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Application of core–shell technology for determination of retinol and alpha-tocopherol in breast milk

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ABSTRACT

Breast milk is a main source of fat-soluble vitamins for newborns and it is needful to monitor the nutritional status prior to its application. In this work a novel, high-throughput and low-cost method for monitoring of retinol and alpha-tocopherol in breast milk was developed, validated and compared with reference method using monolithic column.

For this purpose five various porous shell and monolithic columns were tested on the basis of relationship between HETP and linear mobile phase velocity, analysis time and consumption of solvents. Finally the core–shell analytical column Kinetex C_{18} (2.6 μm, 100 Å, 100 × 4.6 mm) was chosen as the best and optimal values of flow rate, injection volume and temperature of analysis were established.

The detection of retinol and alpha-tocopherol was carried out at 325 and 295 nm, respectively by diode array detector. The LOD 0.004 μmol/L and 0.078 μmol/L, the LOQ 0.012 μmol/L and 0.182 μmol/L for retinol and alpha-tocopherol, respectively were calculated. The validation data showed good linearity, repeatability of retention time with RSD 0.22% and 0.12%, repeatability of peak area with RSD 6.94% and 1.75%, recovery 114.1–116.3% and 99.0–108.6% for retinol and alpha-tocopherol, respectively. Moreover, the newly developed method substantially decreased the solvent consumption by about 263 mL per 100 samples with the total time of analysis 1.75 min in comparison with analysis time 1.80 of the reference method.

1. Introduction

Oxidative stress, defined as a disturbance in the balance between the production of reactive oxygen species (free radicals) and antioxidant defences leading to tissue injury [1], plays an important role in the pathogenesis of numerous degenerative or chronic diseases such as atherosclerosis, arthritis, neurodegenerative disorders, coronary heart disease, allergy or cancer [2–9]. Of the non-enzymatic antioxidants, fat-soluble antioxidant vitamins represent an important class that includes all-trans-retinol and alpha-tocopherol [10].

Maternal breast milk must supply enough retinol to meet the needs for growth and for the building up of the liver reserves during the suckling period to ensure adequate retinol status of the infant after weaning. Concentration level of retinol in breast-milk is correlated significantly with the levels in the plasma of mothers and infants [11]. Retinol plays roles in cell–cell communication, cellular differentiation, and regulation of cell growth and apoptosis. Recently, it was proved that very low plasma retinol and RBP concentrations are associated with night blindness (two point mutations I41N and G75D on the RBP gene) [12] or xerophthalmia [13], the progression of normal to mild cognitive impairment to Alzheimer's disease [14].

Alpha-tocopherol is another important antioxidant and protective factor, especially for infants born prematurely. It represents one of the most abundant forms of vitamin E in plasma and breast milk [15]. Preterm infants may be especially prone to develop clinical symptoms such as hemolytic anemia, characterized by an increased susceptibility of red cells to oxygen due to an insufficient supply of alpha-tocopherol [16]. Furthermore, infants can tend to retrolental fibroplasias, intraventricular hémorragie and bronchopulmonary dysplasia as a result of alpha-tocopherol deficiency. Because of the lower alpha-tocopherol concentrations in plasma, the preterm infant has a higher requirement for this

Abbreviations: DAD, diode array detector; HETP, Height Equivalent to a Theoretical Plate; *R*, recovery; RBP, retinol-binding protein; R_S, peak resolution; T_f, tailing factor

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vitamin than the full-term infant. The tocopherol content of human milk depends on many factors, such as the stage of lactation and maternal diet [17].

The transfer of fat-soluble vitamins via the placenta is limited and plasma levels are usually low in newborn babies. Colostrum, rich in fat-soluble vitamins, is one important source for newborn or preterm infants [16] and that is why it is needful to monitor concentrations of vitamins before its application.

Several methods have been proposed for the measurement liposoluble vitamins levels. HPLC, using ultraviolet/visible (UV/Vis) or fluorescence detection and particle or monolithic columns, are currently used to measure retinol and alpha-tocopherol in different matrices [2,4,13,17,18]. Clinical laboratories have considerable interest in utilization the new methods with time- and cost-reduction, as permitted, among other things development of new stationary phases.

During last few years new brands of porous shell columns were being commercialized: Halo (Advanced Materials Technology, DE, USA) in 2006, Poroshell (Agilent, Little River, DE, USA), Ascentis Express (Supelco, Bellefonte, USA) and Kinetex (Phenomenex, Torrance, CA, USA) in 2009 [19] and four next generation columns were released in 2011, namely: Accucore (Thermo Fisher Scientific, Waltham, MA, USA), Nucleoshell (Macherey-Nagel, Düren, Germany), SunShell (Sunniest, Marl, Germany) and Aeris (Phenomenex, Torrance, CA, USA) [20–23].

Monolithic column consist of a single rod of porous material with several unique features in terms of permeability and efficiency. These materials were originally developed by Hjerten et al., Svec and Frechet, Tanaka and Nakanishi during the 1990s [24]. Commercial silica monolith columns are the Chromolith (Merck KgGa, Darmstadt, Germany) and Onyx (Phenomenex, Torrance, California) based upon technology licensed from Merck. The 1st monolith generation has been sold as Chromolith Performance columns by Merck since 2000, and the 2nd generation was announced at the HPLC 2011 conference in Budapest to be introduced to the market as Chromolith High-Resolution columns [25,26]. The silica monolith columns have a bimodal pore size distribution; therefore they have a higher permeability than columns packed with particles having the same size as the skeleton elements of these monolithic columns and allow higher mobile phase velocities to achieve high efficiency or high speed [18,27–29]. The special porous character of the monolithic column allows relatively high flow rates (1–9 mL/min) while keeping the backpressure low. Due to the favorable properties of monolithic materials, the risk of destruction and damage of the column by movement of the sorbent inside the column is eliminated and reliability as well as reproducibility of the analysis are improved [18]. The kinetic efficiency of commercially available the 1st generation monolith columns is comparable to columns packed with 3–4 μm particles [27].

With traditional fully porous 3 μm and 5 μm particles, efficiency decreases significantly as flow rate increases. Speeding up the analysis by increasing the flow rate is, in most cases, accompanied by loss of resolution and sensitivity. Smaller fully porous sub-2 μm particles provide faster chromatographic separations at low HETP but require higher pressure capable instrumentation. Apparently these features have triggered the explosion of interest in the use of small particles and short columns to improve the speed of separation [30].

Another alternative to improve separation efficiencies and speed without reducing particle size is the use of superficially porous particles, also termed porous shell particles. Technology of porous shell particles offers the ultra-high efficiency of sub-3 μm and sub-2 μm particles without generating excessive column backpressure and without updating HPLC instrumentation. Analytes spend less time diffusing into and out of the pores as they travel through the column. This shorter diffusion path allows for faster mass transfer [31]. The commercialization of porous shell particles presents a new option for HPLC bioanalysis. The 2.7 μm particles of Ascentis Express columns consist of 1.7 μm nonporous solid silica inner core surrounded by 0.5 μm porous silica layer [27,32–34]. The core–shell technology of Kinetex columns produces the 2.6 μm particles, which consist of 1.9 μm nonporous core and 0.35 μm porous silica layer. This technology is using sol–gel processing techniques that incorporate nano-structuring technology; a durable, homogeneous porous shell is grown on a solid silica core [27,35]. As a result Kinetex columns provide roughly 3× the efficiency of 5 μm fully porous particles and 2× the efficiency of 3 μm fully porous particles without the need for specialized, high pressure instrumentation. A major benefit of the shell particles is the small diffusion path 0.5 μm for Ascentis Express and 0.35 μm for Kinetex. In theory, decreasing the thickness of the porous layer influence a decrease of the C term in the van Deemter plot, which is the cause of different results obtained during analysis [36].

The main purpose of this work was to develop a novel, high-throughput and low-cost method for monitoring of retinol and alpha-tocopherol in the breast milk. Great emphasis was placed on reducing the analysis time and consumption of mobile phase. The advantages and disadvantages of monolithic and porous-shell separation technologies were examined. The separation method was validated and together with pre-separation liquid–liquid extraction procedure was used for determination of liposoluble vitamins in the breast milk.

2. Materials and methods

2.1. Chemicals and columns

The monitored analytes DL-all-rac-tocopherol, purity ≥ 96% and retinol, purity ≥ 99% were purchased from Sigma Aldrich (Prague, Czech Republic). Ethanol absolute for analysis from MERCK (Darmstadt, Germany), potassium hydroxide pellets pure Ph. from AppliChem (Chemos, Prague, Czech Republic), L-ascorbic acid from Sigma Aldrich (Prague, Czech Republic) and distilled water GORO (Prague, Czech Republic) were needed for the extraction process. n-Hexan multisolvent HPLC grade ASC UV–vis from Scharlau (Sentmenat, Spain) and methanol super gradient from LAB-SCAN analytical sciences (Lach:ner, Neratovice, Czech Republic) were needed for the preparation of standard solutions.

Chromolith Performance RP-18e 100 mm × 4.6 mm and 50 mm × 4.6 mm were purchased from MERCK (Darmstadt, Germany), Kinetex C_{18} core–shell columns (100 Å, size of shell particles 2.6 μm, 100 mm × 4.6 mm and 50 mm × 4.6 mm) were purchased from Phenomenex (Torrance, USA) and Ascentis Express C_{18} fused-core columns (100 Å, 2.7 μm, 100 mm × 4.6 mm and 50 mm × 4.6 mm) were purchased from Supelco (Bellefonte, USA).

2.2. Instrumentation and software

All measurements were performed using the HPLC set Prominence LC 20 (Shimadzu, Kyoto, Japan) equipped with Diode array detector SPD-M20A with deuterium lamp, wavelength range 190–800 nm and flow cell with optical path length 10 mm and volume 10 μL, Fluorescence detector RF-10 AXL, Rack changer/C—autosampler for microtitrate plates, Autosampler SIL/20 AC with injection volume range 1–100 μL, Degasser DGU-20A5, two Pumps LC20-AB with flow rate setting range 0.1–10 mL/min, Column oven CTO-20 AC with temperature setting range 4–85 °C and Communication bus module CBM-20 A. The whole

HPLC system was controlled by LabSolution—LCsolution v. 1.25 SP1

Lab Dancer (IKA WERKE, Staufen, Germany) for quick mixing between working steps, Laboratory shaker LT-1 (Kavalier, Sázava, Czech Republic) for horizontal mixing, Transsonic Ultrasonic Cleaning Units TP 680 DP (Elma, Germany), Thermostat Stericell 55 (BMT, Brno, Czech Republic) for heating during saponification and Centrifuge Eppendorf 5810-R (Hamburg, Germany) with Swing-bucket Rotor A-4-62 for separation of organic and aqueous layer were used during liquid–liquid extraction procedure of liposoluble vitamins from breast milk.

2.3. Preparation of standard solutions and samples

Stock solutions of retinol and alpha-tocopherol with concentration 2 mmol/L were prepared by dissolving in methanol and n-hexane, respectively. Calibration solutions of retinol were subsequently diluted from stock solution by methanol to concentrations 7.0, 4.9, 2.45, 0.98, 0.294 and 0.0588 μmol/L. Calibration solutions of alpha-tocopherol were subsequently diluted from stock solution by methanol to concentrations 50.0, 35.0, 17.5, 7.0, 2.1 and 0.42 μmol/L. The calibration curve was created out of six concentration levels. Retinol and alpha-tocopherol solutions were stored at −25 and +4 °C, respectively.

Sample preparation procedure of breast milk consisted of deproteinization, saponification and liquid–liquid extraction steps carried out in one screw top glass tube. Ethanol was used for deproteinization of 500 μL breast milk (in ratio 4:1, v-v). 1 mL of 0.1 mol/L of ascorbic acid was added before second step of sample preparation to avoid oxidation of vitamins. Saponification effect of 1 mL of potassium hydroxide with concentration 10 mol/L was optimized by controlled conditions (30 min, 80 °C and protected from light). After cooling down to laboratory temperature vitamins were extracted by 2 mL of n-hexane and contemporary 5 min of vigorous shaking. The extraction was supported by 1 mL of distilled water added into the mixture. The organic and water layer were separated by centrifugation (10 min, 3220g, 4 °C). 1500 μL Of organic phase was evaporated and the residue was dissolved in 375 μL of methanol mobile phase [37]. All samples were taken from Milk bank of University Hospital in Hradec Králové (Czech Republic).

3. Chromatographic conditions

3.1. Reference method employing monolithic column

Separation of vitamins was performed using a monolithic column Chromolith Performance RP-18e, 100 mm × 4.6 mm. As the mobile phase 100% methanol was used at the flow rate 2.5 mL min/L and column pressure 7.4 MPa (1069 PSI). The column oven CTO-20 AC was utilized to set temperature of analytical column at 25 °C. The injection volume was 20 μL. The DAD detection of retinol and alpha-tocopherol was carried out at 325 nm and 295 nm, respectively. The total time of analysis was 1.8 min [18,43].

3.2. New method employing core–shell column

Separation of vitamins was performed using a Kinetex C_{18} (100 Å, 2.6 μm, 100 mm × 4.6 mm). As the mobile phase 100% methanol was used at the flow rate 1.5 mL min/L and column pressure 12.5 MPa (1816 PSI). The column oven CTO-20 AC was utilized to set temperature of analytical column at 49 °C. The injection volume was 5 μL. The DAD detection of retinol and alpha-tocopherol was carried out at 325 nm and 295 nm, respectively. The total time of analysis was 1.75 min.

4. Results and discussion

4.1. Parameters of system suitability test

The method validation was performed according to the 5th edition of European Pharmacopoeia and Validating chromatographic methods—a practical guide by David M. Bliesner including Guidance for Industry on Analytical Procedures and Methods Validation (Food and Drug Administration—FDA) and also Q2 Validation of Analytical Procedures: Text and Methodology (The Tripartite International Conference on Harmonization—ICH) and Modern HPLC for Practicing Scientist by Michael W. Dong [38–42].

The calculated values for expression of the column efficiency such as number of theoretical plates (N), Height Equivalent to a Theoretical Plate (HETP), tailing factor (T_f), peak resolution (R_S), separation factor (α) are summarized in Table 1.

4.2. Comparison of columns

Different chromatographic conditions, summarized in Table 2, were applied on five columns under study. Data obtained during columns testing were compared with validation parameters of previously developed method using monolithic column Chromolith Performance RP-18e, 100 mm × 4.6 mm (Merck, Darmstadt, Germany) [18,43]. Two ranges of flow rate were chosen due to the difference of stationary phases. For Chromolith Performance higher range of flow rate from 0.9 to 2.5 mL/min was elected. For porous shell columns Ascentis Express and Kinetex lower range of flow rate from 0.5 to 2.0 mL/min was elected due to the high pressure generated at higher flow rate. Mobile phase composition, injection volume 20 μl and analysis temperature 25 °C remained unchanged. Short analysis time, low consumption of solvent, satisfactory parameters of system suitability played a major role for the evaluation of the columns (Table 1 and Figs. 1–4). Separation column Kinetex C_{18} (100 Å, 2.6 μm, 100 mm × 4.6 mm, Phenomenex, Torrance, USA) with flow rate 1.5 mL/min was chosen for further testing (Fig. 5). Injection volume in the range of 1 to 20 μL was tested as a next parameter. The injection was four times decreased from 20 μL to 5 μL and consequently the LOD

Table 1
Comparison of system suitability tests.

Column	Chromolith RP-18e (100 × 4.6 mm)[a]		Kinetex C_{18} (100 × 4.6 mm)[b]	
Compound	Retinol	Alpha-tocopherol	Retinol	Alpha-tocopherol
V_I (μL)	20		5	
T (°C)	25		49	
F (mL/min)	2.5		1.5	
t_R (min)	0.81	1.46	0.87	1.50
A	61,070	25,526	24,422	10,753
H	26,118	7984	10,382	4007
N	30,098.5	52,94	32,65	74,62
HETP (μm)	33.25	19	31	13
T_f	1.68	1.52	1.75	1.42
R_S	1.4	3.5	2.6	4.9
α	2.1		1.4	
W (min)	0.17	0.20	0.12	0.13
t_A (min)	1.80		1.75	
V_S (mL)	450		263	

V_I: injection volume (μL), T: temperature (°C), F: flow rate (mL/min), t_R: retention time (min), A: peak area, H: peak height, N: number of theoretical plates, HETP: Height Equivalent to a Theoretical Plate (μm), T_f: tailing factor, R_S: peak resolution, α: separation factor, W: width of peak at baseline (min), t_A: time of analysis (min), V_S: consumption of solvent (mL)/100 samples.

[a] Reference method with monolithic column [43].

[b] Developed method with core–shell column.

Table 2
Characteristics of stationary phases and chromatographic conditions.

Separation column	Chromolith[a] RP-18e	Chromolith [b] RP-18e	Ascentis[b] Express C_{18}		Kinetex C_{18}[b]	
Column dimension	100 × 4.6 mm	50 × 4.6 mm	100 × 4.6 mm	50 × 4.6 mm	100 × 4.6 mm	50 × 4.6 mm
Macropore size (μm)	2		–		–	
Mesopore size (nm)	13		–		–	
Particle size (μm)	–	–	2.7		2.6	
Porous shell (μm)	–	–	0.5		0.35	
Solid core (μm)	–	–	1.7		1.9	
Average pore size (Å)	130		90		100	
Surface area (m^2/g)	300		150		200	
Temperature stability (°C)	45		60		60	
Pressure stability (MPa)	20		60		60	
pH stability	2.0–7.5		2.0–9.0		1.5–10	
Injection volume (μL)	20		20		1–20	20
Temperature (°C)	25		25		25–55	25
Mobile phase	100% Methanol		100% Methanol		100% Methanol	
Flow rate (mL/min)	2.5	0.9–2.5	0.5–2.0		0.5–2.0	
Column pressure	7.4 MPa (1069 PSI)	0.8–3.2 MPa (113–464 PSI)	4.8–18.2 MPa (703–2633 PSI)	2.6–10.7 MPa (374–1557 PSI)	5.0–20.0 MPa (725–2900 PSI)	2.9–11.9 MPa (422–1723 PSI)
Time of analysis (min)	1.80	1.06–3.17	2.61–8.98	1.37–4.7	1.81–6.4	1.02–3.34
Detection (nm)	295 nm for alpha-tocopherol and 325 nm for retinol					

[a] Reference method with Chromolith Performance RP-18e (100 × 4.6 mm) [43].
[b] Tested monolithic and porous shell columns.

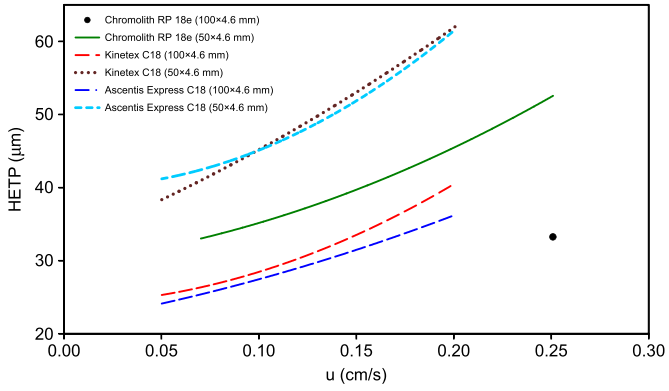

Fig. 1. Van Deemter plot of retinol—comparison of five HPLC columns (HETP vs. *u*—linear mobile phase velocity). Black point represents reference method with monolithic column Chromolith RP 18e 100 mm × 4.6 mm for comparison with the others.

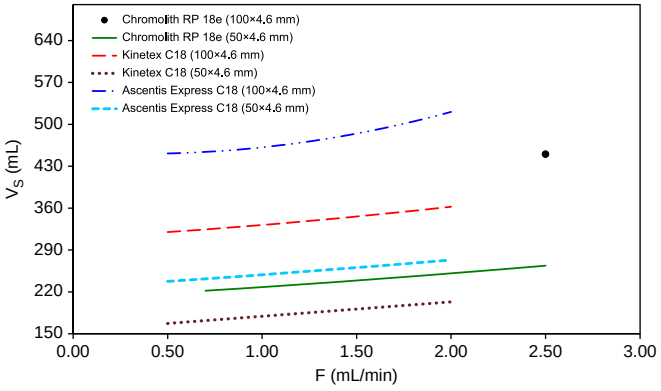

Fig. 3. Graph expressing the dependence of solvent consumption on flow rate in 100 measured samples. Black point represents reference method with monolithic column Chromolith RP 18e 100 mm × 4.6 mm for comparison with the others.

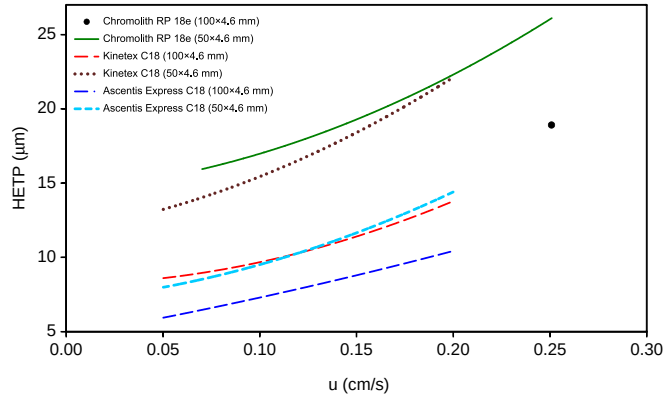

Fig. 2. Van Deemter plot of alpha-tocopherol—comparison of five HPLC columns (HETP vs. *u*—linear mobile phase velocity). Black point represents reference method with monolithic column Chromolith RP 18e 100 mm × 4.6 mm for comparison with the others.

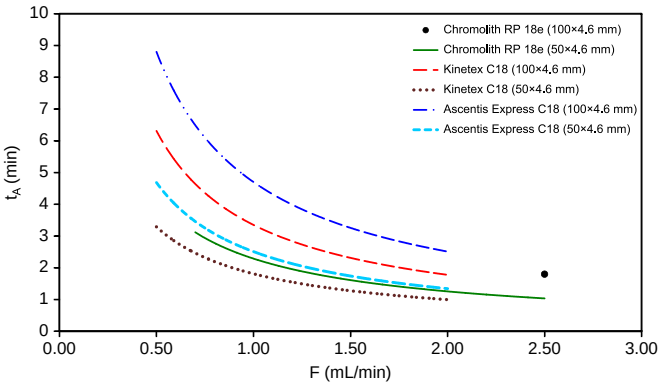

Fig. 4. Graph expressing the dependence of analysis time on flow rate. Black point represents reference method with monolithic column Chromolith RP 18e 100 mm × 4.6 mm for comparison with the others.

0.004 μmol/L, the LOQ 0.012 μmol/L and the LOD 0.078 μmol/L, the LOQ 0.182 μmol/L for retinol and alpha-tocopherol, respectively were calculated. Reduction of injection did not have a negative effect on the limits in comparison with the reference method (Table 3). The analysis temperature range from 25 °C to 55 °C was tested as a last step. Increasing of the temperature up to 49 °C

positively affected back pressure which was decreased from 2234 PSI (15.4 MPa) to 1740 PSI (12.0 MPa) (Fig. 6). As the mobile phase 100% methanol was used and the detection of retinol and alpha-tocopherol was carried out at 325 and 295 nm, respectively by diode array detector (Table 2).

4.3. Method validation

Validation of method using core–shell column technology was carried out by calculating the following parameters: linearity, accuracy, precision, limit of detection and limit of quantification.

Linearity was expressed as the confidence limit around the slope of the regression line and established for retinol and alpha-tocopherol. Six concentration levels were measured six times in the range of 0.0558–7.0 μmol/L for retinol and 0.42–50.0 μmol/L for alpha-tocopherol using Kinetex C_{18} (100 Å, 2.6 μm, 100 mm × 4.6 mm) and calibration curve was constructed for both analytes. The resulting plot slope (a), intercept (b) and determination coefficient (R^2) were calculated from the regression equation using Microsoft Excel 2003. Linear regression equations and determination coefficients obtained from software are shown in Table 3.

Accuracy was expressed as the closeness of agreement between a true value and an actual value found. Accuracy of the method was calculated as the recovery determined from three measurements of three levels of spiked breast milk (0.92, 1.91, 3.87 μmol/L of retinol standard and 7.29, 15.29, 28.61 μmol/L of alpha-tocopherol standard).

Precision was expressed as the closeness of agreement between a series of measurements obtained from multiple aliquots of a homogenous sample under prescribed conditions. Repeatability was expressed as the relative standard deviation (RSD) of peak area and retention time calculated from data which were obtained by analyzing of six samples of human milk.

Limit of detection (LOD) was the lowest amount of analyte in a sample that could be detected but not necessarily quantitated as

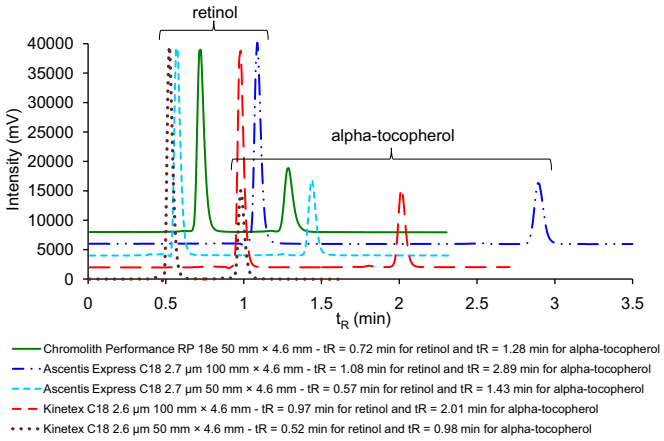

Fig. 5. Comparison of columns under chromatographic conditions: injection volume 20 μl, flow rate 1.5 mL/min, mobile phase 100% methanol, analysis temperature 25 °C, detection 295 nm for alpha-tocopherol and 325 nm for retinol.

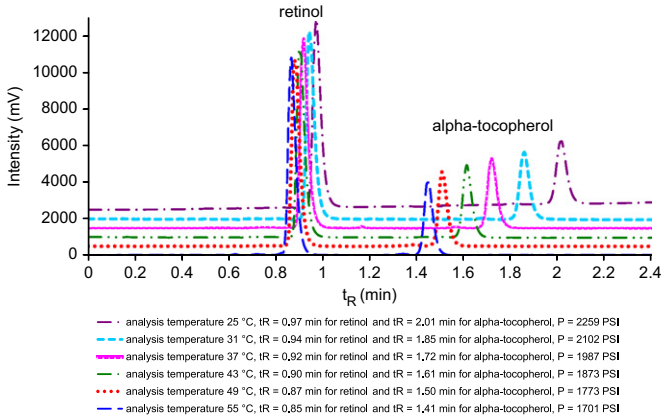

Fig. 6. Evaluation of different temperatures under chromatographic conditions: Kinetex C_{18} 2.6 μm 100 mm × 4.6 mm, injection volume 5 μl, flow rate 1.5 mL/min, mobile phase 100% methanol, detection 295 nm for alpha-tocopherol and 325 nm for retinol.

Table 3
Comparison of validation parameters for Kinetex C_{18} and Chromolith Performance RP-18e columns.

Column	Chromolith RP-18e (100 × 4.6 mm)[a]		Kinetex C_{18} (100 × 4.6 mm)[b]	
Compound	Retinol	Alpha-tocopherol	Retinol	Alpha-tocopherol
Calibration range (μmol/L)	0.25–10.00	0.5–50	0.0558 – 7.0	0.42–50.0
Regression equation	y=24,488.31x+7277.149	y=1380.7x–85.139	y=10,479.78x–140.03	y=566.89x–17.86
Determination coefficient R^2[c]	0.9998	0.9999	0.9999	0.9999
LOD (μmol/L)	0.13	0.09	0.004	0.078
LOQ (μmol/L)	0.27	0.19	0.012	0.182
Repeatability (% RSD)				
Of t_R	–	–	0.32[d]	0.19[d]
Of peak area	–	–	0.25[d]	0.98[d]
Of t_R	0.41[e]	0.83[e]	0.22[f]	0.12[f]
Of peak area	5.65[e]	5.51[e]	6.94[f]	1.75[f]
Accuracy % R (% RSD)				
Level 1	93.9 (5.65)[g]	73.4 (5.51)[g]	116.3 (1.87)[h]	99.0 (2.12)[h]
Level 2	–	–	115.2 (3.43)[h]	103.8 (3.05)[h]
Level 3	–	–	114.1 (1.51)[h]	108.6 (1.14)[h]

[a] Validation parameters of reference method with monolithic column [43].
[b] Validation parameters of developed method with core–shell column.
[c] Linearity was measured in six concentration levels.
[d] RSD of retention time and peak area repeatability of standard calibration solution (retinol c=0.98 μmol/L and alpha-tocopherol c=7.0 μmol/L).
[e] Retention time and peak area repeatability of lyophilized human serum [43].
[f] Retention time and peak area repeatability of breast milk measured six times.
[g] Accuracy of chromsystems control serum, n=3 [43].
[h] Accuracy of spiked breast milk, n=3, each level measured in triplet. R: recovery.

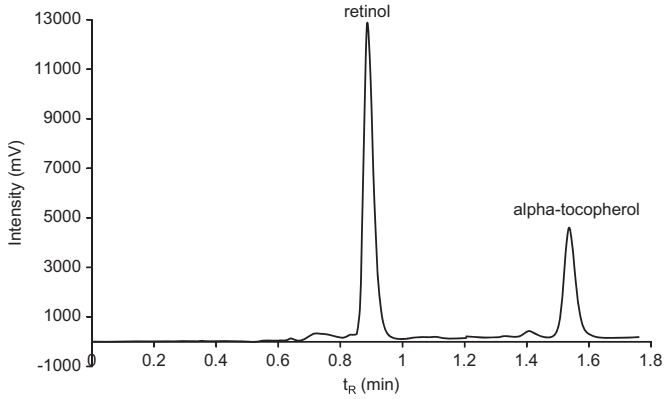

Fig. 7. Chromatogram of retinol (t_R=0.88 min, c=2.63 μmol/L) and alpha-tocopherol (t_R=1.53 min, c=20.36 μmol/L) extracted from breast milk and analyzed by Kinetex C_{18} column (2.6 μm, 100 Å, 100 mm × 4.6 mm) and chromatographic conditions: flow rate 1.5 mL/min, injection volume 5 μL, temperature 49 °C, mobile phase 100% methanol, detection 295 nm for alpha-tocopherol and 325 nm for retinol, total time of analysis 1.75 min.

an exact value. LOD were based on signal-to-noise ratio (3:1) and expressed as the concentration of analytes. Limit of quantification (LOQ) was the lowest amount of analyte in sample that could be quantitatively determined with suitable precision and accuracy. LOQ were generally calculated from a determination of signal-to-noise ratio (10:1) and expressed as the concentration of analytes.

There was a significant improvement in repeatability and recovery after liquid–liquid extraction from breast milk and the limits for retinol and alpha-tocopherol RSD < 15% and recovery 100 ± 25% were fulfilled. All results of method validation are shown in Table 3 [38,40].

4.4. Method application

The newly developed HPLC method using Kinetex C_{18} (100 Å, 2.6 μm, 100 mm × 4.6 mm) column was applied for determination of retinol and alpha-tocopherol in samples of breast milk in order to monitor the nutritional status prior to application for newborn (Fig. 7). Moreover, the method can be employed for determination of liposoluble vitamins in various biological matrices such as serum, cholesterol fractions and erythrocyte membrane for monitoring of antioxidant activity in patients with atherosclerosis and during anticancer therapy.

5. Conclusion

The main idea of this work was to develop a novel HPLC method for determination of liposoluble vitamins retinol and alpha-tocopherol in breast milk with better separation parameters, moreover low-cost, in comparison with reference method using monolithic column [18,43]. Great emphasis was placed on reducing analysis time and mobile phase consumption.

Five selected columns with monolithic and core–shell stationary phases, Chromolith Performance RP-18e 50 mm × 4.6 mm, Kinetex C_{18} core–shell columns (100 Å, size of shell particles 2.6 μm, 100 mm × 4.6 mm and 50 mm × 4.6 mm) and Ascentis Express C_{18} fused-core columns (100 Å, 2.7 μm, 100 mm × 4.6 mm and 50 mm × 4.6 mm) were tested and compared with Chromolith Performance RP-18e 100 mm × 4.6 mm column.

Using core–shell technology improved the method validation parameters and also the consumption of sample and organic solvents were reduced if compared with the reference method using monolithic column [43]. Kinetex C_{18} (100 Å, 2.6 μm, 100 mm × 4.6 mm) column with flow rate 1.5 mL/min of 100% methanol was finally chosen for this analysis. The temperature was increased from 25 °C to 49 °C, which had a positive effect on the pressure. The injection volume from 20 μL to 5 μL and mobile phase consumption for 100 samples batch from 450 to 263 mL were decreased in comparison with the reference method using monolithic column Chromolith Performance RP-18e (100 mm × 4.6 mm). Calibration ranges for retinol and alpha-tocopherol were extended towards lower concentration values being from 0.056 to 7.0 μmol/L and from 0.42 to 50.0 μmol/L, respectively. Employing the liquid–liquid extraction of vitamins from breast milk brought significant improvement in the repeatability and recovery and helped to fulfill the limits demanded by FDA.

The newly developed HPLC method was used for analysis of retinol and alpha-tocopherol in 50 real samples of breast milk.

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