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Determination of Vitamin B_5 in a range of fortified food products by reversed-phase liquid chromatography–mass spectrometry with electrospray ionisation

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Abstract

Methods for Vitamin B_5 determination in food products remain limited by their low sensitivity and poor selectivity. Here, we have developed a liquid chromatography–mass spectrometry (LC–MS) method for Vitamin B_5 determination in wide range of fortified food products. Vitamin B_5 was extracted from food samples by heat treatment and analysed by LC–MS in the positive mode using electrospray ionisation (ESI). Vitamin B_5 was quantified using hopantenic acid (HOPA) as internal standard after their separation on a C_{18} narrow-bore column with a gradient of mobile phase made of water/acetonitrile and trifluoroacetic acid (TFA) 0.025%. MS with single ion monitoring mode at mass *m/z* 220 was used for Vitamin B_5 quantification. Calibration curve between 0.5 and 10 μg/ml of Vitamin B_5 was linear (r^2=0.9993) and the detection limit was determined to be 800 pg. The overall quantitative efficiency of the method was evaluated using Nestlé reference sample (infant formula). The intra-assay RSD was 4.8% (n=8), the inter-assay RSD 6.4% (n=4) and the recoveries of the spiked samples were above 95%. Application of the LC–MS method to Vitamin B_5 determination in wide range of fortified food products including three US National Institute of Standards and Technology (NIST) reference samples (RM 8435, RM 8415 and SRM 1546) shows consistent results with those obtained by microbiology and recoveries of Vitamin B_5 between 93 and 104% for the spiked samples.

Keywords: Infant formulas; Food analysis; Vitamins; Pantothenic acid; Hopantenic acid

1. Introduction

Vitamin B_5 (Fig. 1A) is involved in several biological functions and its deficiency is associated with metabolic and energetic disorders in humans [1,2]. Vitamin B_5 exists in foods as bound and free forms. However, the bound form is fully available as a source of Vitamin B_5, because it is converted to free Vitamin B_5 in the intestine by the action of phosphatase and an amylase. This molecule is optically active, but only the D isomer is biologically active and occurs in nature. Calcium D-(+)-pantothenate is the form mainly used for food fortification. This form exhibits greater stability and is less hydroscopic than the free acid. Therefore the analytical results are often expressed as calcium D-(+)-pantothenate, but they can be easily converted to Vitamin B_5. The amount of Vitamin B_5 varies drastically between different food products. For example, the Vitamin B_5 content in milk is between 0.4 and 4 mg/kg [1] whereas in yeast and pork liver, this amount could reach 200 mg/kg [1].

During the last years, methods such as microbiology [3,4], immunoassay [5–7] and radioimmuno assay (RIA) [8] consisting in the determination of Vitamin B_5 without its prior separation were developed. Microbiology method based on the turbidimetric growth of *Lactobacillus plantarum* response has been approved by the Association of Analytical Communities (AOAC) as the official method for Vitamin B_5 determination in food products. Although microbiology has been successfully applied to the determination of Vitamin B_5 in premixes [4], the selectivity of the method remains limited for complex samples where the matrices components could interfere with the growth of the *Lactobacillus plantarum*. In addition, the method is time consuming and 2 to 3 days are needed from the samples reception until reporting the analysis results. With the respect to RIA and immunoassay, the selectivity is very high, but these methods are not sensitive enough to determine low level of Vitamin

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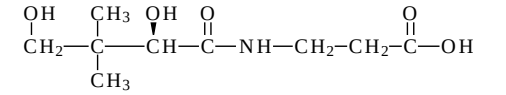
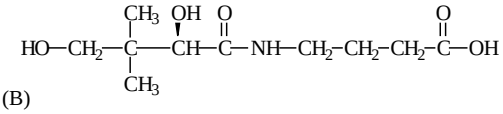


(A)



(B)

Fig. 1. Structures of: (A) Vitamin B_5 and (B) HOPA used in this study as internal standard.

B_5 in various food matrices. In addition, the production of anti bodies is cumbersome and cross-reactions between the latter and the component of the food matrices to be analysed are not excluded. To match the criteria of high selectivity and sensitivity the isolation of Vitamin B_5 prior its determination appears to be very important. HPLC–UV has been used for Vitamin B_5 determination in food products such as milk powders [9], infant formulae [10] and multivitamin tablets [11]. Although the sensitivity is adequate to determine low level of Vitamin B_5 in the product tested, the lack of selectivity due to the maximum of Vitamin B_5 absorption at about 200 nm where several compounds can interfere with the detection hampers the extension of the method to a wider range of products. Gas chromatography (GC) with flame ionisation detection has been used for the determination of Vitamin B_5 in food products [12], also Banno et al. [13] applied GC–MS for the analysis of Vitamin B_5 and one of its metabolites HOPA (Fig. 1B) in biological fluid. However, these methods require a derivatisation step, as Vitamin B_5 is not volatile enough for direct GC. Little use has been made of LC–MS in Vitamin B_5 analysis. To our knowledge, only one study reported the analysis of Vitamin B_5 by LC–MS with particle beam and electrochemical ionisation on multivitamin preparations [14], but this approach was successful only for a very limited number of matrices.

The introduction of atmospheric pressure ionisation techniques greatly expanded the number of compounds and matrices that can be analysed by LC–MS. With the development of ESI interface it is now possible to combine the high selectivity of MS detection with the better sensitivity gained by the use of narrow bore columns for the determination of added Vitamin B_5 in different food matrices. Recently, a stable isotope dilution assay has been developed for the quantification of Vitamin B_5 by liquid chromatography–tandem mass spectrometry [15]. To our knowledge no studies dealing with the analysis of Vitamin B_5 by LC–MS has been published so far. Thus, the purpose of the present study was to develop a method for the determination of Vitamin B_5 in a wide range of food products including: petfood, breakfast cereals, infant cereals without milk, health care product, hypoallergenic infant formula, soya based product and milk based infant formulae. The proposed method combines a rapid sample preparation prior to the separation of Vitamin B_5 by reversed-phase chromatography on a narrow bore column with ESI–MS detection.

2. Experimental

2.1. Chemicals

D-Pantothenic acid calcium salt was purchased from Fluka (Buchs, Switzerland). Acetonitrile and trifluoroacetic acid were obtained from Merck (Geneva, Switzerland). Hopantenic acid was custom synthesised by InterBio-Screen (Chemogolovka, Russia). Water was purified using a Milli-Q system from Millipore (Le Mont-sur-Lausanne, Switzerland).

2.2. Food samples

Because of the difficulty to obtain different levels of certified materials, precision studies were performed on one Nestlé reference sample (infant formula) used within the Nestlé organization as control samples. Petfood, breakfast cereals, infant cereals without milk, health care product, hypoallergenic infant formula, soya based product, milk based infant formula and three NIST reference samples (milk powder RM 8435, egg powder RM 8415 and meat homogenate SRM 1546) from the US National Institute of Standard and Technology (NIST, Gaithersburg, MD, USA) were used for this study.

2.3. Solutions preparation

For the calibration curve, a stock solution of Vitamin B_5 at 500 μg/ml was made by dissolving 54.4 mg of calcium pantothenate in 100 ml of distilled water. No correction was made for the moisture content of the pure pantothenate standard. Aliquots of this solution were withdrawn and spiked with HOPA (2.5 μg/ml) to obtain different points for the calibration curve. The aqueous eluent (pH 2.6) was prepared as followed: to a 1 l flask 10 ml of pure acetonitrile and 250 μl of TFA were added and then the flask was filled to the mark with distilled water. For the organic eluent, in a 1 l flask, 10 ml of aqueous phase were added and then filled up to the mark with acetonitrile.

2.4. Food sample preparation

A well-homogenised food sample (50 g) was dissolved in 100 ml of warm distilled water (45 °C) and thoroughly mixed to obtain homogeneous slurry. A portion of the above mixture (9 g) was accurately weighed into a 250 ml erlenmeyer and 45 ml of distilled water (45 °C) was added to the solution under agitation; the latter mixture was then spiked with HOPA (500 μl of 500 μg/ml stock solution). The Erlenmeyer was then covered with aluminium foil and autoclaved at 103 °C for 20 min. The solution was cooled

down until the room temperature was reached and was then quantitatively transferred into a volumetric 100 ml flask; the flask was filled to the mark with distilled water (45 °C) and the latter mixture was shaken well. Prior to the injection onto the LC–MS system the solution was filtered through a Millipore filter 0.22 μm.

2.5. LC–MS

The analysis of sample was performed by LC using an Agilent 1100 HPLC system (Agilent Technology, Urdorf, Switzerland) with a mass-selective detection system (Agilent Technology). Sample injections of 20 μl were made from an Agilent 1100 Series auto-sampler (Agilent Technology). The chromatographic separations were performed on a C_{18}, 3 μm, 150 mm × 3.0 mm column (YMC Europe), with a flow rate of 0.5 ml/min. The mobile phase was a series steps of gradient consisting of water and 0.025% of TFA pH 2.6 (solvent A) and acetonitrile (solvent B) that follows: range 0–3.5 min 100:0, range 3.5–11 min 75:25, range 11–19 min 65:35, range 19–20 min 90:10 and range 20–26 min 100:0 (v/v). The column was further equilibrated for 4 min before the next injection of samples. The column effluent was monitored by MS with positive ESI and SIM mode. The operating conditions were 140 V for the fragmentor voltage, 10 l/min for the drying gas flow, 350 °C for the drying gas temperature, 30 psig for the nebuliser pressure and the capillary voltage was set to 4500 V. Quantifiers ions used for Vitamin B_5 and HOPA were 220 and 234, respectively.

3. Results and discussion

The main objective of this paper was to develop a novel method to monitor and quantify Vitamin B_5 in a wider range of food products including: petfood, breakfast cereals, infant cereals without milk, health care product, hypoallergenic infant formula, soya based product and milk based infant formulae and to compare the results obtained with those found with the existing official method.

3.1. LC–MS development

Liquid chromatography with UV detection has been applied to the detection of Vitamin B_5. Eluent made of phosphate buffer were used for Vitamin B_5 separation [9–11]. Although giving a better resolution between Vitamin B_5 and the interferences in food products, these eluents are not compatible with MS analysis. Thus, a mobile phase (pH of 2.6) made of acetonitrile and water acidified with TFA was used in the present study. No ion suppression was found with TFA under our experimental conditions (data not shown). In addition, good resolution between Vitamin B_5 and HOPA peaks were achieved as it is shown in Fig. 2. The

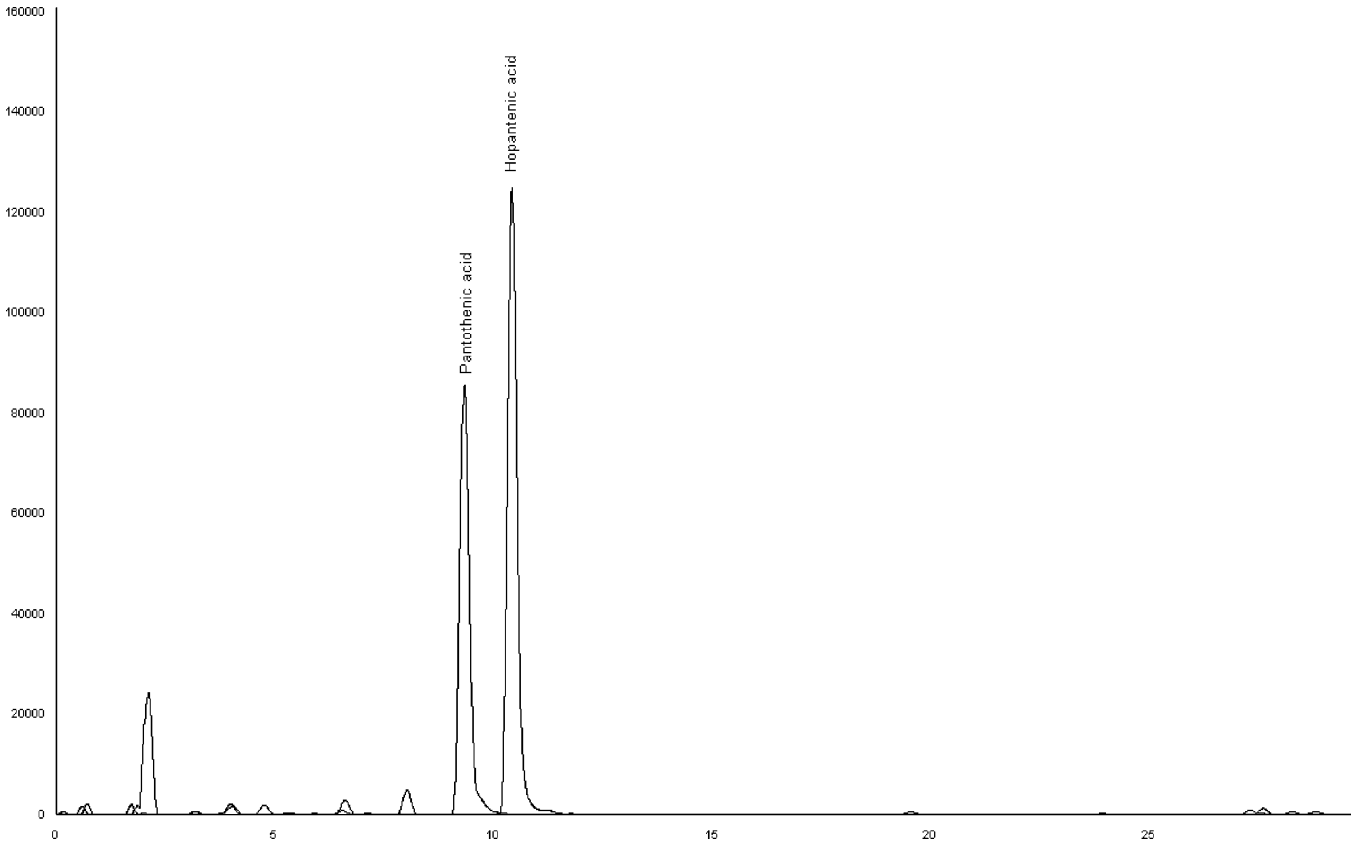


Fig. 2. LC–MS chromatogram of Vitamin B_5 (pantothenic acid) and HOPA. The LC–MS conditions are described in the Section 2. Time scale in min.

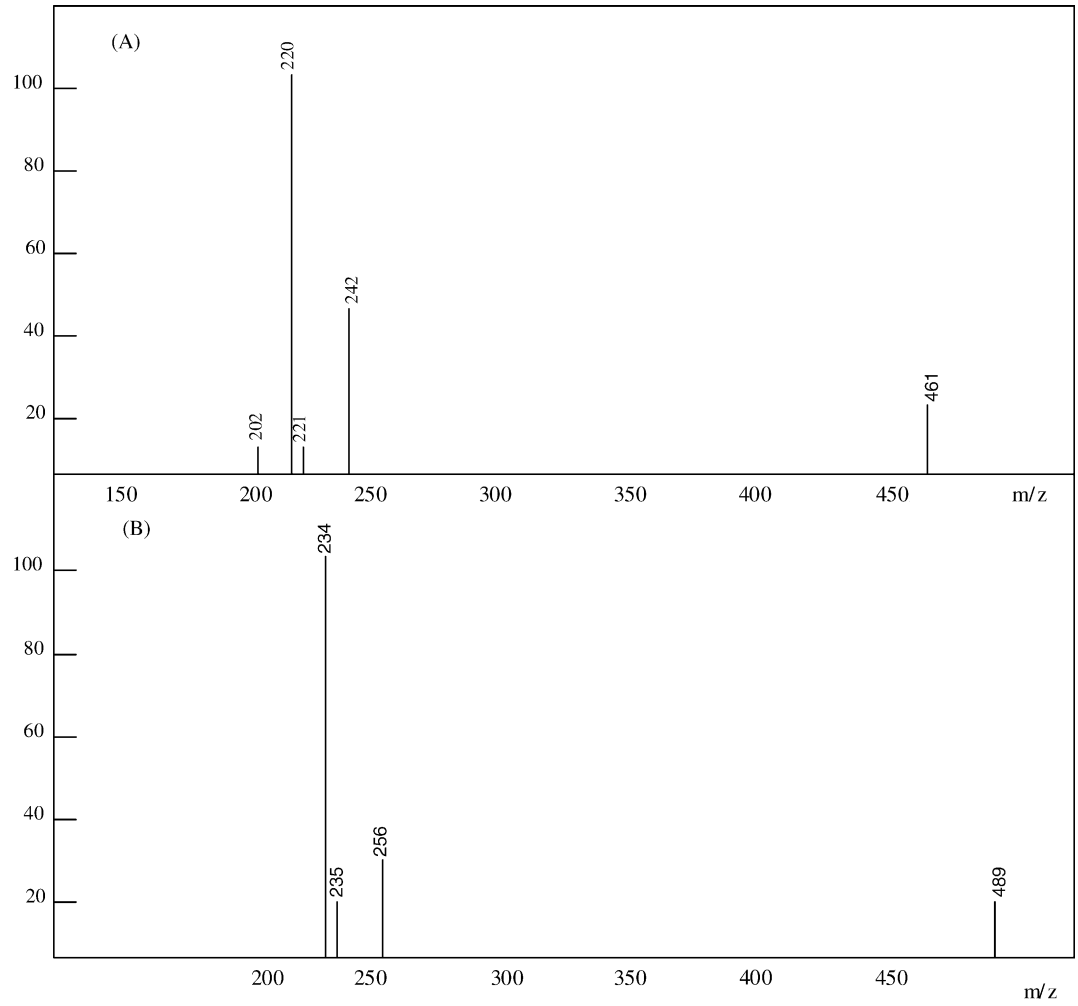


Fig. 3. Mass spectra showing the full scan or total ion current of: (A) Vitamin B_5 and (B) HOPA.

detection of Vitamin B_5 and that of its internal standard was performed by ESI in positive mode. The full scan spectrum of Vitamin B_5 displays a predominant ions at *m/z* 220 which corresponds to the protonated ion $[M + H]+$ of Vitamin B_5. In addition, three ions at *m/z* 202, 242 and 461 whose structures correspond to a water loss $[M - H_2O + H]^+$ and sodium adducts $[M + Na]+$and $[2M + Na]+$, respectively were found (Fig. 3A). The full scan spectrum of HOPA depicts a predominant ion at *m/z* 234 and minor ones at *m/z* 256 and 489 whose structures correspond to $[M + H]+$, $[M + Na]+$ and $[2M + Na]+$, respectively (Fig. 3B).

The SIM analysis using the ions at *m/z* 220 for Vitamin B_5 determination and the one at *m/z* 234 for HOPA identification of all products tested yield clear chromatograms where Vitamin B_5 and HOPA peaks can be unambiguously distinguished. In contrast when using LC–UV absorbance detection, Vitamin B_5 and HOPA peaks were not always well resolved from interferences. This is illustrated in Fig. 4 where a comparison is made between LC–UV and LC–MS chromatograms of an extract of a hypoallergenic infant formula. The particularity of this product is that proteins are enzymatically hydrolysed to peptides, which absorb in the low UV range. It was found to be one of the most complex matrices for analysis of Vitamin B_5 by LC–UV absorbance. Comparing the retention time with those of the standards, and obtaining MS spectra allows the identification of Vitamin B_5 in different products analysed.

3.2. *Vitamin B_5 quantification*

Internal standards are desirable when the analysis is performed by mass spectrometry. Deuterated analogues are commonly used as internal standards as they are essentially identical in chemical and chromatographic properties to the respective unlabelled compounds whilst being readily distinguishable by mass spectrometry because of their mass difference. For Vitamin B_5 determination, deuterated compounds are not available. Thus, HOPA a non-labelled compound is the employed internal standard (IS) as it closely matches chemical and physical properties of Vitamin B_5 [13].

Endogenous Vitamin B_5 in different food samples was quantified by means of an external calibration curve in the concentration range from 0.50 to 10 μg/ml of Vitamin B_5 and corresponding HOPA concentration was set

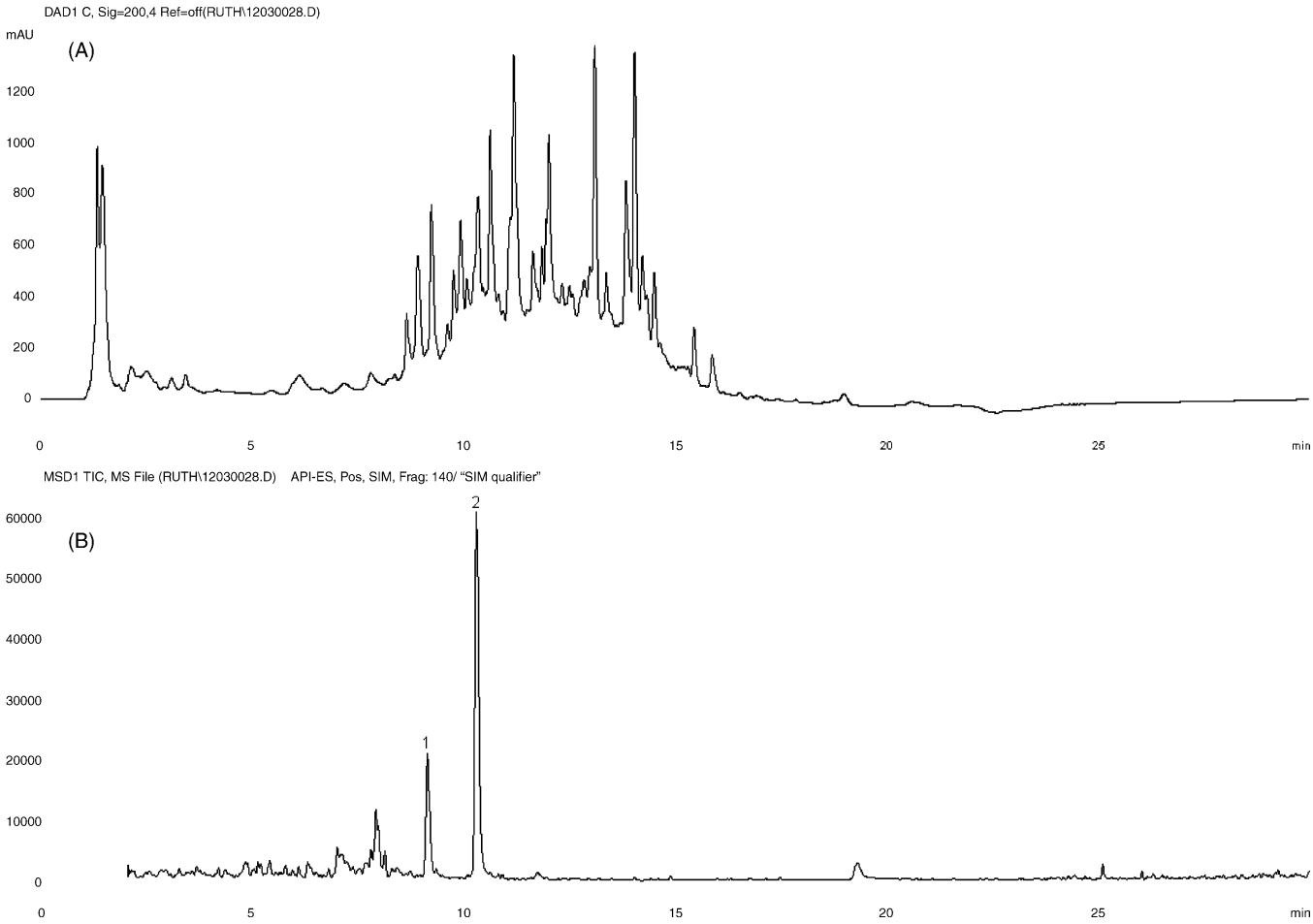


Fig. 4. (A) LC–UV chromatogram and (B) SIM chromatogram of one of the product used in this study (hypoallergenic product) spiked with hopantenic acid. 1: Vitamin B_5 and 2: HOPA.

Table 1
Calibration curve data and quantification parameters for the analysis of Vitamin B_5 in food products by LC–MS

Slope	0.2192
Intercept	0.035
Regression (R^2)	0.9993
Limit of quantification (μg/ml)	0.25
Limit of detection (pg)	800
Injection volume (μl)	20

to 2.5 μg/ml. Minimum of five Vitamin B_5 concentrations were used to generate the calibration curve that was linear ($r^2 = 0.9993$). The limit of detection (LOD) of the method as it is shown in Table 1 was determined to be 800 pg on column and the limit of quantification (LOQ) was 0.25 μg/ml, which is equivalent to 0.28 mg/100 g in a dry food product. To determine the within-day precision, the Nestlé reference sample was analysed eight times on the same day under the

Table 2
Recovery, precisions data and comparison of the results obtained for Vitamin B_5 on a Nestlé reference sample (infant formula) by LC–MS and official microbiological methods

	Recovery (%)	Precision-RSD (%)		Reference values (mg/100 g)	LC–MS (mg/100 g)
		Within-day	Between-day		
Vitamin B_5	95	4.8 (n=8)	6.4 (n=3)	6.0 ± 0.7[a]	6.1 ± 0.163

Vitamin B_5 was quantified by comparing the ratio of the ion of the vitamin to the internal standard and calculating their concentrations from ratio of vitamin/internal standard of known concentration of vitamin.

[a] Values obtained by 13 different Nestlé laboratories.

Table 3
Recoveries and comparison between LC–MS and microbiological methods for Vitamin B_5 determination in various food samples

Samples	Spiked (μg/ml)	Recovery (%)	Conc. (mg/100 g) LC–MS	MBA
Petfood	1.0	104	1.2 ± 0.09 (n=3)	1.42 ± 0.03 (n=3)
Health care product	1.4	102	2.3 ± 0.03 (n=3)	2.1 ± 0.09 (n=3)
Infant cereal without milk	2.5	93	2.5 ± 0.05 (n=3)	2.2 ± 0.01 (n=3)
Hypoallergenic infant formula	5	100	3.4 ± 0.04 (n=3)	3.7 ± 0.1 (n=3)
Breakfast cereals	6.5	94	8.7 ± 0.70 (n=3)	8.8 ± 0.03 (n=3)
Infant formula milk based			5.0 ± 0.04 (n=3)	4.2
Soya based			3.4 ± 0.01 (n=3)	4.4 ± 0.2 (n=3)
Milk and cereal infant formula			1.4/1.3[a]	1.8/1.7[a]
Lactose free infant formula			2.6/2.7[a]	2.9/2.8[a]
RM 8435			3.1/3.4[a]	2.6 ± 0.5[b]
SRM 1546			0.30/0.4[a]	0.58 ± 0.07[b]
RM 8415			10.4/11.3[a]	9.1[c]

[a] Values obtained on two different LC–MS or MBA data points.
[b] Reference values.
[c] Information value.

same conditions; the precision (RSD) obtained for Vitamin B_5 4.8%. Similarly the between-day precision (RSD) obtained on the same sample analysed on four different days was 6.4%. To test the recovery of the method, extraction was carried out at one spike concentration equivalent to 140% of the original amount of Vitamin B_5 and the recovery was 95%. These results are summarised in Table 2.

In order to demonstrate the applicability of our method for Vitamin B_5 determination, several fortified food products including three NIST reference samples (milk powder RM 8435, egg powder RM 8415 and meat homogenate SRM 1546) were analysed by LC–MS. Also the recoveries were checked at multiple levels by spiking different food matrices with variable amounts of Vitamin B_5. As it is shown in Table 3, the recoveries ranged from 93 to 104%. In addition, a reasonable correlation was found between the LC–MS and MBA procedures except for the NIST sample RM 8415. It should be noted that the values given for the later sample was not certified and this value just represents the determination performed by few laboratories. In food products Vitamin B_5 occurs in the free form, but may also be bound as coenzyme A or as acyl carrier protein. Thus, an enzymatic treatment is commonly used to release the bound forms of Vitamin B_5. In the present study, only the free form of Vitamin B_5 was measured, as it is the major source of Vitamin B_5 in supplemented products. The three SRM products may contain both free and bound Vitamin B_5 and that can explain why the value that we found by LC–MS for the SRM 1546 sample was inferior to the reference value.

4. Conclusion

Our data demonstrate that single quadrupole mass spectrometry could be applied for the determination of Vitamin B_5 with accuracy in a wide range of fortified food products. The usefulness of LC–MS for vitamin analysis shown in this study will open the door of new applications such as simultaneous quantification of B-group Vitamins in food products in a single assay. This would simplify the food samples preparation and consequently enhances the vitamin throughput analysis in the food area.

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