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Rapid sample preparation procedure for determination of retinol and α-tocopherol in human breast milk

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ABSTRACT

The liposoluble vitamins (retinol and α-tocopherol) concentration in human breast milk is of a cardinal knowledge especially for nutrition of prematurely born. It enables the feeding optimization of these important micronutrients for preterm infants.

The novel rapid liquid–liquid extraction procedure for human breast milk investigation was developed and validated according to FDA guidelines. The recovery of retinol was 82–90% measured at three concentration levels 1.0, 2.5 and 5.0 μmol/L, for α-tocopherol 92–109% at concentration levels 2.5, 5.0 and 10.0 μmol/L. The repeatability of extraction procedure expressed as relative standard deviation was 3.26% for retinol and 4.79% for α-tocopherol.

Developed extraction procedure was applied on 120 human breast milk samples. The separation of vitamins was completed using advantages of a monolithic column which accomplished demands of acceleration made by modern bio-analytical HPLC methodology. The analytes of interest were detected by diode-array detector at wavelengths 325 nm for retinol and 290 nm for α-tocopherol.

1. Introduction

The World Health Organization recommends full breastfeeding for the first 6 months of life. Milk is the sole source of liquids, nutrition, vitamins, minerals and immunity oligosaccharides during this time. Concentrations of breast milk constituents are influenced by several factors including stage of lactation, breastfeeding routine, age and others. The liposoluble vitamins concentration in human breast milk is of a cardinal knowledge especially for nutrition of prematurely born. Therefore rapid, uncomplicated and inexpensive sample preparation and quantification procedure for human breast milk investigation is demanded.

The protective role of α-tocopherol, the major fat soluble antioxidant of the body, is the best achieved at plasma concentration observed in adults [1]. Connections between α-tocopherol deficiency and higher risk of oxidants stress [2,3], haematological [4] and neuromuscular [5] diseases are well known. Many events leading to overproduction of free radicals may easily induce oxidative stress in the earliest phases of human life [6,7]. An effort to minimize free radical production and promote the development of adequate antioxidant systems through an adequate nutritional strategy is the main task of modern neonatology. Prematurity is associated with protracted oxidative stress [8,9] and mother's milk helps to reduce the consequences by providing protective substance [10].

Retinol is involved in the regulation and promotion of growth and differentiation of epithelial cells and the cells of many tissues (skin, mucous membranes, blood vessel walls and the cornea) therefore the need for retinol is critical during periods of rapid growth and tissue development [11]. It also maintains the integrity of respiratory tract epithelial cells [12,13]. Retinol is also essential to ensure a good functioning of the adaptive immunity, deficiency diminishes antibody-mediated response and consequent changes in mucosal epithelial regeneration [14]. Blood concentrations below 0.70 μmol/L have been considered deficient in premature infants and concentrations below 0.35 μmol/L indicate severe deficiency and depleted liver reserves.

Milk concentrations of major constituents such as fat and protein can differ substantially. The total lipid content increases significantly with time of lactation from 1.99 ± 0.25 g/dL in colostrum up to 3.89 ± 0.28 g/dL in mature milk [15]. Protein content of colostral, transitional and mature human milk differs from 1.88 ± 0.4 g/dL to 1.35 ± 0.3 g/dL, respectively. Statistical variation

Abbreviations: AOAC, association of official analytical chemists'; FDA, food and drug administration; LLE, liquid–liquid extraction; RSD, relative standard deviation; SD, standard deviation; t_R, retention time of analyte.

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($P < 0.05$) was observed also with effect of diurnality or nocturnality [16]. Therefore sample preparation procedure has to be robust and effective at major constituent removal (saponification and deproteinization). To complicate the situation even more, also the concentrations of vitamins and other micronutrients can be highly variable. Retinol [17] and α-tocopherol [18] concentrations decreases from colostrums (5–7 μmol/L and 22.3–44.2 μmol/L) to transitional milk (3–5 μmol/L and 10.4–16.3 μmol/L) and finally to mature milk (1.4–2.6 μmol/L and 7.2–10.9 μmol/L). The vitamin levels in human breast milk and vitamin supplementation were investigated many times [19–23] in different countries.

During last 5 years several approaches for retinol and α-tocopherol determination were published but HPLC/UHPLC is dominant [24–27]. The aim of modern analytical method is to accomplish demand of acceleration. In comparison with classical particle columns the usage of a monolithic column enables shortening of analysis because of better mass transfer properties ensured by monolithic skeleton. The structure of monoliths favors high-speed separations while maintaining low back pressure.

Liquid–liquid extraction (LLE) is still frequently used for sample treatment although in the last 10 years there is an obvious tendency to substitute it by modern extraction procedures as solid phase extraction (SPE), microextraction by packed sorbent (MEPS) or even to skip the sample pretreatment. Adequate conditions must be applied to free up retinol and α-tocopherol from milk matrix components and lipophylic milieu (lipoproteins, fat droplets) [28], which most create competitive conditions for organic extraction solvent. Enzymatic [29] or supercritical fluid extraction [30] has been described nevertheless classical extraction by n-hexane after saponification by basic condition [31–34] or without saponification [35,36] was utilized many times for a sample treatment.

Various samples (mainly with higher fat content) necessitate saponification. Completing of fat digestion depends mainly on applied time and temperature. The determination of liposoluble vitamins in milk is often realized after lipid elimination (saponification) [26,37]. A complete saponification process takes overnight at room temperature as used by traditional methods [38,39], but increasing temperature to 80 °C [40,41] enables shortening of time up to 30 min. Association of Official Analytical Chemists' (AOAC) International provides fully validated reference methods for retinol [42] and α-tocopherol [43] in which saponification takes place at 70 °C and for 25 or 30 min. Necessity of saponification, which is in principle additional step and therefore source of errors during sample treatment, was tested several times.

The objective of this work was to develop and validate appropriate extraction procedure for retinol and α-tocopherol from human breast milk. The procedure was expected to meet the demands of modern analytical procedure: simplicity, accessibility, robustness and specificity.

2. Materials and methods

2.1. Chemicals

Standards of retinol and DL-α-tocopherol were purchased from Sigma–Aldrich (Prague, Czech Republic).

Reagent water was purified by reverse osmosis and filtration Ultrapure water system, Goro (Prague, Czech Republic). Methanol, gradient HPLC-grade used as a mobile phase and n-hexane were purchased from Scharlau Chemie (Sentmenat, Spain). Absolute ethanol and acetonitrile gradient grade for liquid chromatography were obtained from Merck (Darmstadt, Germany).

Potassium hydroxide—pellets, pure Ph. Eur., NF was purchased from AppliChem GmbH (Darmstadt, Germany) and L-ascorbic acid purris p.a. from Sigma–Aldrich (Prague, Czech Republic). Hydrochloric acid 35% p.a. was obtained from Penta (Prague, Czech republic).

Stock solutions of retinol and α-tocopherol were prepared as follows. Retinol stock solution was prepared at the concentration 2 mmol/L in methanol and then diluted to the calibration solutions. α-Tocopherol standard was firstly dissolved in n-hexane (2 mmol/L), consecutive dilutions were made by methanol.

Solutions of potassium hydroxide at concentrations 5, 10 and 15 mol/L and L-ascorbic acid (0.1 mol/L) were prepared by dissolving weighted amount of substance in distilled water.

Hydrochloric acid 1 mol/L was prepared by dilution of concentrated (35%) acid using distilled water. Distilled water pH values (6.2, 5.2, 4.2 and 3.2) were adjusted by 1 mol/L hydrochloric acid. Changes were monitored by pH meter.

2.2. Instrumentation

Orbital shaker LabDancer V IKA (Staufen, Germany) was utilized for stirring before sample deproteinization as well as saponification and the horizontal laboratory shaker LT1 Kavalier (Votice, Czech Republic) for extraction procedure. Thermo block, Transsonic Ultrasonic Cleaning Units TP 680 DP Elma (Singen, Germany) and thermostat Stericell BMT (Brno, Czech Republic) were utilized for heating during saponification. Centrifuge 5810-R Eppendorf (Prague, Czech Republic) accommodated with Swing-bucket Rotor A–4–62 was used for separation of n-hexan and aqueous layer during liquid–liquid extraction. The laboratory vacuum evaporator Concentrator 5301 Eppendorf (Hamburg, Germany) was used for evaporation of organic layer after extraction. pH meter Argus χ Sentron (Roden, Netherlands) was utilized for pH adjusting.

Target analytes retinol and α-tocopherol were quantified on the HPLC device Prominence Shimadzu (Kyoto, Japan) composed by Rack changer/C autosampler for microtitrate plates, Degasser DGU-20A5, Column Oven CTO – 20 AC, Diode array detector SPD – M20A and Communication bus module CBM-20A.

2.3. Sample pretreatment

Samples of human breast milk were obtained from University Hospital Hradec Kralove. The University Hospital ethical committee approved the protocol of this study.

Milk samples were stored frozen at −28 °C. Before the extraction material was left to thaw and homogenized by constant mixing at water bath warmed up to 37 °C. Homogenized volume of human breast milk was partitioned to obtain representative analytical sample.

2.4. HPLC determination

Liposoluble vitamins retinol and α-tocopherol were measured by HPLC method [44], which was recently optimized for biological material analysis in Research laboratory of Department of Metabolic Care and Gerontology, Teaching Hospital Hradec Kralove. Separation of vitamins was performed using the Chromolith Performance RP-18e, 100 mm × 4.6 mm monolithic column Merck (Darmstadt, Germany). As the mobile phase 100% methanol was used at the flow rate of 2.5 mL/min. The DAD detection of retinol and α-tocopherol was carried out at 325 and 295 nm, respectively. Limit of detection and limit of quantification were 0.13 and 0.27 μmol/L for retinol, for α-tocopherol 0.09 and 0.19 μmol/L [44].

Table 1
Selection of the optimal deproteinization reagent.

Deproteinization reagent	Retinol $c \pm SD$[a] (μmol/L)	α-Tocopherol $c \pm SD$[a] (μmol/L)
Acetonitrile	0.9 ± 0.2	12 ± 0.5
Methanol	1.8 ± 0.1	23 ± 1.0
Ethanol	2.3 ± 0.1	22 ± 0.4

[a] Measured concentration of vitamins ± standard deviation ($n = 3$).

3. Results and discussion

3.1. Optimization of sample preparation procedure

Presented sample preparation procedure refers to human breast milk contents and was designed to reflect wide range of individual concentration differences. The preparation procedure consists of deproteinization, saponification and liquid–liquid extraction steps. All procedure steps were carried out in one screw top glass tube to minimized losses in extraction process and maximized recovery of determined vitamins.

3.1.1. Selection of deproteinization reagent

Concentrations of α-tocopherol were not significantly influenced by alcoholic deproteinization reagents (methanol and ethanol) while measured levels of retinol were significantly variant. According to these results (Table 1) acetonitrile was assessed as unsuitable contrary to the ethanol, which was selected as the appropriate deproteinization reagent.

3.1.2. Optimization of saponification conditions

Potassium hydroxide solution (KOH) was chosen to remove lipids from the sample. Influence of increasing concentration (5, 10 or 15 mol/L) was tested. The addition of an antioxidant (ascorbic acid, 0.1 mol/L, 1 mL) was employed to protect vitamins against oxidation during the saponification, 1 mL of ethanol was used for deproteinization. Samples were saponified by different concentrations of KOH (1 mL), the mixtures were heated for 30 min at 80 °C. The extraction preceded the same manner as already mentioned.

The optimal concentration of KOH was selected in agreement with results of repeatability expressed by % RSD (Table 2). Repeatability of extraction after saponification by 5 and 15 mol/L KOH showed higher % RSD for retinol than results obtained after saponification by 10 mol/L KOH as contrasted to results for α-tocopherol. 10 mol/L solution was chosen owing to relative standard deviation (RSD) of measured concentration, for retinol 0.50–0.58 μmol/L, RSD 7.94% and for α-tocopherol (6.11–7.16 μmol/L, RSD 7.93%).

The optimal volume (1.0, 1.5 and 2.0 mL) of potassium hydroxide (10 mol/L) was tested. The 0.5 mL of breast milk was mixed with 2 mL of ethanol, 1 mL of ascorbic acid (0.1 mol/L) and different volumes of potassium hydroxide. Higher volumes did not resulted in higher concentrations of measured analytes. Therefore 1 mL of potassium hydroxide was selected as sufficient.

Also the prolongation of saponification time (30, 40 and 50 min) was investigated. Previously selected conditions were used. Prolongation did not bring statistical significant differences. Three different possibilities of warming up system were compared (thermo block, thermostat and water bath). The best results for both determined vitamins were obtained when using thermostat, the samples were protected from light.

Table 2
Selection of the optimal concentration of potassium hydroxide.

	KOH (mol/L)	5	10	15
Retinol	c[a] (μmol/L)	0.40	0.54	0.41
	% RSD	10.5	7.9	18.1
α-Tocopherol	c[a] (μmol/L)	7.3	6.6	6.9
	% RSD	1.4	7.9	4.8

[a] Measured concentration of vitamin ($n = 6$).

Table 3
Influence of water addition to the sample on the determined concentration.

water (mL)	0	1
Retinol $c \pm SD$[a] (% RSD)	1.6 ± 0.2 (14.8)	2.3 ± 0.2 (6.8)
α-Tocopherol $c \pm SD$ (% RSD)[a]	2.5 ± 0.1 (4.9)	3.5 ± 0.04 (1.2)

[a] Measured concentration of vitamins ± standard deviation, $n = 3$.

3.1.3. Extraction

Retinol and α-tocopherol were extracted with 2 mL of n-hexane by vigorous shaking. The organic and water layers were separated by centrifugation (3220 × *g*, 10 min, 4 °C). Than 1.5 mL of organic layer phase was evaporated and the residue was dissolved in 375 μL of methanol.

Prolongation of shaking time from 5 min to 10 or 15 min was tested. Longer time of extraction did not resulted in significantly better extraction effect. Therefore time of 5 min was chosen as sufficient and moreover total time of sample preparation was not made than necessary.

Efficiency of extraction was supported by addition of 1 mL of distilled water (Table 3). Measured concentration of both vitamins significantly increased and repeatability of the extraction procedure was eminently improved.

Influence of pH of added water was tested (Table 4). Different water pH (6.2, 5.2, 4.2 and 3.2) was added into the extraction tube together with n-hexane. Water at pH 5.2 was chosen as optimal since concentration of retinol measured at others pH were almost 20% lower, for α-tocopherol losses were about 10%.

3.2. Validation of the extraction procedure

The developed sample preparation procedure was validated for recovery, repeatability and precision according to FDA guidelines [45].

3.2.1. Analyte recovery

Spiked human breast milk samples were used for accuracy (recovery) of validation test. Determinated vitamins were already present in the unspiked sample matrix therefore concentrations of spiked vitamins were corrected by subtracting the blank. The human breast milk samples were spiked by assistance of deproteinization reagent at the concentrations matching calibration range. FDA [45] recommended criteria for recovery are from 75% to 125%.

Samples of human breast milk were spiked by retinol at the concentrations 1.0, 2.5 and 5.0 μmol/L and by α-tocopherol at the concentrations 2.5, 5.0 and 10.0 μmol/L. For each concentration level six individual samples were prepared. The results are summarized in Table 5 showing all recovery values in the range 82–110% thus having satisfied the FDA requirements.

Recovery of both vitamins fulfills FDA recommended criteria therefore the tested extraction procedure is suitable for analysis of human breast milk.

3.2.2. Repeatability

The method precision characteristic (repeatability) was validated with six samples of breast milk. The precision was expressed by % relative standard deviation (% RSD). FDA [45] recommended criteria are % RSD not greater than 15%.

The intra-day repeatability of extraction procedure was tested. Average concentration of retinol was 1.24 ± 0.04 μmol/L (3% RSD).

Table 4
Influence of pH on the extraction.

pH of water	6.2	5.2	4.2	3.2
Retinol $c \pm SD^a$ (D^b)	1.1 ± 0.2 (84)	1.4 ± 0.3 (100)	1.1 ± 0.1 (78)	1.1 ± 0.1 (81)
α-Tocopherol $c \pm SD^a$ (D^b)	11.6 ± 1.1 (93)	12.4 ± 1.0 (100)	10.9 ± 0.7 (88)	10.8 ± 0.6 (88)

[a] Measured concentration of vitamins ± standard deviation, $n = 4$.
[b] Percentage decrease of concentration.

Table 5
Validation of the extraction process.

		Retinol			α-Tocopherol		
Recovery	c [a] (μmol/L)	1.0	2.5	5.0	2.5	5.0	10.0
	recovery (%)	86	82	90	109	95	92
	% RSD	10	4	7	0.5	2	2
Repeatability	$c \pm SD^b$ (μmol/L)	1.24 ± 0.04			7.7 ± 0.4		
	% RSD	3			5		
Precision	$c \pm SD^c$ (μmol/L)	0.80 ± 0.08			6.3 ± 0.4		
	% RSD	10			7		

	Day	**1**[f]	**2**[g]	**3**[h]	**4**[i]	**1**[f]	**2**[g]	**3**[h]	**4**[i]
Inter-day repeatability	$c \pm SD$ (μmol/L)	0.78 ± 0.04	0.77 ± 0.07	0.61 ± 0.05	0.61 ± 0.03	6.0 ± 0.4	5.0 ± 0.3	5.2 ± 0.3	5.4 ± 0.5
	c_d [d] (μmol/L)	–	0.01	0.17	0.18	–	0.6	0.7	0.5
	D [e] (%)	–	1.07	22.0	22.6	–	9.5	11.8	9.0
	% RSD	5	7	14	14	6	8	8	8

[a] Concentration of vitamins which were used for milk samples spiking ($n = 6$).
[b] Average concentration of vitamins ± standard deviation ($n = 6$).
[c] Average concentration of vitamins ± standard deviation ($n = 30$).
[d] Decrease of concentration (μmol/L) correlated to value of day 1.
[e] Percentage decrease of concentration.
[f] Average values and standard deviation calculated from 6 samples.
[g] Average values calculated from 12 samples.
[h] Average values calculated from 18 samples.
[i] Average values calculated from 24 samples.

Average concentration of α-tocopherol was 7.7 ± 0.4 μmol/L (5% RSD). Repeatability of both vitamins fulfills FDA recommended criteria (% RSD ≤ 15%).

Inter-day repeatability was determined during 4 consecutive days. 25 mL of pasteurized and frozen human breast milk was warmed up to 37 °C and pipetted under constant shaking (to obtain aliquot homogeneous samples) into the 1.5 mL ependorf tubes. Six samples were processed by the extraction procedure and measured. Obtained results correspond to the values day 1. The rest of aliquots was stored (−28 °C) and determined by HPLC in subsequent days (days 2, 3 and 4). Obtained concentrations of retinol and α-tocopherol are summarized in Table 5.

Inter-day repeatability was evaluated by calculation of % RSD of measured concentrations. Standard deviation was correlated to the measured concentration of day 1 and number of samples for calculation has increased from 6 (day 1) to 12, 18 and finally 24 (day 4). Repeatability of both vitamins meets recommendation of FDA, nevertheless α-tocopherol showed stable values (6–8% RSD). On the contrary the retinol values showed relevant increasing tendency of % RSD corresponding to decrease of concentration.

3.2.3. Precision

Interpersonal precision was investigated by two different analysts during one day. Each of them extracted 15 individual samples of breast milk. The % RSD of all measured concentrations ($n = 30$) for retinol (0.80 ± 0.08 μmol/L) was 10% and for α-tocopherol (6.3 ± 0.4 μmol/L) it was 7%.

3.3. Method application

Randomized group of 27 women (age 29 ± 4 years) at different lactating stages (1–2, 3–4, 5–6 and 9–12 months postpartum) was selected for the screening of vitamins. The samples about 25 mL were stored maximally 2 years at −86 °C than the whole volume was warmed up to laboratory temperature and consequently pasteurized. Homogeneous aliquots pipetted under constant shaking into the 1.5 mL eppendorf tubes were stored till measurement at −28 °C.

The differences among samples of 12 women originated from four lactating stages were tested by statistical software NCSS (Kaysville, USA), non-parametric paired t-test (Wilcoxon) was used. The results of analyses are shown in Table 6. Concentrations of retinol ($n = 12$) proved statistical differences at 5%

Table 6
Changing of retinol concentration during lactating stages.

Month postpartum	1–2	3–4	5–6	9–12
Retinol $c \pm SD^a$ (μmol/L)	1.6 ± 1.0	1.1 ± 0.9	0.8 ± 0.4	0.6 ± 0.4
t-Test paired (p^b)		0.432591	0.037633	0.010787
α-Tocopherol $c \pm SD$ (μmol/L)[a]	9.2 ± 3.3	8.7 ± 3.9	8.4 ± 3.5	9.3 ± 3.1
t-Test paired (p^b)		0.724082	0.666137	0.504903

[a] Concentrations of measured vitamins ± standard deviation ($n = 12$).
[b] Statistical evaluation.

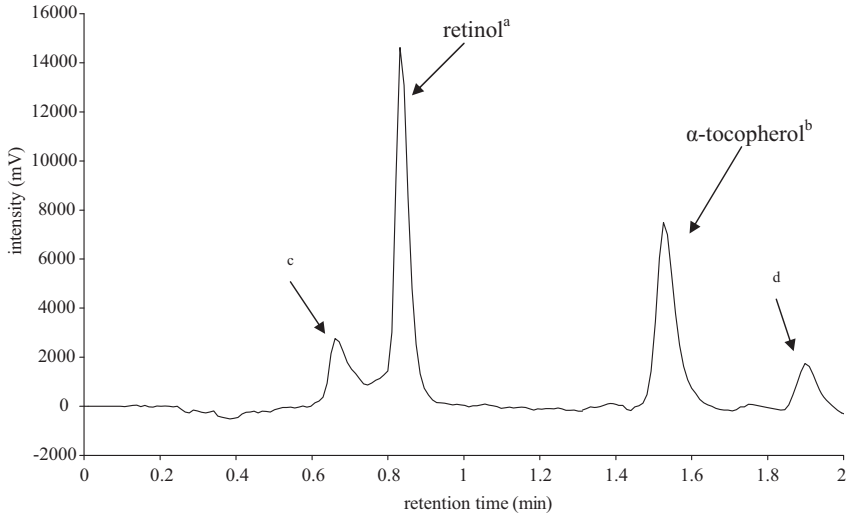

Fig. 1. Chromatogram of real sample human breast milk (lactating stage: 4 months). [a]Retinol (t_R = 0.83 min, c = 1.17 μmol/L, detected at 325 nm). [b]α-Tocopherol (t_R = 1.52 min, c = 21.22 μmol/L detected at 295 nm). [c,d]Peaks of impurity. Void time (t_0) was measured in retention time 0.59 min.

significance among stages 1–2/5–6 and 1–2/9–12 on the contrary of α-tocopherol (n = 12). A typical chromatogram of retinol and α-tocopherol in real sample of human breast milk (lactating stage—3 months postpartum) is depicted in Fig. 1.

4. Conclusion

Rapid sample preparation procedure for determination of retinol and α-tocopherol in human breast milk was developed and validated. Extraction recovery of both vitamins fulfils FDA recommended criteria (retinol 86 ± 4% and α-tocopherol 99 ± 10%) as well as repeatability (retinol 1.24 ± 0.04 μmol/L, RSD 3% and α-tocopherol 7.7 ± 0.4 μmol/L, RSD 5%). Carrying out of the extraction process has only a little workmanship requirements on a technician. Preparation of 10 samples is possible to complete during 90 min.

HPLC separation was performed using the monolithic column the Chromolith Performance RP-18e, 100 mm × 4.6 mm and 100% methanol as the mobile phase at a flow rate of 2.5 mL/min were utilized for the determination of retinol and α-tocopherol. The detection was carried out at 325 and 295 nm, respectively. The time of analysis was 2.0 min.

The newly developed extraction method was used for analysis of real breast milk samples. Randomized group of 27 women was selected for retinol and α-tocopherol human breast milk screening. The study group (n = 12) implied mothers at different lactating stages (1–2, 3–4, 5–6 and 9–12 months postpartum). Concentrations of retinol proved statistical differences at 5% significance among stages 1–2/5–6 and 1–2/9–12 on the contrary of α-tocopherol.

According to FDA guidelines, the newly developed extraction procedure was evaluated as suitable for analysis of human breast milk.

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