrosorb Si 60, 5 μm, 125 × 4 mm) was used. The mobile phase consisted of *n*-hexane/dioxane (97:3, v/v) at a flow rate of 1.0 mL/min. Injected volume was 50 μL. The detector was set to excitation at 292 nm; emission was measured at 326 nm; gain, 1; attenuation, 1; response, 3 s. Retention time for α-tocopherol was 5.5 min. Other compounds of the vitamin E group, for example, β-, γ-, and δ-tocopherol and tocotrienols, are usually present in only traces in the beverages investigated and have not been quantified. The following retention times were determined using standard solutions: β-tocopherol, 8.5 min; γ-tocopherol, 9.5 min; and δ-tocopherol, 15 min.

Validation Experiments. *Linearity of the Chromatographic Systems (**Table 1**).* Vitamin standard solutions of different concentrations were analyzed in duplicate each. The LOQ for *all-trans*-retinol equaled the lower limit of the linear range. The LOQ for *all-rac*-α-tocopherol was below the linear range investigated and has not been determined experimentally. The detection limit for both vitamins was determined at a signal to noise ratio of 3:1. The upper limit of the linear range has not been determined.

Accuracy by Recovery. Recovery experiments were carried out using a beverage with a very low vitamin content (orange juice, sample D). Amounts added covered ranges of 0.12−5.64 μg of *all-trans*-retinol and 1.95−96.45 mg of α-tocopherol (**Table 2**). Additionally, three different beverages (samples A−C) were spiked at two different levels each, corresponding to 0.83 and 1.67 μg of *all-trans*-retinol and 40 and 80 μg of α-tocopherol, respectively (**Table 3**). Samples were analyzed as described under Sample Preparation with the following deviations. Vitamins were added as their acetates dissolved in EtOH (1.0 mL) directly to the sample in a reaction tube. Only 4 mL of ascorbic acid solution was added to spiked samples. Control samples contained water (2 mL) instead of a beverage. All experiments were carried out in duplicate.

RESULTS AND DISCUSSION

Development of the Method. The current investigation focused on the development of a reliable and cost-efficient laboratory working procedure for the determination of vitamins A and E in beverages. Special emphasis was put on reduction of laboratory working time and organic solvent consumption. Key improvements of the new method are a one-pot sample preparation featuring a microwave-assisted saponification and an optimized single-step extraction procedure (**Figure 1**).

The use of microwave energy for sample preparation has become increasingly popular in analytical chemistry (*9*, *10*).

Table 2. Results of Recovery Experiments with *all-trans*-Retinol[a] and α-Tocopherol[a] Added to Orange Juice (Sample D) and Blank Samples ($n = 2$)

	spike level				
	I	II	III	IV	V
		all-trans-retinol			
amount added, μg	0.12	0.28	1.41	2.82	5.64
recovery from orange juice, % ± SD	78.5 ± 6.5	92.1 ± 2.3	91.3 ± 3.4	92.6 ± 0.6	95.9 ± 0.9
recovery from blank sample, % ± SD	86.6 ± 9.3	nt[b]	94.7 ± 1.9	nt	97.5 ± 1.0
		α-tocopherol			
amount added, μg	1.95	4.8	24.1	48.25	96.45
recovery from orange juice, % ± SD	101.7 ± 2.7	106.3 ± 4.4	99.7 ± 0.3	98.8 ± 0.4	99.8 ± 0.0
recovery from blank sample, % ± SD	98.7 ± 2.7	nt	100.5 ± 0.6	nt	100.6 ± 1.3

[a] Vitamins were added as their acetates dissolved in EtOH. [b] nt, not tested.

Table 3. Results of Recovery Experiments with *all-trans*-Retinol[a] and α-Tocopherol[a] Using Three Different Beverages ($n = 2$)

	all-trans-retinol		α-tocopherol	
	spike level I	spike level II	spike level I	spike level II
amount added, μg	0.83	1.67	40.0	80.0
sample A recovery, % ± SD	99.1 ± 1.7	100.6 ± 0.1	103.8 ± 0.4	102.8 ± 0.2
sample B recovery, % ± SD	101.1 ± 1.3	95.6 ± 0.7	96.5 ± 1.2	95.5 ± 1.0
sample C recovery, % ± SD	103.0 ± 2.2	101.8 ± 0.3	97.0 ± 6.4	98.6 ± 0.2

[a] Vitamins were added as their acetates dissolved in EtOH.

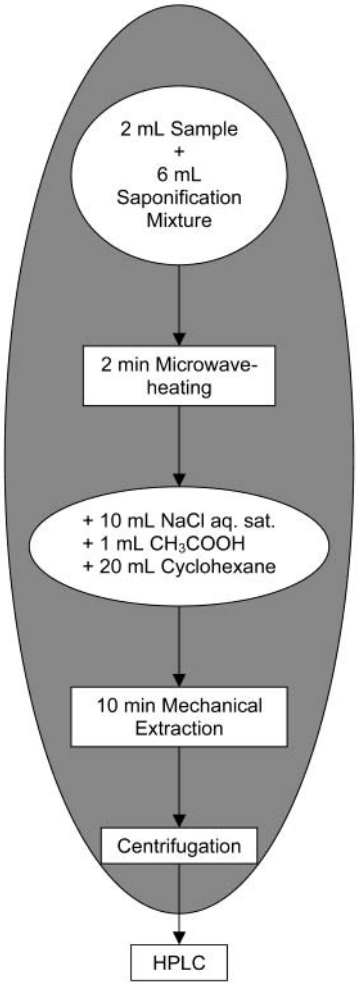

Figure 1. Scheme of the sample workup procedure. All steps within the shaded oval are carried out in the same reaction vial.

Examples of this technique used for the analysis of vitamins include the extraction of vitamins A, D, and E from premixed feed (*11*) and the saponification of vitamin A during an FIA method for retinol determination in tablets (*12*). Benefits of a microwave-assisted extraction and saponifcation include a faster reaction with improved reproducibility and lower expenditures for solvents when compared with working under reflux. In our case, saponification time could be reduced from the 30 min necessary for conventional workup to only 2 min using microwave energy. Additionally, no sample transfer prior to extraction of the vitamins with an organic solvent was necessary; the solvent could be added directly into the saponification vial.

The efficacy of vitamin extraction from the saponified sample prior to HPLC analysis could be markedly improved by adding a saturated sodium chloride solution and by choosing cyclohexane as an extraction solvent, allowing the extraction procedure to be reduced to a single step. Neutralization of the digest helped to prevent the formation of stable emulsions. After centrifugation, the supernatant organic layer could be directly used for HPLC analysis without further purification or concentration.

Table 4. Quantitation of Vitamin Contents in Beverages ($n = 6$)

sample[a]	α-tocopherol, mg/L (RSD, %)	13-*cis*-retinol, μg/L (RSD, %)	*all-trans*-retinol, μg/L (RSD, %)
A	0.8[b] (12.4)	<LOD	399 (2.7)
B	15.7 (0.5)	<LOD	147 (4.1)
C	49.5 (1.4)	82[b] (11.7)	1476 (4.6)

[a] Samples are detailed under Materials and Methods. [b] Value below the range of the method.

Method Validation. The new method has been validated with respect to linearity of the chromatographic systems, accuracy by recovery, and precision.

The linearity of the chromatographic systems and the detection limits for the two vitamins were determined by analysis of standard solutions (**Table 1**). For each vitamin, the linear range determined was found to cover the respective concentration range expected for beverages during routine analysis.

The accuracy of the method was investigated by two different sets of recovery experiments. The first experiment covered a broad concentration range and was carried out using a beverage with a low genuine vitamin content (orange juice, **Table 2**). Amounts added approximately equaled vitamin contents in a beverage of 60−2820 μg/L *all-trans*-retinol and 1−48 mg/L α-tocopherol. Control experiments with water instead of beverages were also carried out. Except for the lowest *all-trans*-retinol addition investigated, recoveries from both water and orange juice were similar and ranged from 91.3 to 97.5%. Recoveries of α-tocopherol were slightly higher and with one exception equaled 100 ± 2%. These results were confirmed in a second experiment with three different beverages, which were spiked at two different levels each (**Table 3**). Vitamin amounts added were below the genuine content of the sample with the highest content (cf. **Table 4**) and equaled concentrations in a beverage of 415 and 830 μg/L *all-trans*-retinol and 20 and 40 mg/L α-tocopherol, respectively. Recoveries ranged from 95.5 to 103.8% and demonstrated the applicability of the method for a broader selection of samples. Thus, the following working ranges for the method, corresponding to the vitamin content of the samples, have been deduced from the two experiments: *all-trans*-retinol, 140−2820 μg/L; α-tocopherol, 1−48 mg/L.

The precision of the method has been investigated by analyzing three different beverages sixfold each (**Table 4**). Overall, the precision can be characterized by relative standard deviations of <2% for α-tocopherol and <5% for *all-trans*-retinol.

In conclusion, a new workup procedure for the rapid determination of vitamins A and E in beverages has been developed and validated. The use of a microwave-assisted saponification and a single-step extraction led to a simplified

and miniaturized procedure that has been successfully applied in routine analysis in our department.

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