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Quantification of selected fat soluble vitamins and carotenoids in infant formula and dietary supplements using fast liquid chromatography coupled with tandem mass spectrometry

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ABSTRACT

A sensitive and fast analytical method has been developed and validated for the simultaneous identification and quantification of nine fat soluble vitamins and their derivatives (retinol, retinyl acetate, retinyl palmitate, cholecalciferol (D3), α-, β-, γ-, and δ-tocopherol, α-tocopheryl acetate) along with three carotenoids (β-carotene, lutein and zeaxanthin) in dietary supplements and infant formulas using a fast liquid chromatography (UFLC) system coupled with tandem mass spectrometry. Separation was performed within 15 min on a YMC C_{30} column (100 mm × 2 mm I.D., 3 μm) using methanol:water (90:10 v/v) and *tert*-butyl methyl ether (TBME):methanol (80:20 v/v) as the mobile phase with gradient elution. Quantification was carried out by multiple reaction monitoring in combination with information dependent acquisition (IDA) using atmospheric pressure chemical ionization (APCI) in the positive mode. Enhanced product ion scan was used and the mass spectrum of each target compound confirmed their presence in the analyzed extracts. Extraction recovery for all analytes spiked at two levels ranged from 90% to 105%. The overall intra-day precision values for the method based on the relative standard deviation for three QC levels were below 6% for all analytes. The accuracy ranged from 87.6% to 113.5%. A selection of multivitamin supplements and infant formulas available on the Canadian market was analyzed and the results showed good agreement with the values declared on the labels.

1. Introduction

Vitamins are a chemically heterogeneous class of compounds which play an important role in human nutrition. They are classified into water-soluble, including the vitamin B complex and vitamin C, and fat-soluble vitamins (FSVs) comprising of vitamins A, D, E and K. In foods, FSV can be detected in different chemical forms with differing biological activity. A balanced diet should be sufficient to provide the required amounts of vitamins to the body. However, due to losses during processing and storage, and also because certain population groups are in need of higher vitamin intakes, various multivitamin supplements have appeared in the marketplace. Although vitamins are essential nutrients, supplementary dietary intake of vitamins may result in adverse chronic and acute effects such as osteoporosis and hip fracture which resulted from hypervitaminosis A (Penniston & Tanumihardjo, 2006). Standardized testing to assess content uniformity and to describe cross-product variation among the existing formulations is essential for regulatory bodies and industry. Furthermore, the regulatory requirements for precise labeling on infant foods fortified with vitamins and dietary supplements require reliable analytical methods for the quantitative analysis of vitamins in foods.

Different methods have been reported for the analysis of FSVs in various types of matrices including food and feed products, biological samples, and pharmaceuticals. Most methods are based on high-performance liquid chromatography (Escriva, Esteve, Farre, & Frigola, 2002; Gentili et al., 2012; Mendoza, Pons, Bargallo, & Lopez-Sabater, 2003) but only a few were developed for the simultaneous determination of FSVs. In a recent review on methods of FSV analysis, the need for developing analytical methods to identify and quantify several vitamins and related compounds such as carotenoids in a single chromatographic run was emphasized (Blake, 2007). However, difficulties exist for the simultaneous determination of FSV. First, vitamins are present as structurally related vitamers and synthetic forms, which require highly efficient separation methods. Second, concentrations of different vitamins vary greatly even in one sample, which requires analytical methods with high sensitivity and selectivity and applicable for large dynamic range. Moreover, the large number of components present in foods and multivitamin dietary supplements implies the risk of interference.

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Simultaneous quantification of vitamins A, D_3 and E in fortified infant formula has been achieved using liquid chromatography coupled with atmospheric pressure chemical ionization mass spectrometry (LC-APCI-MS) in the positive ion mode. The sample was saponified and FSVs were concentrated using solid-phase extraction (SPE). The extracted vitamins were separated using normal-phase stationary phases and detected with selected ion monitoring (SIM) mass spectrometry (Heudi, Trisconi, & Blake, 2004). However, reversed-phase systems are preferred over normal-phase HPLC due to their improved column stability. A procedure for the determination of encapsulated vitamin A acetate, vitamin A palmitate, vitamin E acetate, vitamin D_3 and vitamin K was developed by Stary, Cruz, Donomai, Monfardini, and Vargas (1989). FSVs were extracted into hexane and separated by HPLC on a reversed-phase C_{18} phase with ultraviolet (UV) detection. For physiological samples such as human plasma, vitamins A, E, 25(OH) D_2 and 25(OH) D_3 were separated on a RP-C_{18} column with gradient elution and all compounds were eluted in less than 15 min (Alvarez & De Mazancourt, 2001). Vitamins A, E and β-carotene in bovine milk were simultaneously separated on RP-C_{18} column using methanol:water solvent system in about 30 min (Plozza, Trenerry, & Caridi, 2012) but the separation of vitamin D_3 was not included and reported as a separate method (Trenerry, Plozza, Caridi, & Murphy, 2011). Another LC/MS method was described for the determination of vitamin A, vitamin A acetate, vitamin A palmitate, vitamin E (α-tocopherol), vitamin E acetate, and coenzyme Q10 in multivitamin dietary supplements using a RP-C_{30} stationary phase. This C_{30} stationary phase was shown to outperform RP-C_{18} phases in the separation of geometric and positional isomers of tocopherols. The vitamins were extracted with light petroleum, ethyl acetate and methanol without saponification and quantified by LC-(APCI)-MS Coupling to APCI-MS provided high sensitivity and selectivity and excluded possible interferences in these complex samples. This was also the first application of LC-APCI-MS analysis for vitamin A and vitamin E in multivitamin supplement capsules and tablets on a C_{30} column, but vitamin D quantification was not included in this study (Breithaupt & Kraut, 2006).

Ultra-performance liquid chromatography (UPLC) was used for the determination of FSVs. Citova et al. (2007) described a UPLC-DAD method to analyze retinol and α-tocopherol in human serum. Paliakov et al. (2009) developed a rapid quantitative UPLC method with UV detection to determine vitamins A, E (α- and γ-tocopherol), β-carotene and coenzyme Q10 in human serum. The separation was achieved using C_{18} columns with a run time of 2 min.

Developing fast and reliable analytical methods to determine FSVs is of great importance in terms of nutritional and regulatory perspectives. Therefore, the objective of this work was to establish a sensitive and selective analytical method using fast liquid chromatographic system coupled to APCI-MS/MS for the simultaneous separation and quantification of selected FSV and carotenoids in dietary supplements and infant formulas.

2. Experimental

2.1. Chemicals

Methanol, *tert*-butyl methyl ether (TBME), *n*-hexane, ethyl acetate, ethanol, and HPLC-grade water were purchased from Fisher Scientific (Ottawa, ON, Canada). Retinol (>99.0% purity), retinyl acetate (~90.0% purity), retinyl palmitate, (±)-α-tocopheryl acetate (>96.0% purity), cholecalciferol (>98.0% purity), and butylated hydroxy toluene (BHT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Vitamin E derivatives [(±)-α-tocopherol (≥95.0% purity), β-tocopherol (≥95.0% purity), γ-tocopherol (≥95.0% purity), and δ-tocopherol (≥95.0% purity)] were purchased from Calbiochem (Darmstadt, Germany). β-Carotene (96.0% purity), zeaxanthin (97% purity), and lutein (96% purity) were from CaroteNature (Lupsingen, Switzerland).

2.2. Samples

Three different brands of commercial infant formulas and four different multivitamin supplements were purchased at a local health food store (Edmonton, AB, Canada). The infant formula and multivitamin supplement are mixtures of water soluble and fat soluble vitamins, and minerals. All samples were stored at 21 °C in their original package and analyses were completed within two weeks.

2.3. Extraction of fat soluble vitamins

2.3.1. Dietary supplements

All multivitamin supplements were in tablet form. For sample preparation, 15–20 tablets were ground using a small grinder until a fine powder was obtained and stored in sealed glass bottles covered with aluminum foil. Quantities of 200 mg were accurately weighed into a glass testing tube covered with aluminum foil to protect samples from light. For the extraction of FSVs, hexane:ethyl acetate (9:1, v/v, with 0.01% butylated hydroxytoluene (BHT)) was used (Kamao et al., 2007). After addition of 5 mL of the extraction solvent, the sample was mixed thoroughly for 30 s using a vortex mixer and placed in an ultrasonic bath for 15 min at 21 °C. After centrifugation at 3000 rpm for 5 min, the organic layer was transferred to a 50 mL round flask, and another 5 mL of extraction solution was added and the above extraction procedure was repeated. In total, four extractions were performed and the combined organic extracts were evaporated under nitrogen. The residue was dissolved in methanol and made up to 5 mL in a volumetric flask. The extract was then filtered with a 0.2 μm nylon syringe filter (Mandel Scientific, Guelph, ON, Canada) and stored in nitrogen purged amber glass vials at −20 °C until analysis. To protect the samples from light during extraction, all glassware used was covered with aluminum foil and dim light conditions were used. Amber glass vials were used for storage and analysis purposes.

2.3.2. Infant formula

Extraction was carried out according to the procedure described by Huang, LaLuzerne, Winters, and Sullivan (2009) with minor modifications. For this purpose, 10 g of infant formula was accurately weighed into an Erlenmeyer flask covered with aluminum foil. Subsequently, 40 mL of ethanol with 2% pyrogallol and 20 mL of 50% aqueous KOH solution were added, and the sample was mixed thoroughly, purged with nitrogen and left for overnight saponification at room temperature using a mechanical shaker at a speed of 200 rpm. For the extraction of FSVs, approximately 30 mL of hexane stabilized with 0.01% BHT was added to the mixture. The sample was allowed to shake for another 30 min. The organic upper phase was transferred to a round bottom flask and the lower phase was extracted three more times with hexane. The organic upper phases were combined and dried under nitrogen. The residue was dissolved in methanol and made up to 5 mL in a volumetric flask. The samples were then filtered as described above and stored in amber glass vials at −20 °C.

2.4. Instrumentation and chromatographic conditions

2.4.1. UFLC-DAD

Samples were analyzed using a Shimadzu UFLC-XR system (Shimadzu, Kyoto, Japan) equipped with a DGU-20A3 degasser, LC-20AD XR binary pump, SIL-20AC XR autosampler, CTO-20AC column oven, a SPD-M20A diode array detector, and a communication bus module CBM-20A. The autosampler temperature was maintained at 15 °C during analyses. The separation of analytes was performed on a polymeric YMC C_{30} reversed-phase column, 100 mm × 2.0 mm I.D. and 3 μm particle size (YMC America, Allentown, PA, USA) operated at 21 °C. The injection volume was 3 μL. The composition of mobile phase A was methanol:water (90:10, v/v); mobile phase B consisted of TBME:methanol (80:20, v/v). The gradient program was 0–8 min