Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Chromatography A, 1114 (2006) 132–137
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Determination of γ- and α-tocopherols in human milk by a direct high-performance liquid chromatographic method with UV–vis detection and comparison with evaporative light scattering detection☆

M. Romeu-Nadal, S. Morera-Pons, A.I. Castellote, M.C. López-Sabater*

Department Nutrició i Bromatologia, Centre de Referència en Tecnologia dels Aliments (CeRTA), Facultat de Farmàcia, Universitat de Barcelona. Avda. Joan XXIII s/n, E-08028 Barcelona, Spain

Received 19 December 2005; received in revised form 17 February 2006; accepted 17 February 2006
Available online 30 March 2006

Abstract

A rapid direct method (Method I) for measuring γ- and α-tocopherols in human milk was developed and validated using reversed-phase high-performance liquid chromatography with ultraviolet/visible (UV–vis) detection. Human milk, with an internal standard (α-tocopherol acetate) added, was diluted in hexane. The chromatographic system consisted of a short column (50 mm × 2.1 mm I.D., 3 μm particle size) that allowed the separation of the γ- and α-tocopherols in less than 6 min. The new direct method (Method I) was compared with other methods. Method II (saponification with ultraviolet/visible detection) determined 24% and 22% less γ- and α-tocopherols, respectively. Method III (saponification with evaporative light scattering detection) gave the same values for α-tocopherol content as Method II. However, the amount of sample used in the application of Method III was higher than that used in Method II. Furthermore, Method I uses smaller amounts of solvents, and it is simpler and faster than Methods II or III. Only a small volume of sample is needed, which is an additional advantage for biological assays.

Keywords: Human milk; Tocopherol; Vitamin E; HPLC; ELSD; Saponification

1. Introduction

The term Vitamin E refers to a group of tocopherols (α, β, γ, and δ), which differ in structure and biopotency. α-Tocopherol is the most active, whereas the activity of the other tocopherols is some 70–95% less. Vitamin E is the main lipophilic antioxidant, which inhibits peroxidation of polyunsaturated fatty acids in cell membranes [1,2].

Vitamin E is essential for infants, particularly preterm neonates. Because their transport capacity for Vitamin E is low and their lipoprotein metabolism is immature, neonates have very low levels of plasma Vitamin E [3,4]. Preterm infants may be especially prone to develop clinical symptoms such as hemolytic anemia, retrolental fibroplasias, intraventricular hemorrhage and bronchopulmonary dysplasia as a result of Vitamin E deficiency. Because of the lower α-tocopherol concentrations in plasma, the preterm infant has a higher requirement for Vitamin E than the full-term infant. The content of Vitamin E in human milk would influence the Vitamin E status in breast-fed infants.

The tocopherol content of human milk depends on many factors, such as the stage of lactation and maternal diet. The methods used to take samples and to measure the content will affect the results [5–10].

Several methods have been developed to measure tocopherol levels [11–14]. High-performance liquid chromatography (HPLC), using fluorescence [6,15–26] or ultraviolet/visible (UV–vis) [27–33] detection, are currently used to measure α-tocopherol in food. Some HPLC methods use an evaporative light scattering detection (ELSD) [19,21,34] or an electrochemical detector [35–38].

In milk, dairy products and infant formulas, reversed-phase HPLC (RP-HPLC) after saponification is in general use [18,27–29,33,37,39]. Normal-phase HPLC (NP-HPLC) with fluorescence detection is also used in infant formulas [6,15,16,20,40].

☆ Presented at the 11th Meeting on Instrumental Analysis, Barcelona, 15–17 November 2005.

* Corresponding author. Tel.: +34 93 4024512; fax: +34 93 4035931.
E-mail address: mclopez@ub.edu (M.C. López-Sabater).

0021-9673/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2006.02.049

Saponification entails multiple solvent extraction, drying and concentration steps but tocopherols are sensitive to light and air. Thus, such complex procedures may lead to measurement errors [41]. In addition, long exposure to alkaline conditions in saponification significantly decreases tocopherol levels [42,43]. Moreover, most procedures for the saponification of dairy products use high concentrations of potassium hydroxide (50–80%, w/v). Therefore, sample preparation is the key step of the analyses.

Direct lipid extraction without saponification using NP-HPLC with fluorescence detection [20,22–25,44] and RP-HPLC with UV–vis detection [45] have been used to measure tocopherols in infant formulas. The NP-HPLC system with ultraviolet detection has been studied for use in human milk [46].

Here we attempted to develop and validate a rapid, direct and simple RP-HPLC method with UV–vis detection (Method I) to measure γ- and α-tocopherols in human milk. The method is based on the use of a short reversed-phase column suitable for routine analyses of large amounts of samples, which obviates saponification, and may shorten the analysis time. It was compared with two methods that use saponification: one with UV–vis detection (Method II), which measured both γ- and α-tocopherol, and the other with ELSD (Method III) which measured α-tocopherol.

2. Experimental

2.1. Collection of breast milk

Samples of human milk were collected from both breasts by a Chicco manual breast pump (Chicco, Italy), following the manufacturer's instructions, from healthy mothers aged 20–35 years, at the Extraction Unit of the Department. Informed consent was obtained from the participants. All the mothers had had a full term pregnancy. Mature human milk was collected in sterile opaque bottles during the first expression in the morning. The milk from different mothers was immediately pooled, and aliquots of 5 ml were transferred to plastic tubes. This volume was enough to allow the analysis of three methods in triplicate on the same day. The methods could thus be compared. Aliquots were stored at −80 °C for no longer than one month before the analysis.

2.2. Chemicals and reagents

Stock standard solutions were prepared by dissolving γ- and α-tocopherol in dichloromethane/acetonitrile (3:1) and stored at −20 °C in dark bottles for up to a month. The γ-tocopherol and the α-tocopherol acetate standard were obtained from Sigma (St. Louis, MO, USA). The α-tocopherol standard, with a purity of 98.6%, and ascorbic acid standard were purchased from Merck (Darmstadt, Germany).

HPLC-grade acetonitrile, HPLC-grade dichloromethane and HPLC-grade methanol were purchased from SDS (Peypin, France), and the *n*-hexane and the light petroleum (20–75 °C) were supplied by Panreac (Barcelona, Spain). The Milli-Q water was purified by passing it through a Millipore Compact Milli-Q water system (Bedford, MA, USA).

2.3. Assay procedure

2.3.1. Method I

The direct method (Method I) to determine γ- and α-tocopherols was developed and validated following a modification of the method described by Brennan et al. [47].

The aliquots of human milk were thawed to around 22 °C in a water bath, protected from light, and then mixed. Five hundred microliters of human milk and 100 μl of the internal standard solution (0.25 mg/ml of α-tocopherol acetate in dichloromethane/acetonitrile (3:1)) were poured into a glass tube. The mixture was shaken mechanically for 1 min. One thousand and five hundred microliters of *n*-hexane was then added and the mixture was shaken for further 1 min and centrifuged at 10 °C (10 min, 3000 × *g*). The organic phase was evaporated off under nitrogen and the residue was reconstituted in 100 μl of a dichloromethane/acetonitrile (3:1) solution. The resulting solution was passed through a nylon filter (0.22 μm pore) (Teknokroma, Barcelona, Spain) and transferred to vial inserts and placed in amber vials for analysis by HPLC.

2.3.2. Method II

The γ- and α-tocopherols were measured using saponification, following a modification of the method described by Cayuela et al. [26]. The aliquots of human milk were thawed to around 22 °C in a water bath, protected from light, and then mixed. One milliliter of human milk and 30 mg of ascorbic acid were poured into glass tubes. Later, 1.6 ml of saponification solution, constituted by 8.5% potassium hydroxide in methanol, and 0.8 ml of methanol were added. The air was removed from the tubes by displacement with nitrogen. The solution was stirred and placed in a water bath at 70 °C for 30 min. The tubes were shaken rigorously every 10 min during the saponification. After 30 min, the tubes were cooled under tap water and 1.6 ml of light petroleum was added to each tube. The procedure was repeated twice. The three ether extracts were combined in a new tube. The new, combined ether extract was repeatedly washed in distilled water until the water was neutral to 1% phenolphthalein solution (no visible pink). The organic phase containing γ- and α-tocopherols was finally evaporated completely under nitrogen. The tocopherols were reconstituted in 100 μl of dichloromethane/acetonitrile (3:1) solution and filtered as described for Method I. Samples were then transferred to vial inserts and placed in amber vials for analysis by HPLC.

2.3.3. Method III

The α-tocopherol was measured using saponification, following a modification of the method described by Cayuela et al. [26]. The aliquots of human milk were thawed to around 22 °C in a water bath, protected from light, and then mixed. Five milliliters of human milk and 120 mg of ascorbic acid were poured into glass tubes. Later, 8 ml of saponification solution, constituted by 8.5% potassium hydroxide in methanol, and 4 ml of methanol were added. The air was removed from the tubes by