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Simultaneous quantification of Vitamins A, D_3 and E in fortified infant formulae by liquid chromatography–mass spectrometry

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Abstract

A novel method for the simultaneous quantification of Vitamins A, D_3 and E in fortified infant formulae has been developed using isocratic normal-phase liquid chromatography with positive atmospheric pressure chemical ionization mass spectrometry (LC–APCI-MS). Food products were saponified and the vitamins were extracted by solid-phase extraction (SPE) on a Chromabond XTR® cartridge. Quantification of Vitamins D_3 and E were performed with Vitamin D_2 and 5,7-dimethyltocol (DMT) as internal standards (IS), respectively while no IS was used for Vitamin A. Detection of the vitamins was made in the selected ion monitoring (SIM) mode. MS calibration curves were linear between 0.15 and 12 mg/l for Vitamin A, 5–400 μg/l for Vitamin D_3 and 0.25–20 mg/l for Vitamin E with regression coefficient $r^2 > 0.996$ and the limits of detection were below 1.4 ng. The repeatability (CV) obtained on a reference dietetic infant formula was 2.3% for Vitamin A, 2.6% for Vitamin E and 5.9% for Vitamin D_3. The between-day variations (CV) over 6 days were in the ranges of 2.4–6.9% for the three vitamins. The mean recoveries from a reference infant formula spiked with all three vitamins ranged from 96 to 105% with a relative standard error less than 9%. The applicability of the method was demonstrated by analyzing a set of infant formula and infant cereals; similar results were obtained with the LC–MS method and reference HPLC methods.

Keywords: Food analysis; Vitamins

1. Introduction

Fat-soluble vitamins have important roles in several functions of the human body, such as vision (Vitamin A), calcium absorption (Vitamin D) and antioxidative protection in cell membranes (Vitamin E). Reliable, sensitive and rapid methods for fat-soluble vitamins determination in fortified food products are essential for nutritional reasons. Currently, Vitamins A and E are determined in food products by HPLC with UV and fluorescence detections, respectively [1,2] while Vitamin D_3 is analyzed separately by HPLC-UV after a preparative chromatography step [3]. In recent years, however, there has been particular emphasis on simultaneous analysis of fat-soluble vitamins especially with HPLC techniques. Using this approach, HPLC with UV detection has been applied for the simultaneous determination of Vitamins A, D_3 and E in the plasma of cattle [4] and humans [5–8] after proteins precipitation and liquid–liquid extraction. Application of this technique to food analysis is not straightforward, as the presence of high fat content and several other compounds in the products requires a sample preparation that involves saponification and isolation of the fat-soluble vitamins. Nevertheless, several authors have used HPLC coupled with either UV or electrochemical detection for simultaneous determination of Vitamins A, D_3 and E in animal feeds [9], pharmaceutical preparation [10] and milk products [11–14]. Although these techniques were selective and sensitive enough to determine low levels of fat-soluble vitamins in the products tested, the extension of the method to complex food matrices remains problematical. In this context, for the unambiguous identification of fat-soluble vitamins, MS is beginning to be recognized as an advantageous detection system.

Because of their poor volatility, most of the fat-soluble vitamins are not suitable for determination by GC–MS. Particle beam LC–MS has been used to identify Vitamins A and E in infant formula powder [15] as well as for the simultaneous determination of Vitamins A, D_3 and E in pharmaceutical

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preparations [16]. However, this technique is not applicable to a wide range of matrices. The lack of protonation sites on fat-soluble vitamins makes their analyses by LC–MS with electrospray ionization difficult, however, reacting the vitamins with silver ions enhances the sensitivity of the method [17,18]. LC–MS with APCI appears to be a promising alternative method. Several authors have used this technique for the analysis of individual vitamins such as: Vitamin A in human plasma [19,20] and in rat tissues [21,22] and Vitamin E in human plasma [23,24]. In the food area, only a few authors reported the use of LC–APCI-MS for vitamins analysis, for example, Huck et al. [25] have used the method to monitor carotenoids content in vegetables and Vitamin D_3 has been quantified in multi-vitamin tablets [26]. More recently we have applied the technique to monitor Vitamin E (α-tocopherol) in infant formulae [27]. To our knowledge, no studies about the simultaneous quantification of Vitamins A, D_3 and E by LC–APCI-MS have been published so far. The aim of this study was to develop and validate a LC–MS method for the simultaneous quantification of Vitamins A, D_3 and E in milk-based infant formulae and infant cereals.

2. Experimental

2.1. Chemicals

Vitamin A (all-*trans*-retinol), Vitamin D_2 (ergocalciferol), Vitamin D_3, Vitamin E (α-tocopherol), takadiastase, sodium 1-pentanesulfonate, sodium sulphide, sodium ascorbate, potassium hydroxide and butylated hydroxytoluene (BHT, 2,6-di-*tert*-butyl-p-cresol) were obtained from Fluka (Buchs, Switzerland). 5,7-Dimethyltocol (DMT) was obtained from Matreya Inc. (Pleasant Gap, PA, USA). Vitamin A_2 was a generous gift from Hofmann (Hofmann La Roche, Basle, Switzerland). 13-*cis*-Retinol was purchased from Sigma. HPLC-grade *n*-hexane, ethanol, 2-propanol and dioxan were obtained from Merck (Geneva, Switzerland). Water was purified using a Milli-Q system from Millipore (Le Mont-sur-Lausanne, Switzerland).

2.2. Preparation of standards

Since fat-soluble vitamins are light sensitive, precautions were taken to minimize exposure of the extracted solutions to daylight by using either amber glassware or ordinary glassware protected with aluminium foil. Approximately 40–50 mg each of Vitamins A, A_2, D_2, D_3, E, 13-*cis*-retinol and DMT were dissolved in ethanol and made up to 100 ml as separate stock solutions. Then, 10 ml of Vitamin A and 13-*cis*-retinol stock solutions, 5 ml of Vitamin E stock solution, and 100 μl of Vitamin D_3 stock solution were added to a 100 ml volumetric flask, the solvent was evaporated and the residue was dissolved in 100 ml of the mobile phase. The latter solution was then diluted with the mobile phase at several concentration levels and spiked with appropriate amount of the internal standards Vitamin D_2 and DMT. Eight calibration points with a concentration ranging from 0.15–12 mg/l (Vitamin A), 5–400 μg/l (Vitamin D_3) and 0.25–20 mg/l (Vitamin E) were used to determine the linearity of the method for each vitamin.

2.3. Samples and reference materials

Six commercial powdered dietetic infant formulae and infant cereals, whose fat contents were about 25%, were used. One certified reference material (milk powder, SRM/RM 1846) from National Institute & Standard Technology (NIST, Gaithersburg, MD, USA) and one Nestlé reference material (NRM) a dietetic milk powder (DDP2) of well-characterized vitamins content were also used.

2.4. Sample preparation

2.4.1. Saponification

The saponification procedure was similar to that described in the official CEN methods for Vitamins A, D_3 and E determination [1–3]. Briefly, a well-homogenized food sample (50 g) was dissolved in 100 ml of warm distilled water (40 °C) and thoroughly mixed to obtain homogeneous slurry. A portion of the above mixture (30 g) was accurately weighed into a 250 ml Erlenmeyer flask and spiked with the internal standard solution containing Vitamin D_2 and DMT. For starch containing products, about 0.2 g of takadiastase was added and the solution was incubated for 30 min at 45 °C. To the latter solutions, were added the following reagents under agitation: 7 g of potassium hydroxide, 50 ml of ethanol, 1 g of sodium sulphide and 1 g of sodium ascorbate. The solution was mixed under a nitrogen steam and then heated under reflux at 85 °C for 30 min. After cooling, the solution was quantitatively transferred in a 100 ml volumetric flask and 2 g of sodium 1-pentanesulfonate were added under agitation. The solution was made up to 100 ml volume with water. Because the method that we applied for the sample preparation was based on a validated CEN method we did not want to change too much the procedure thus, we did not explore into details the function of reagent such as sodium 1-pentanesulfonate and sodium sulphide, added to the sample preparation.

2.4.2. Extraction

The CEN methods [1–3] describe solvent-extraction procedures to isolate the fat-soluble vitamins from the saponified products. However the solid-phase extraction (SPE) was found to be considerably more rapid and efficient and was preferred. The choice of cartridge type is critical and a Chromabond XTR®, 70 ml cartridge (Macherey-Nagel, Düren, Germany) gave the highest and most reproducible recoveries. Twenty milliliters of the saponified sample solution were poured onto a Chromabond cartridge and allowed to absorb for 15 min. The vitamins were eluted, using 100 ml of *n*-hexane (containing 5 mg of BHT). The eluate

was evaporated under a stream of nitrogen and the residue was dissolved in 4 ml of HPLC mobile phase. When the concentration of either Vitamin A or E was found to be too high the extract was diluted appropriately with the HPLC mobile phase. The final solution was filtered through a 0.45 μm membrane filter (Millipore, Bedford, MA, USA).

2.5. Liquid chromatography

Sample injections of 40 μl were made from an Agilent 1100 Series auto-sampler (Agilent Technology Inc., Urdorf, Switzerland). LC analyses were performed with an Agilent 1100 HPLC system using an UV-diode array detector. The samples were separated isocratically on a silica-based column (Nucleosil 100-5, 250 mm × 4.6 mm i.d., Macherey-Nagel, Düren, Germany) at a flow rate of 1.45 ml/min with a mobile phase of hexane/dioxan/2-propanol (96.7:3:0.3, v/v/v).

2.6. Mass spectrometry

Analysis of LC flow was performed on-line by a mass spectrometry detector (MSD) system (Agilent Technology Inc., Urdorf, Switzerland) equipped with an APCI source. The optimum operating conditions were found to be 200 V for the fragmentor voltage, 5 l/min for the drying gas flow, 60 psig for the nebulizer pressure, 350 °C for both drying gas and vaporizer temperatures. The capillary voltage and corona current were set to 3000 V and 4.0 μA, respectively. SIM was used for the quantification of vitamins and the ions monitored were as follows: *m/z* 269.1 for Vitamin A and 13-*cis*-retinol, *m/z* 367.2 for Vitamin D_3, *m/z* 379.1 for Vitamin D_2, *m/z* 431.2 for Vitamin E and *m/z* 417.2 for DMT.

2.7. Statistics

The repeatability and intermediate reproducibility of the method were evaluated using an in-house statistical program making use of the robust-statistics concept of Rousseew and Croux [28].

3. Results and discussion

3.1. Mass spectrometry of Vitamins A, D_3 and E in APCI mode

Because fat-soluble vitamins lack protonation sites, the sensitivity obtained by electrospray ionization is insufficient for their detection in food products, particularly for Vitamin D_3. It is not possible to apply UV detection for the simultaneous determination of fat-soluble vitamins since the sensitivity and selectivity at wavelength below 280 nm is inadequate. To overcome these difficulties, MS detection after APCI was applied. The most suitable was the positive APCI mode giving the spectra for Vitamins A, D_3, E and Vitamin D_2, and DMT displayed in Figs. 1–3.

The mass spectrum of Vitamin A shows a predominant protonated molecule at *m/z* 269.1 which can be assigned

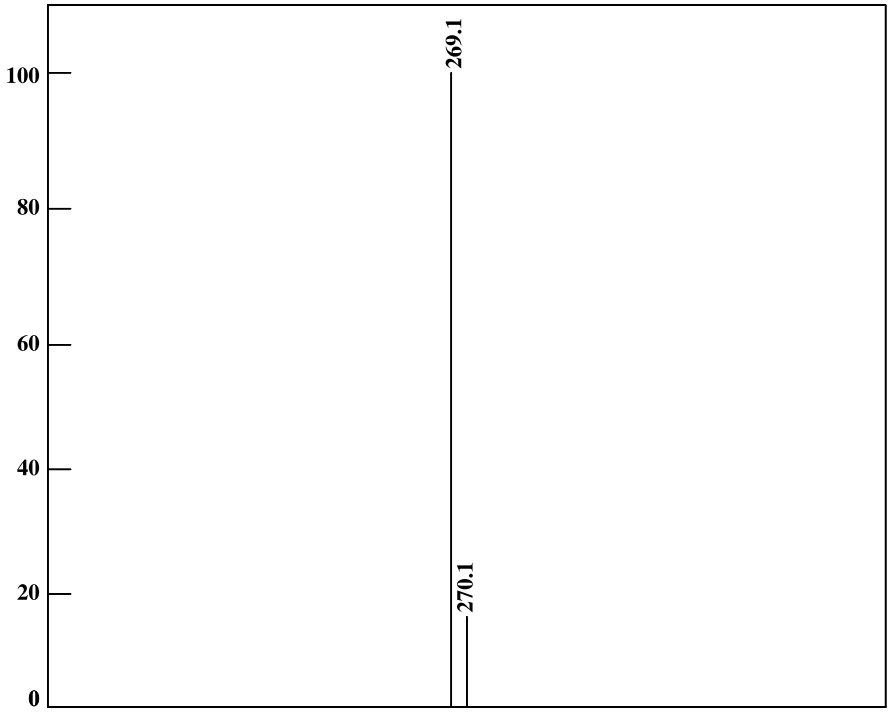

Fig. 1. Mass spectra of Vitamin A in positive APCI mode.

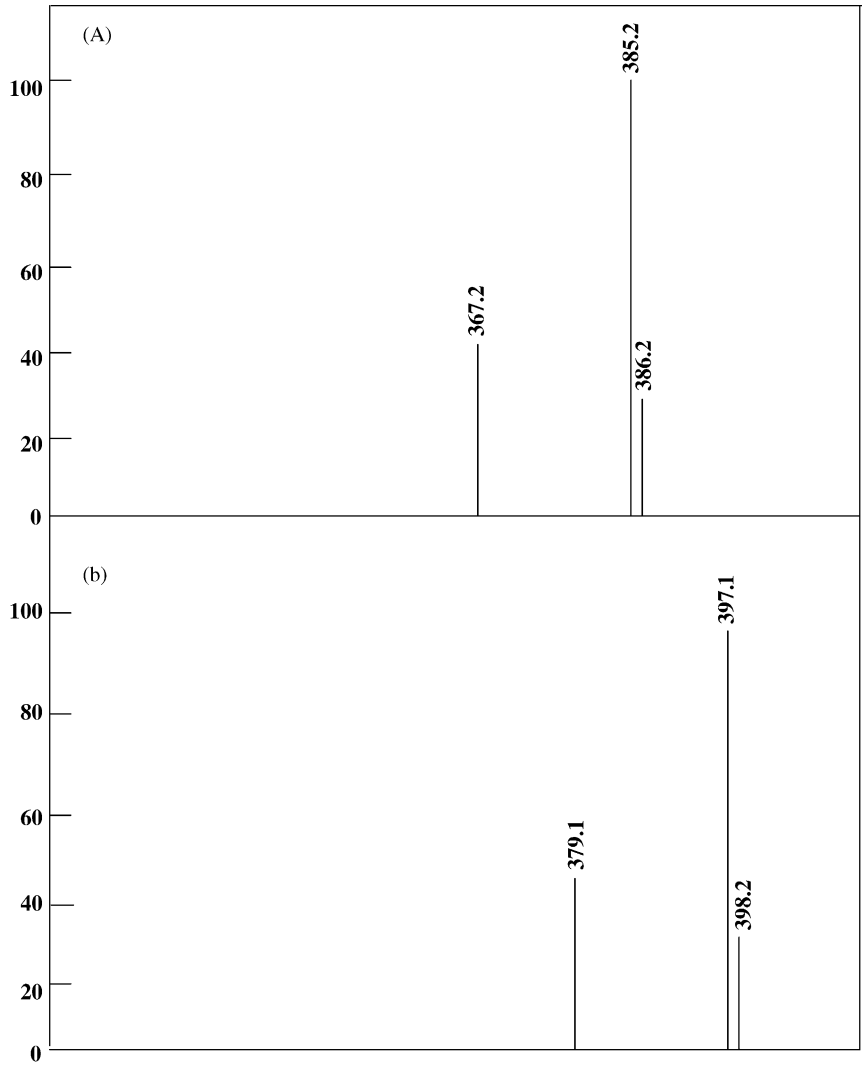

Fig. 2. Mass spectrum of (A) Vitamin D_3 and (B) Vitamin D_2 in positive APCI mode.

to $[M–H_2O + H]^+$, under our experimental conditions no parent ion was found (Fig. 1). The mass spectrum of Vitamin D_3 yielded a protonated molecule $[M + H]^+$ at *m*/*z* 385.2 and a fragment $[M–H_2O + H]^+$ at *m*/*z* 367.2 (Fig. 2A). Similarly, Vitamin D_2 gave signals at *m*/*z* 397.1 and 379.1 (Fig. 2B). The identical pattern found by MS between the signals of Vitamins D_3 and D_2 justify the choice of D_2 as IS for Vitamin D_3 analysis. The full scan spectrum of Vitamin E displayed predominant cluster ions at *m*/*z* 429–432 (Fig. 3A) and a minor ion at *m*/*z* 165 (not shown), which is attributed to the loss of the phytyl chain. Analogously, DMT yielded predominant cluster ions at *m*/*z* 416–418 (Fig. 3B) and a minor ion at *m*/*z* 151 (not shown). Although the protonated molecular ion $[M + H]^+$ of Vitamin E gives a mass of 431 we found in APCI mode three additional ions at *m*/*z* 429, 430 and 432. The presence of these ions have been also found by Lauridsen et al. [23] when using APCI to monitor Vitamin E and they attributed their formation to the electron oxidation by the corona discharge in the source and to the protonation and dehydrogenation of Vitamin E. The same mechanism can also explain the pattern found in the MS spectrum of DMT.

3.2. LC–MS analysis of Vitamins A, D_3 and E in fortified food products

The LC separation obtained with the silica column using APCI-MS detection in the isocratic mode provides a good separation of all vitamins in a standard mixed solution except for Vitamins D_2 and D_3, which co-elute. However, the use of the SIM signal at *m*/*z* 385.2 and 397.1 (parent ions) or at *m*/*z* 367.2 and 379.1 (fragment ions) allowed the deconvolution of the two peaks, and thus to discriminate between the Vitamins D_2 and D_3 peaks (Fig. 4). The use of isocratic conditions offers the advantage that the mobile phase composition is constant during the analysis, thus the vitamins and their respective internal standards are ionized under the same conditions.

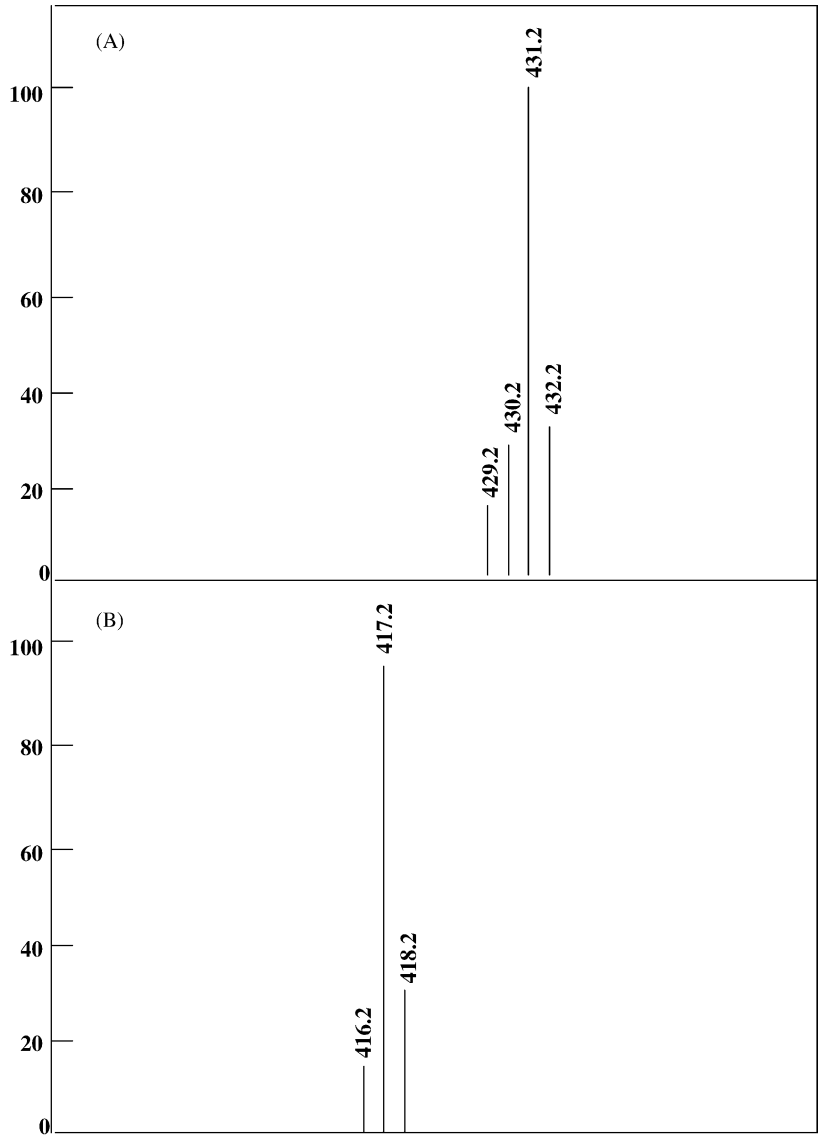

Fig. 3. Mass spectrum of (A) Vitamin E and (B) 5,7-dimethyltocol in positive APCI mode.

In order to demonstrate the applicability of our LC–MS method to the simultaneous identification of fat-soluble Vitamins, several fortified food products were analyzed. Prior to LC–MS analysis the products were saponified and purified on a Chromabond XTR cartridge. The use of more conventional SPE cartridges such as C_{18} or Oasis HLB (Waters, Milford, MA, USA) was problematical since the retention and elution conditions for each Vitamin were difficult to optimize for the whole range of matrices tested (data not shown). In addition, the polar solvents were not suitable to completely dissolve all the fat-soluble vitamins present in the residue after the SPE procedure particularly for Vitamins D_3 and E. The SIM chromatograms of fat-soluble vitamins obtained for the products tested were similar thus, an example of reference material DDP2 chromatogram is shown (Fig. 5). It can be seen that the SIM signal is devoid of interferences for the predominant ion at *m*/*z* 431.2 (Vitamin E), 417.2 (DMT) and for the ion at *m*/*z* 269.2 (Vitamin A). Total Vitamin A in food products is usually calculated as the sum of all-*trans*- and 13-*cis*-retinols. It is noteworthy that 13-*cis*-retinol which is an isomer of Vitamin A (all-*trans*-retinol) exhibits about 75% of the activity of the all-*trans* form. Under our experimental conditions, the MS spectra of Vitamin A and that of 13-*cis*-retinol displayed the same protonated ion at *m*/*z* 269.1, but the retention time (Rt) of these two vitamins were different as it is shown in Fig. 5. The SIM analyses of Vitamins D_2 and D_3 using their predominant ions was not applicable because of peaks interfering with the signal at *m*/*z* 385.2 and 397.1. A separate MS-MS analysis showed that the interfering peaks were due to the presence of a high content of sterols in these products whose spectra reveal the presence of fragment ions at *m*/*z* 385.2 and 397.1. Thus, the fragment ions at *m*/*z* 367.2 and 379.1, for Vitamins D_3 and D_2, respectively were used

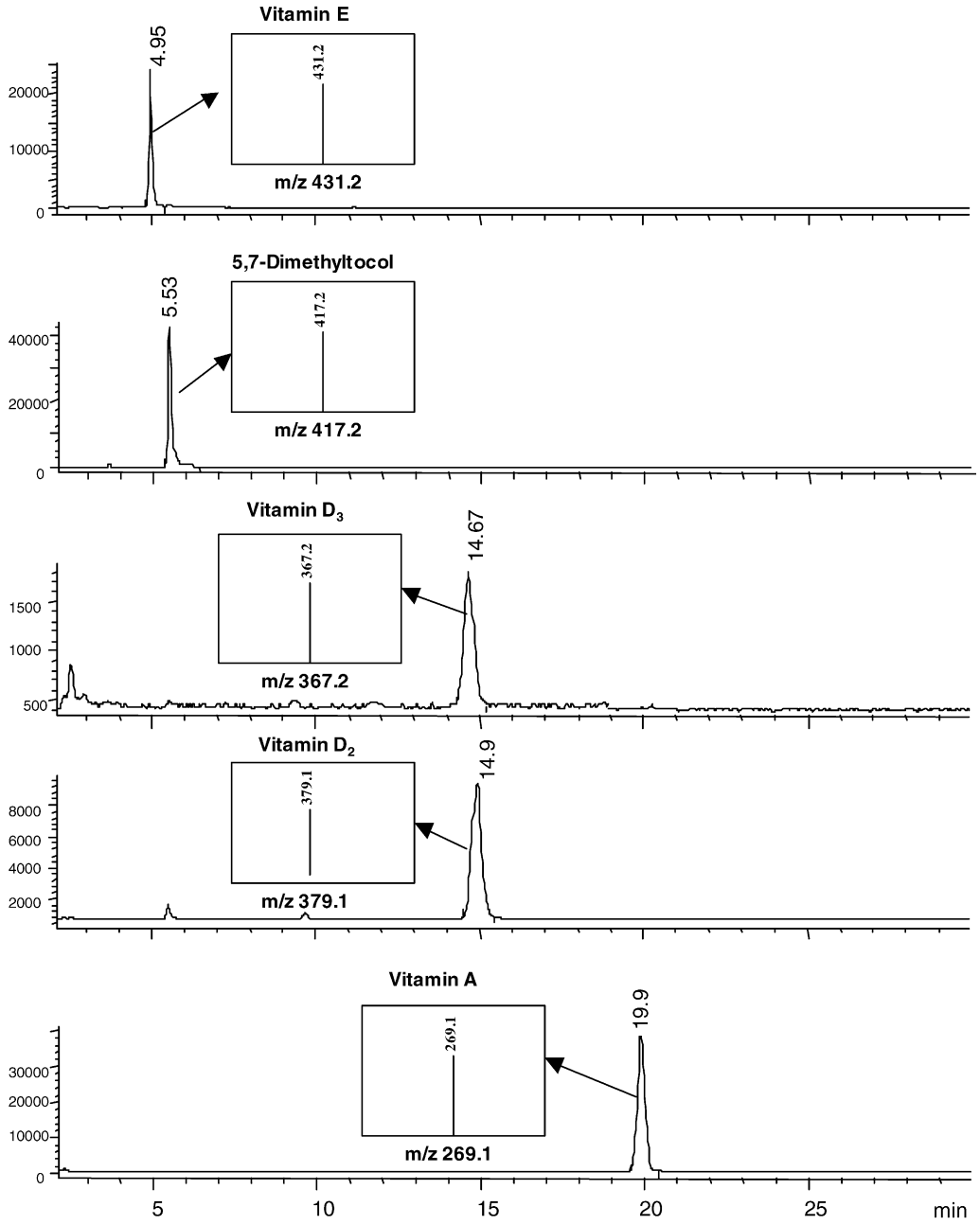

Fig. 4. SIM chromatograms obtained from a standard solution of: Vitamin E (2.5 mg/l), 5,7-dimethyltocol (5 mg/l), Vitamin D_3 (50 μg/l), Vitamin D_2 (250 μg/l) and Vitamin A (1.5 mg/l). For LC–MS conditions, see Section 2.6.

for their analysis as no interferences were found. Some of these sterols were also present in the SIM chromatograms of Vitamins D_2 and D_3 obtained at *m/z* 367.2 and 379.3 in food samples (Fig. 5) but with Rt different from the desired vitamins. Consequently, comparing the Rt with those of the standards, and obtaining MS spectra allows the identification of each vitamin in different products.

3.3. Internal standards linearity and limit of detection

The quantification of fat-soluble vitamins by HPLC is usually performed by the external standardization method. However the use of an internal standard (IS) is desirable to compensate for any analyte loss occurring during sample preparation steps or from signal variation during LC–MS analysis. Vitamin D_2 and DMT were used as IS in this study because they closely match Vitamin D_3 and E in their chemical and physical properties. Generally, the IS used for Vitamin A determination are Vitamin A acetate or palmitate, however these compounds were not suitable for our study, as the saponification procedure will transform them into Vitamin A. An alternative approach was reported by Tanumihardjo and Penniston [29] who used 3,4-didehydroretinyl acetate as IS for Vitamin A analysis

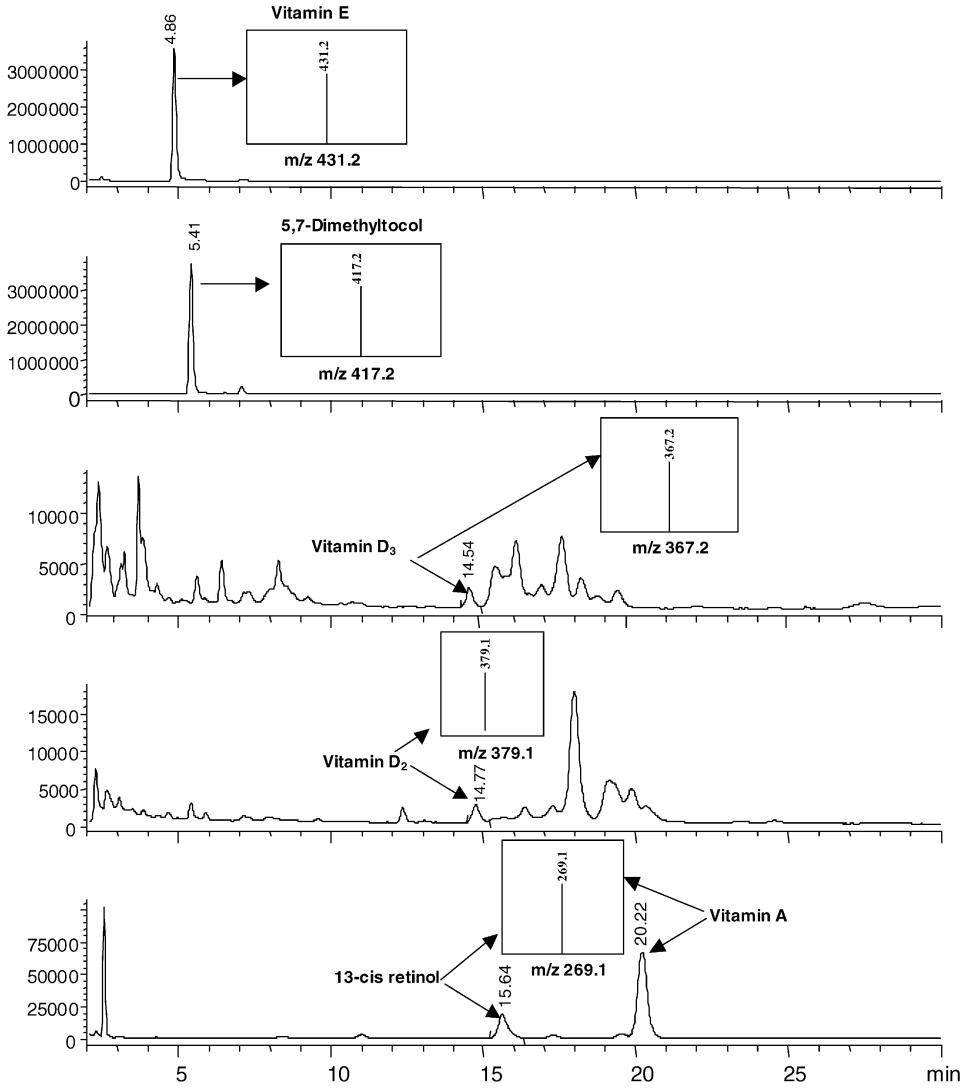

Fig. 5. SIM chromatograms of Vitamins A, D_3 and E extracted from dietetic infant formula (DDP2) spiked with IS—Vitamin D_2 (20 μg/l) and 5,7-dimethyltocol (5 mg/l). For LC–MS conditions, see Section 2.6.

in breast milk by HPLC. The authors report that it can be added to milk before saponification and is carried through the analysis as dehydroretinol (Vitamin A_2). Vitamin A_2 and 3,4-didehydroretinyl acetate are not commercially available and the stock solution of Vitamin A_2 that we received contained around 20%. DMT was not explored as internal standard for Vitamin A as the response factor of these two compounds by MS was different and we do not know the behavior of these two compounds during the sample preparation. The quantification of Vitamin A was performed without any IS. However, in the present work, a reference sample with known values of Vitamin A was introduced in each of the analysis in order to check the accuracy of our method.

The calibration curves plotted from the ratio of the selected ions to the internal standard versus vitamin concentration were linear (Table 1). It should be noted that we did not observe a suppression effect of the co-eluting Vitamins D_2 and D_3 as the signal of Vitamin D_2 remained constant with the increasing of Vitamin D_3 concentration and vice versa. Because of the linearity of our method, a single point calibration was used for the quantification of each vitamin. The results found with this approach were not significantly

Table 1
LC–MS parameters for the quantification of Vitamins A, D_3 and E by LC–APCI-MS

Parameters	A	D_3	E
Range	0.15–12 mg/l	5–400 μg/l	0.25–20 mg/l
Slope	39847	0.0065	0.1039
Intercept	3346.4	−0.0031	0.0296
Regression (r^2)	0.9994	0.9997	0.9961
LOD (ng)	1.4	0.08	0.25
LOQ (ng)	3	0.2	1
Injection volume (μl)	40	40	40

The concentrations of the IS Vitamin D_2 and DMT were 250 μg/l and 5 mg/l, respectively. The data given here take into account the ratio vitamin/internal standard for Vitamin D_3 or E. For Vitamin A, no correction with an IS (Vitamin A_2) was made.

Table 2
Recoveries, precisions data and comparison of the results obtained for Vitamins A, D_3 and E on a reference infant formula (DDP2) by LC–MS and official HPLC methods

	Recovery (%)[a]	Precision CV (%)[a]		Reference values[b]	LC–MS[a]
		Within-day	Between-day		
Vitamin A	102 ± 8	2.3	2.4	2080 ± 390 ($n = 27$)	2350 ± 90
Vitamin D_3	105 ± 7	5.9	6.9	500 ± 70 ($n = 19$)	530 ± 30
Vitamin E	96 ± 9	2.6	3.5	9.1 ± 1.7 ($n = 25$)	9.2 ± 0.3

Vitamins were quantified by comparing the ratio of the ion of each vitamin to the internal standard and calculating their concentrations from ratio of vitamin/internal standard of known concentration of vitamin. For Vitamin A quantification, no correction was made with IS.
Note: *n*, number of laboratories.
[a] The values given here are the mean of six different experiments.
[b] The concentrations of Vitamins A and D_3 are given in IU/100 g of sample and Vitamin E is expressed in mg/100 g of sample.

different from those obtained by the calibration graph (data not shown). The calibration points used for Vitamins A, D_3 and E quantification were: 1.5 mg/l, 125 μg/l and 5 mg/l, respectively. The limit of detection of all vitamins were below 1.4 ng (Table 1), which was sufficient to analyze the products tested whose Vitamin concentrations were in the range of 0.5–2 mg/100 g (Vitamin A), 6–15 μg/100 g (Vitamin D_3) and 3–30 mg/100 g (Vitamin E).

3.4. Method validation

Because of the difficulty to obtain different level of certified materials, precision studies were performed on one Nestlé reference sample (infant formula) used within the Nestlé organization as control samples. To determine the within-day precision, the product was analyzed six times on the same day under the same conditions; the precision (CV) obtained for all three vitamins was below 6% (Table 2). Similarly, the between-day precision (CV) obtained on the same sample analyzed on 6 different days was less than 7%. To test the recovery of the method, extraction was carried out at one spike concentration, equivalent to 140% of the original amount of each vitamin; and this procedure was repeated six times. The spike recoveries, obtained after the saponification and the cleanup procedure, ranged between 96 and 105%. In addition the values of Vitamins A, D_3 and E obtained by LC–MS for reference infant formula (DDP2) were in good agreement with those found by the reference HPLC methods. These data also indicate that the use of an internal standard for Vitamin A was not essential to obtain accurate values (Table 2). The applicability of the method was demonstrated by analyzing six commercial infant formulae and infant cereals products as well as a SRM milk powder. As can be seen in Fig. 6, the mean values obtained for Vitamins A, D_3 and E on two separate LC–MS analyses were in good agreement with those found by the reference LC-UV or LC-fluorescence procedures for all the products tested. In addition, a *t*-test performed on the different data obtained after the determination of Vitamins A, D_3 and E in different food products by the two methods demonstrated that the two sets values were comparable and that the bias was not significantly different from 0 at 95% confidence.

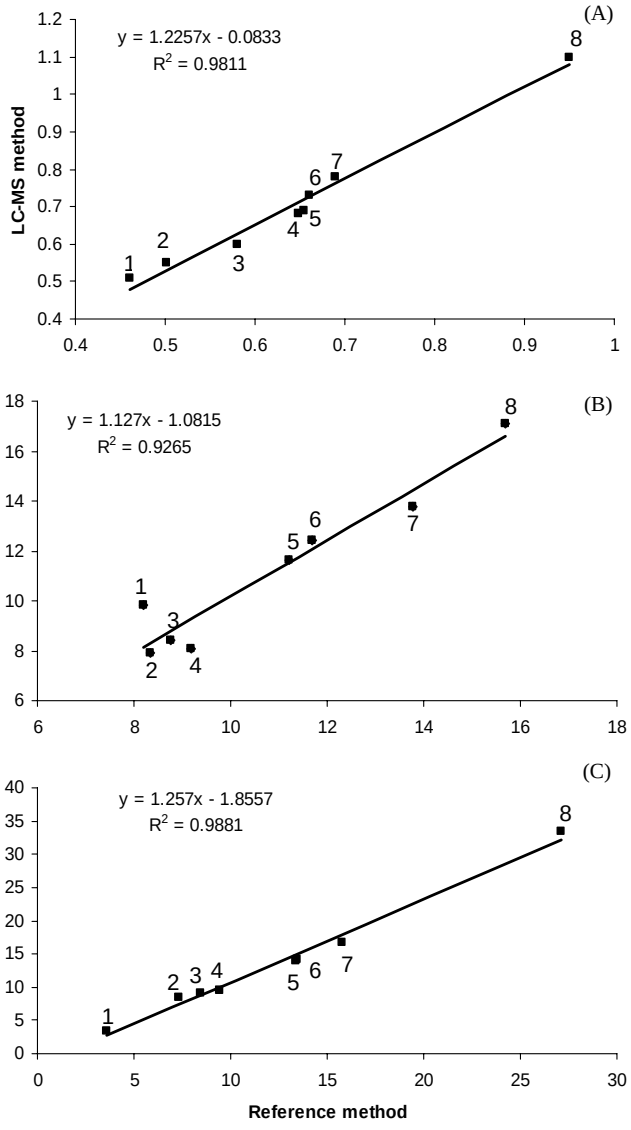

Fig. 6. Relationship between the concentrations of (A) Vitamin A, (B) Vitamin D_3 and (C) Vitamin E found by LC–MS and those obtained by the reference methods HPLC-UV or FLD on different products tested. *Key:* (1) Infant cereal with milk powder; (2) soya-based infant formula; (3) starch-containing infant formula; (4) NRM, dietetic infant formula DDP2; (5) infant formula; (6) infant formula hypoallergenic; (7) infant formula hypoallergenic; and (8) SRM/milk powder, RM 1846.

Consequently, our method is adequate to determine low level of Vitamins A, D_3 and E at different levels in a range of food matrices and the validation performed at a single level could be also applied at multiple levels.

4. Conclusion

A quantitative method has been developed for the simultaneous determination of Vitamins A, D_3 and E in infant formulae and infant cereals using LC–APCI-MS detection. The proposed method would considerably reduce the time required for sample preparation procedures and therefore increase the analytical throughput. The use of LC coupled to a single quadrupole MS instrument shows considerable potential for routine analysis of fat–soluble vitamins in infant formula.

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