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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