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An evaluation of *cis*- and *trans*-retinol contents in juices using dispersive liquid–liquid microextraction coupled to liquid chromatography with fluorimetric detection

Pilar Viñas, María Bravo-Bravo, Ignacio López-García, Manuel Hernández-Córdoba *

Department of Analytical Chemistry, Faculty of Chemistry, Regional Campus of International Excellence "Campus Mare Nostrum", University of Murcia, E-30071 Murcia, Spain

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ABSTRACT

This study describes a method for coupling dispersive liquid–liquid microextraction (DLLME) and normal-phase liquid chromatography (NP-LC) with fluorescence detection for vitamin A determination with the view to developing a new green sample preparation technique. Parameters affecting DLLME, including the nature and volume of both extractant and disperser solvents, salt addition and time and speed of the centrifugation step, were optimized. The sample was saponified according to European Standards to convert all forms of vitamin A to retinol. For microextraction, 8 mL water were placed in a glass tube with conical bottom and the saponified sample consisting of 2 mL of the methanolic extract containing 100 μL tetrachloroethane was rapidly injected by syringe, thereby forming a cloudy solution. Phase separation was performed by centrifugation, and a volume of 20 μL of the sedimented phase was analyzed by NP-LC. The enrichment factor, calculated as the ratio between the slopes of DLLME-LC and direct LC, was 50 ± 3. The matrix effect was evaluated for different juice samples, and it was concluded that sample quantification can be carried out by aqueous calibration when the standards are also submitted to saponification. The proposed method was applied for determining both *cis*- and *trans*-retinol isomers in commercial juices of different types. The intraday and interday precisions were lower than 6% in terms of relative standard deviation. The method was validated using two certified reference materials.

1. Introduction

Some functional foods in the drinks sector are marketed as so-called ACE juices. These are soft drinks based on fruit and vegetable juices to which the antioxidant vitamins A, C and E have been added [1]. Food products enriched with vitamins are assumed to present health benefits against oxidation and the ageing [2]. All the main antioxidants are found in plant foods, which explains the need to include fruits, legumes, vegetables and cereals in the diet [3]. When a food product is enriched with vitamins, a quality control programme must be established to ensure that the product contains the declared levels. However, an excess of several vitamins may have harmful effects.

Vitamin A plays an essential role in vision, and also acts in the body as an antioxidant, providing protection against the risk of certain cancers. There are two sources of dietary vitamin A. Active forms are obtained from animal products and include retinal, retinol and retinyl esters, whose hydrolysis results in retinol, which may exist in *trans*- or *cis*-configuration. Precursors, also known as provitamins, are obtained from fruits and vegetables containing pigments, known as carotenoids, the most well-known being β-carotene [4]. For this reason, amounts of vitamin A are measured in retinal equivalents (RE). Pure retinol is extremely sensitive to oxidization and, when prepared as a dietary supplement, it is stabilized as the ester derivatives. Preform vitamin A is a nutrient of high toxic potential when consumed in excess [5]. The EU Commission [6] established the requisite relative to the nutrient vitamin A in vegetable juices as a final content in the product no lower than 100 μg RE/100 kcal (annex II). The compounds permitted to be added as vitamin A are retinol, retinyl acetate, retinyl palmitate and β-carotene (annex IV). The maximum content of vitamin A in cereal-based products and baby foods is 180 μg RE/100 kcal (annex VI).

Vitamin A has mainly been analyzed in food samples by liquid chromatography (LC) using different detectors, such as diode array, fluorescence, electrochemical or mass spectrometry [7–29]. It was sometimes analyzed together with other liposoluble vitamins. The complex matrix of food samples and the low vitamin concentrations expected mean it is necessary to include isolation and preconcentration steps in the analytical procedure.

* Corresponding author. Tel.: +34 868887406; fax: +34 868887682.
E-mail address: hcordoba@um.es (M. Hernández-Córdoba).

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Saponification and/or solvent extraction in relatively low polarity organic solvents, as in the case of the standardized method of analysis [30], are classical methods of vitamin A analysis. However, these methods are tedious and also require large amounts of organic solvents, as the samples are first saponified using methanol, before being extracted with organic solvents and the procedure is repeated 3–4 times. The combined extracts are washed with water and then evaporated.

Some innovative extraction techniques, such as cloud point extraction [31] and microwave-assisted extraction [32] have been used for the preconcentration of vitamins.

Liquid-phase microextraction (LPME) includes several miniaturized techniques based on the extraction of analytes in a liquid phase using very low amounts of organic solvents [33]. Dispersive liquid–liquid microextraction (DLLME) is a very simple and rapid technique which uses very low volumes of extraction solvent and has successfully been applied to the extraction and determination of organic compounds in aqueous samples, providing high enrichment factors [34]. Among its advantages is the absence of cross-memory effects and the fact the analyses take less time. A small amount of a water-immiscible extraction solvent is dissolved in a water-soluble solvent and is then rapidly injected with a syringe into the aqueous sample. The fast injection of the mixture of organic solvents into the water disperses the water-immiscible solvent in the aqueous mass as small micro-drops, from which the target analytes can be rapidly extracted. The enriched organic phase is then separated from the aqueous sample by centrifugation and directly subjected to chromatography.

As regards the new green sample preparation techniques, no reference has been found to the preconcentration of retinol using DLLME, or to the coupling of LC and DLLME for the vitamin determination. The present study describes a fast and direct method for the sensitive determination of the *cis*- and *trans*-retinol isomers in fruit juices using DLLME-LC-Fluorescence.

2. Experimental

2.1. Reagents

All-*trans*-retinol was obtained from Sigma-Aldrich (St. Louis, MO, USA). A stock solution (100 μg mL^{-1}) was prepared in hexane and stored in amber vials at −20 °C. Working standard solutions were freshly prepared in hexane and stored at 4 °C. Ascorbic acid, potassium hydroxide and hydrochloric acid were purchased from Fluka (Buchs, Switzerland) and Merck (Darmstadt, Germany). Chromatographic quality ethanol, methanol, *n*-hexane, 2-propanol and 1,1,2,2-tetrachloroethane were obtained from Sigma. Water used was previously purified in a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Instrumentation

The LC system consisted of an Agilent 1100 (Agilent, Waldbronn, Germany) quaternary pump (G1311A) operating at room temperature with a flow-rate of 2 mL min^{-1}. The solvents were degassed using an on-line membrane system (Agilent 1100, G1379A). The fluorescence detector was an Agilent FLD (Agilent 1100, G1321A) operating at an excitation wavelength of 325 nm and an emission wavelength of 480 nm. The analytical column used for the normal-phase technique was an LiChrospher Si 60 (25 cm × 0.4 cm × 5 μm) (Agilent). The mobile phase was a 98:2 (v/v) *n*-hexane: 2-propanol mixture. Aliquots of 20 μL were injected manually using a Model 7125-075 Rheodyne injection valve (Rheodyne, Berkeley, CA, USA).

To filter the samples, PVDF filters (0.45 μm) (Teknokroma, Barcelona, Spain) were used. An EBA 20 (Hettich, Tuttlingen, Germany) centrifuge was used at a speed near to the maximum supported by the conical glass tubes, 3000 rpm. The ultrasonic processor UP 200H (Dr. Hielscher, Germany) was used for the experiments carried out using the ultrasound-assisted emulsification microextraction (USAEME) technique.

2.3. Concentration and purity test for vitamin A

The mass concentration of all-*trans*-retinol was calculated considering a molar absorptivity value of 1830 mol L^{-1} cm^{-1}. For an additional check, the absorbance of the standard solution at different wavelengths was measured with 2-propanol as reference. As the ratios between molar absorptivity values at each wavelength and 325 nm did not exceed the permitted values of 0.602 (300 nm), 0.452 (350 nm) and 0.093 (370 nm), the standard substance is suitable for use. On the other hand, the purity test was carried out by injecting a standard solution of all-*trans*-retinol in *n*-hexane into the LC and calculating the correction factor for purity (*P*) using the ratios of the peak area with the standard solution and the sum of both peak areas. Values for *P* were 0.9960 and 0.0040 for all-*trans*-retinol and 13-*cis*-retinol, respectively. Retention times were 4.61 min for 13-*cis*-retinol and 5.76 min for all-*trans*-retinol. Quantification of *cis*-retinol in the juices was carried out considering a value of 1686 mol L^{-1} cm^{-1} for the molar absorptivity of this compound [30].

2.4. Samples and saponification procedure

The samples were different type of juice enriched with vitamin A (ACE juices) containing orange, pineapple, pear, apple–mango, banana–apple and multifruits, soft orange drinks, milk-containing fruit juices and concentrated fruit juices with milk. The method was validated using two reference materials, an infant/adult nutritional formula SRM 1849a supplied by the National Institute of Standards and Technology (NIST) and whole milk powder ERM®-BD600 supplied by the Institute for Reference Materials and Measurements (IRMM).

Samples were submitted to a saponification procedure, as described by the European Standard Committee [30]. All operations were performed in subdued light. Amounts of 0.2–2 mL were measured (depending of the content of the vitamin) and 0.25 g of ascorbic acid, 50 mL of methanol and 5 mL of potassium hydroxide solution (50 g/100 mL) were added. Saponification was carried out in the absence of light, at room temperature overnight (approximately 16 h). Then, the pH of the mixture was adjusted to 6.5–7 using 37% hydrochloric acid. Aliquots were filtered using 0.45 μm PVDF filters for subsequent DLLME-LC, using 2 mL of the methanolic extract containing 100 μL tetrachloroethane. The certified reference materials were analyzed in the same way but using 50 mg of the sample. All samples were saponified in duplicate.

2.5. DLLME procedure

For DLLME, 8 mL water were placed in a 15-mL screw cap glass tube with conical bottom. Then, 2 mL of the methanolic extract from the saponified standard solution or juice sample (dispersive solvent) containing 100 μL of tetrachloroethane (extractant solvent) was rapidly injected into the water solution using a micropipette, and the mixture was again gently shaken manually for several seconds. A cloudy solution consisting of very fine droplets of tetrachloroethane dispersed through the sample solution was formed, and retinol was extracted into the fine droplets. After centrifugation for 2 min at 3000 rpm, the extraction solvent was sedimented at the bottom of the conical tube (volume about 40 ± 10 μL). Twenty microlitres of the sedimented phase were removed with a microsyringe and injected into the NP-LC.