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Separation of water-soluble vitamins by reversed-phase high performance liquid chromatography with ultra-violet detection: Application to polyvitaminated premixes

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Abstract

Nine water-soluble vitamins: [thiamine (B_1), ascorbic acid (C), nicotinamide (PP), pyridoxine (B_6), calcium pantothenate (B_5), folic acid (B_9), cyanocobalamin (B_{12}), riboflavin (B_2) and biotin (B_8)] were separated on a YMC-Pack Pro C_{18} column (250 mm × 4.6 mm, 5 μm particle size) in a single run with a gradient elution of mobile phase consisting of 0.025% trifluoroacetic acid pH 2.6 (solvent A) and acetonitrile (solvent B). The separation was achieved within 17 min with a flow rate of 0.8 ml min^{-1} and the detection was performed at two wavelengths (210 and 275 nm). The calibration graphs plotted with six concentrations of each vitamin were linear with a regression coefficient $R^2 > 0.995$. The method was applied for the quantification of vitamins B_1, C, PP, B_6, B_5, B_9 B_2 and B_8 in polyvitaminated premixes (premixes) used for the fortification of infant nutrition products. The sample preparation involves an aqueous extraction of vitamins and two different samples dilution were used prior the LC-analysis. The specificity of the method was demonstrated by the retention characteristics, UV spectra and by comparing the peak purity with the standard of each vitamin. The repeatability of the method was evaluated at different level of concentrations on 12 premixes and the coefficients of variation (CV_r) were below 6.5%. The values of the intermediate precision (CV_I) were below 9.6% ($n = 6$). The concentrations of vitamins found in premixes with our method were comparable to the declared values, since no bias was found between the two sets of results at 95% confidence. The simplicity of the procedure should make it highly desirable for quality control of premixes in the food industry.

Keywords: Column liquid chromatography; Water-soluble vitamins; Polyvitaminated premixes

1. Introduction

Lack of vitamins can cause serious diseases in humans even though only small concentrations are required to maintain good health [1]. The main source of vitamins for human beings is from food. The human diet does not always contain the amount of vitamins needed for the normal development and maintenance of body functions. Therefore, fortification of certain food products, in particular those which are the sole source of nutrition (infants and clinical nutrition), is needed to ensure an adequate intake of vitamins. Vitamins are also added to anticipate and correct for losses that may occur during food processing or storage. The fortification of food products with vitamins is generally achieved with premixes that contain high concentration of vitamins: thus, there is a need to have fast and reliable analytical methods for their quality control during production and at the end of shelf life.

The official methods for water-soluble vitamins analysis are often based on tedious, and sometimes not completely specific, microbiological assays [2,3]. In addition, vitamins extraction involves pre-treatment through complex chemical reactions followed by individual methods for the determination of each vitamin. During the last decade there has been an increasing interest for the simultaneous determination of vitamin. Thus, methods such as capillary electrophoresis [4–6], micellar electrokinetic chromatography [7–9], micellar liquid chromatography [10,11] and liquid chromatography (LC)

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[12–22] have been developed. Amongst these methods, LC appears promising due to the improvements of both stationary phases and chromatography equipments. Reversed-phase liquid chromatography (RP-LC) without ion-pair reagents [12,13] has been widely applied but problems with low reproducibility in the retention time of some vitamins occurred. RP-LC with ion-pair reagent was also applied but the complex mobile phases associated with this technique make the column equilibration longer and the total run time of the analysis superior to 50 min [15]. LC column with amide-based stationary phase was also used but the total time of analysis remain long (45 min) and the mobile phase associated with the LC-analysis were made of phosphate [23]. However, phosphate buffers are not suitable for mass spectrometry and should be avoided for liquid chromatography/mass spectrometry (LC/MS) applications. Recently few LC methods for the simultaneous analysis of water and/or fat-soluble vitamins with a mobile phase fully compatible with MS [24–27] were developed. In one of the method, the separation of eight water-soluble vitamins was achieved within 30 min and the detection was performed at 280 nm [25]. In another one, water-soluble vitamins were separated in less than 15 min and they were monitored at 280 nm [27]. Surprisingly in this paper, the authors have identified and accurately quantified vitamin B_5 in different food products by UV at 280 nm where this vitamin does not give an UV signal. Vitamin B_8 another water-soluble vitamin was not included in this study. Currently, there is only one study [28] that reports the analysis of vitamin B_8 with the other water-soluble vitamins in a single run. However in the latter work the mobile phases used are not compatible with MS application.

In the present study, the LC-separation of nine water-soluble vitamins: B_1, C, PP, B_6, B_5, B_9, B_{12}, B_2 and B_8 on an endcapped C_{18} column with a mobile phase fully compatible with LC/MS analysis has been achieved within 17 min. The detection has been optimized using a diode array detector and was performed at two wavelengths (275 and 210 nm). In addition, the proposed method has been validated for the simultaneous quantification of vitamins: B_1, C, PP, B_6, B_5, B_9, B_2 and B_8 in premixes that are used for fortification of infant nutrition products.

2. Experimental

2.1. Reagents

All reagents were analytical grade. Thiamine hydrochloride (B_1), D-pantothenic acid calcium salt (B_5) and biotin (B_8), were purchased from Fluka (Buchs, Switzerland). Acetonitrile, ascorbic acid (C), cyanocobalamin (B_{12}), trifluoroacetic acid (TFA), dihydrogen phosphate monohydrate and sodium hydroxide were obtained from Merck (Geneva, Switzerland). Riboflavin (B_2), pyridoxine HCl (B_6) and folic acid (B_9) were from Supelco (Bellefonte, PA, USA). Nicotinamide (PP) was obtained from Sigma (Buchs, Switzerland).

Table 1
Concentration range of vitamins in the premixes used in the present study

Vitamin	Concentration range ($g\,kg^{-1}$)
Vitamin B_1	1.5–8.7
Vitamin C	156–797
Vitamin PP	11–123
Vitamin B_6	1.5–13
Vitamin B_5	5–33
Vitamin B_9	0.3–3.4
Vitamin B_{12}	0.0008–0.003
Vitamin B_2	0.8–7.2
Vitamin B_8	0.09–0.3

Water was purified using a Milli-Q system from Millipore (Le Mont-sur-Lausanne, Switzerland). Polyvitaminated premixes were obtained from DSM nutritional products (Saint-Louis, France).

2.2. Standard preparation

The multi-vitamins stock solution was prepared by weighing in a 100 ml volumetric amber flask, 5 mg of vitamin B_{12}; 10 mg of vitamin B_8; 12.5 mg of vitamins B_2 and B_9; 25 mg of vitamins B_1, B_6, PP and 100 mg of vitamin B_5, and by adding 40 ml of water; the solution was shaken vigorously and 4 ml of NaOH 2 M were added. After complete dissolution of the vitamins, 50 ml of phosphate buffer 1 M (pH 5.5) and 100 mg of vitamin C were added and the solution was made up to the mark with water. Stock standard solution was prepared daily.

2.3. Premixes composition

The premixes selected for the present study are used for the fortification of infant formulae such as milk and cereals based products. They are made of maltodextrine and contain iodine, fat-soluble vitamins (vitamin A-acetate, vitamin D_3, vitamin K_1 and vitamin E-acetate) and water-soluble vitamins: [thiamine mononitrate (B_1), ascorbic acid or sodium ascorbate (C), nicotinamide (PP), pyridoxineHCl (B_6), calcium pantothenate (B_5), folic acid (B_9), cyanocobalamin (B_{12}), riboflavin (B_2) and biotin (B_8)]. Tables 1 and 2 summarize

Table 2
Vitamin concentrations in premixes 2, 10 and 11 used for the evaluation of precision data

Vitamins	Concentration ($g\,kg^{-1}$)		
	Premix 2	Premix 10	Premix 11
B_1	5.2	2.5	2.2
C	488	156	315
PP	73.1	41.1	37.4
B_6	7.7	3.1	3.7
B_5	19.3	8.2	nd
B_9	0.8	0.4	1.6
B_2	2.4	4.6	3.9
B_8	0.13	0.06	0.33

the range of water-soluble vitamin concentrations in the premixes.

2.4. Vitamins extraction

After the homogenization of the whole laboratory sample by mixing, 2 g of premix was weighed in a 100 ml volumetric amber flask, 40 ml of water and 4 ml of NaOH 2 M were added and the suspension was vigorously shaken, then 50 ml of phosphate buffer 1 M (pH 5.5) were added in order to lower the pH of the final solution at 7. The suspension was made up to the mark with water and was sonicated for 10 min in an ultrasonic bath Bransonic 12 (Carouge, Geneva, Switzerland). Aliquot of the latter solution was removed for the quantification of vitamins B_5 and B_8 whereas; a dilution of 20 or 40-fold with water was used for the quantification of vitamins B_1, C, PP, B_2, B_6 and B_9. The solution was filtered through a 0.22 μm Millipore syringe Millex^{TM} GP filter (Bedford, MA, USA) before the LC-analysis.

2.5. LC-UV

Reversed-phase chromatographic columns: Pack Pro (250 mm × 4.6 mm, 5 μm particle size, YMC, Europe, GmbH), Nucleodur pyramid (125 mm × 3 mm, 5 μm particle size, Macherey-Nagel, Düren, Germany), Atlantis (250 mm × 4.6 mm, 5 μm particle size, Waters, Milford, MA, USA) were tested. An Agilent 1100 LC system (Agilent Technology Inc., Urdorf, Switzerland) was connected to a diode array spectrophotometric detector (Agilent Technology Inc., Urdorf, Switzerland) operating at two wavelengths: 210 nm for vitamins B_5, B_8 and B_{12}, and 275 nm for vitamins B_1, C, PP, B_6, B_9 and B_2. Aliquots of 20 μl of sample were injected from an Agilent 1100 Series auto-sampler. The chromatographic separations were performed at flow rates of 0.8 ml min^{-1} on the YMC Pack Pro and Atlantis columns and at 0.25 ml min^{-1} on the Nucleodur pyramid column. The mobile phase was made of: solvent (A) pH 2.6 consisting of an aqueous solution of TFA 0.025%, and solvent (B) acetonitrile. The gradient elution program is described in Section 3.2.

2.6. Statistics

The precision data of the method were evaluated using an in-house statistical program making use of the robust-statistics concept of Rousseew and Croux [29].

3. Results and discussion

3.1. UV detection and chromatographic column selection

Based on the UV spectra of each vitamin shown in Fig. 1, it was not possible to use a single wavelength for the detection of all vitamins. Therefore, vitamins B_8, B_{12} and B_5 were detected at 210 nm. It should pointed out that the better signal-to-noise obtained with our LC mobile phases at optimum flow rate of 0.8 ml min^{-1} allow the detection of vitamin B_{12} at 210 nm, unlike the results of Wongyai [30]. Vitamins B_1, C, PP, B_6, B_9 and B_2 were observed at 275 nm, despite the fact that vitamins PP and C give a higher signal at 210 nm compared to the one at 275 nm. These two vitamins were found at a very high concentration in the premixes in comparison with the other one. Thus, for the quantification of Vitamins PP and C together with the other vitamins it was decided to use the UV signal at 275 nm.

LC is widely used for the separation of water-soluble vitamins in food products. The LC methods have been improved for the retention of polar vitamins by using either ion paring agent or new types of columns. The choice of the column has an influence on the separation of water-soluble vitamins due to the difference in their chemical structures. Consequently, a compromise between the hydrophilic and lipophilic characters of the column stationary phases has to be found. In the present study, three endcapped reversed-phase columns: Nucleodur pyramid, Atlantis and YMC-Pack Pro were compared on the basis of operating at low pH (pH < 3) as well as in the presence of high percentage of water in the mobile phase. These columns were selected because conventional columns without ion-pair reagents often displays stability problems such as sudden decrease of retention time and poor reproducibility of the separation in solvent with percentage of water [12,13]. The data of this comparison show that the column YMC-Pack Pro was the most appropriate for the separation of the water-soluble vitamins studied and this column was selected for the present work. The YMC-Pack Pro C_{18} column is made with highly inert ultrapure, pH neutral silica and the endcapping is based on novel Lewis acid–Lewis base chemistry.

3.2. Water-soluble vitamins separation

Isocratic elution of the vitamins was not possible as the optimal mobile phase in the absence of acetonitrile only allows the separation of vitamins B_1, PP and C. When a higher concentration of organic solvent was used (40% acetonitrile), vitamins B_2 and B_8 were separated, whereas the rest of the vitamins eluted together at the void volume. Therefore, a gradient elution technique was applied to achieve a baseline separation of the vitamins and to shorten the total analysis time. The optimal gradient selected was the following. First, an initial isocratic step with solvent (A) for 5 min, followed by a linear gradient to solvent (B)–solvent (A) (25:75, v/v) mixture during 6 min. Then a second linear gradient to solvent (B)–solvent (A) (40:60, v/v) during 8 min, this mixture being held for 1 min. Finally the initial conditions were re-established in 1 min and held for 4 min.

The chromatographic separation of the nine water-soluble vitamins using this gradient elution program is shown in Fig. 2. The elution order was: vitamins B_1, C, PP, B_6, B_5,

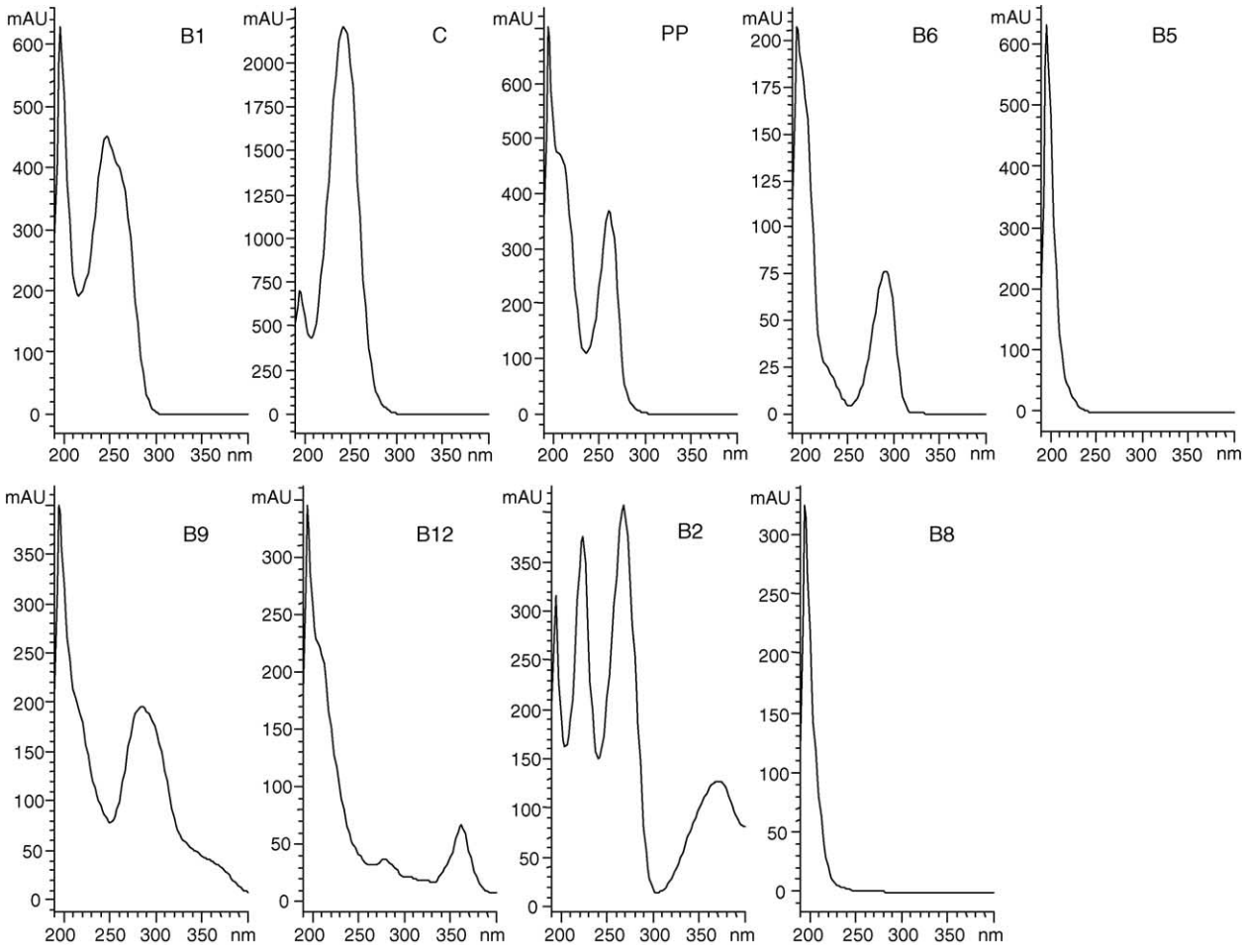

Fig. 1. UV spectra of different water-soluble vitamins used in this study.

B_9, B_{12}, B_2 and B_8. Vitamins were separated to the baseline and eluted as sharp peak within 17 min, which was quicker than the separation achieved in the previous studies [15,20,23]. Moreover, reproducible retention times for the vitamins tested even at high percentage of solvent (A) were observed. This is illustrated by the acceptable standard deviation value of the retention time of vitamin B_1 peak (Table 3). The LC solvents are compatible with MS, which is increasingly developed for the analysis of water-soluble vitamins in food products [31–33]. Consequently, our LC conditions can also be applied to LC/MS for the simultaneous determination of water-soluble vitamins. Indeed, the determination of water-soluble vitamin (i.e. vitamin B_5) by HPLC with fluorescence detection requires a time consuming clean up step

Table 3
Calibration characteristics of water-soluble vitamins

Vitamin	λ (nm)	$R_t \pm$ SD[a] (min)	Slope ± SD[b] (ml μg^{-1})	Intercept	Correlation	Linearity (μg ml^{-1})
B_1	275	3.78 ± 0.13	29.3 ± 0.2	−2.3	0.9999	1.25–50
C[c]	275	6.40 ± 0.11	5.8 ± 0.6	15.2	0.9956	5–200
PP	275	7.55 ± 0.05	14.3 ± 0.3	0.9	0.9997	1.25–50
B_6	275	11.00 ± 0.03	34.0 ± 0.6	−1.1	0.9997	0.125–50
B_5	210	13.90 ± 0.01	7.3 ± 1.0	10.9	0.9999	2.5–500
B_9	275	14.81 ± 0.02	83.2 ± 3.3	−4.6	0.9998	0.6–25
B_{12}	210	15.20 ± 0.02	58.4 ± 1.4	−2.54	0.9994	0.25–10
B_2	275	15.98 ± 0.02	73.2 ± 3.9	31.2	0.9996	0.62–25
B_8	210	16.50 ± 0.02	10.7 ± 0.5	1.21	0.9997	0.5–50

[a] Represents the standard deviation of the retention time ($n = 6$).
[b] Represents the standard deviation of the slope of calibration curves obtained by plotting in triplicate six different concentrations of each vitamin versus the UV response.
[c] For vitamin C the standard deviation of the slope was calculated on duplicate points.

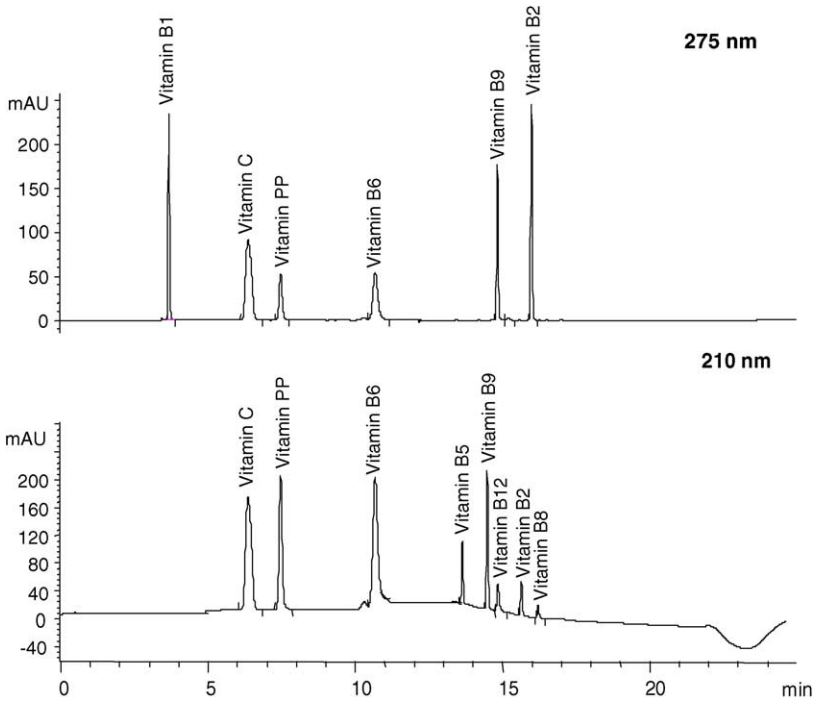

Fig. 2. LC-UV chromatogram of standard mixture of nine water-soluble vitamins: B_1 (25 μg ml^{-1}); C (100 μg ml^{-1}); PP (25 μg ml^{-1}); B_6 (25 μg ml^{-1}); B_5 (25 μg ml^{-1}); B_9 (12.5 μg ml^{-1}); B_{12} (5 μg ml^{-1}); B_2 (12.5 μg ml^{-1}) and B_8 (12.5 μg ml^{-1}). Flow rate: 0.8 ml min^{-1}, injection volume: 20 μl.

(two purifications on solid phase extraction cartridges) [34] while, no cleanup procedure was needed for MS analyses [31,33]. The better selectivity of MS technique over the current LC procedures with UV or fluorescence detection would simplify the sample preparation and enhance the throughput of vitamin analysis.

3.3. Identification of water-soluble vitamins in premixes

Vitamins can be extracted from the premixes in water at room temperature in the presence of sodium hydroxide 2 M. Moreover, the use of sodium hydroxide in the extracting solution increased the recovery of vitamin B_9, which was partially soluble in water. Under the above alkaline condition (the pH on the extraction solution is 13), vitamins B_5, B_1, PP and C were not stable. Thus, the pH of the extraction solution was lowered to 7 with the use of a phosphate buffer 1 M (pH 5.5). At pH 7, vitamins were found to be stable in the premixes solution for at least 10 h. Sample size of 2 g of premixes represent a homogeneous test portion for the premixes studied.

Under our experimental protocol, it was not possible to determine vitamin B_{12} in a single run with the other vitamins in the premixes. As a matter of fact, the concentration of vitamin B_{12} found in premixes (see Table 1) was below the limit of detection (LoD) of our method, which was estimated at 200 ng ml^{-1}. Therefore, the determination of vitamin B_{12} would require different sample weight and sample injection volume onto the column. Consequently, it was decided to exclude vitamin B_{12} from the present study. For the other vitamins, the method was sensitive enough to determine low concentrations in premixes and it was not relevant to determine the LoD of the method. However, because each vitamin was found in different concentration in premixes, two sample dilutions were needed to determine the eight water-soluble vitamins. An example of a typical premix chromatogram is shown in Fig. 3 displaying a baseline separation and excellent resolution of vitamins B_1, B_2, PP, B_5, B_6, B_9 and C. The peaks were identified by comparing both the relative retention times and the UV spectrum of these vitamins to those of the reference ones.

3.4. Calibration graphs and quantification

The calibration curves were obtained by plotting concentration (μg ml^{-1}) against peak area. For each vitamin, a series of six concentration points were prepared and each solution was injected three times except for vitamin C, which was injected twice due to the degradation of vitamin C standard solution. However, we found that vitamin C was more stable in premixes than in the standard solution (data not shown). As shown in Table 3, the curve of each vitamin was linear ($R^2 > 0.995$) in the working range concentration. Quantification of the vitamins in premixes was performed by the external standard method with a single calibration point due to the linearity of method and because the intercept of each calibration curve includes the zero. The results found with this approach were not significantly different from those obtained with the calibration graph (data not shown). The following concentration were used for each vitamin: B_1 (25 μg ml^{-1}), C (100 μg ml^{-1}), PP (25 μg ml^{-1}),

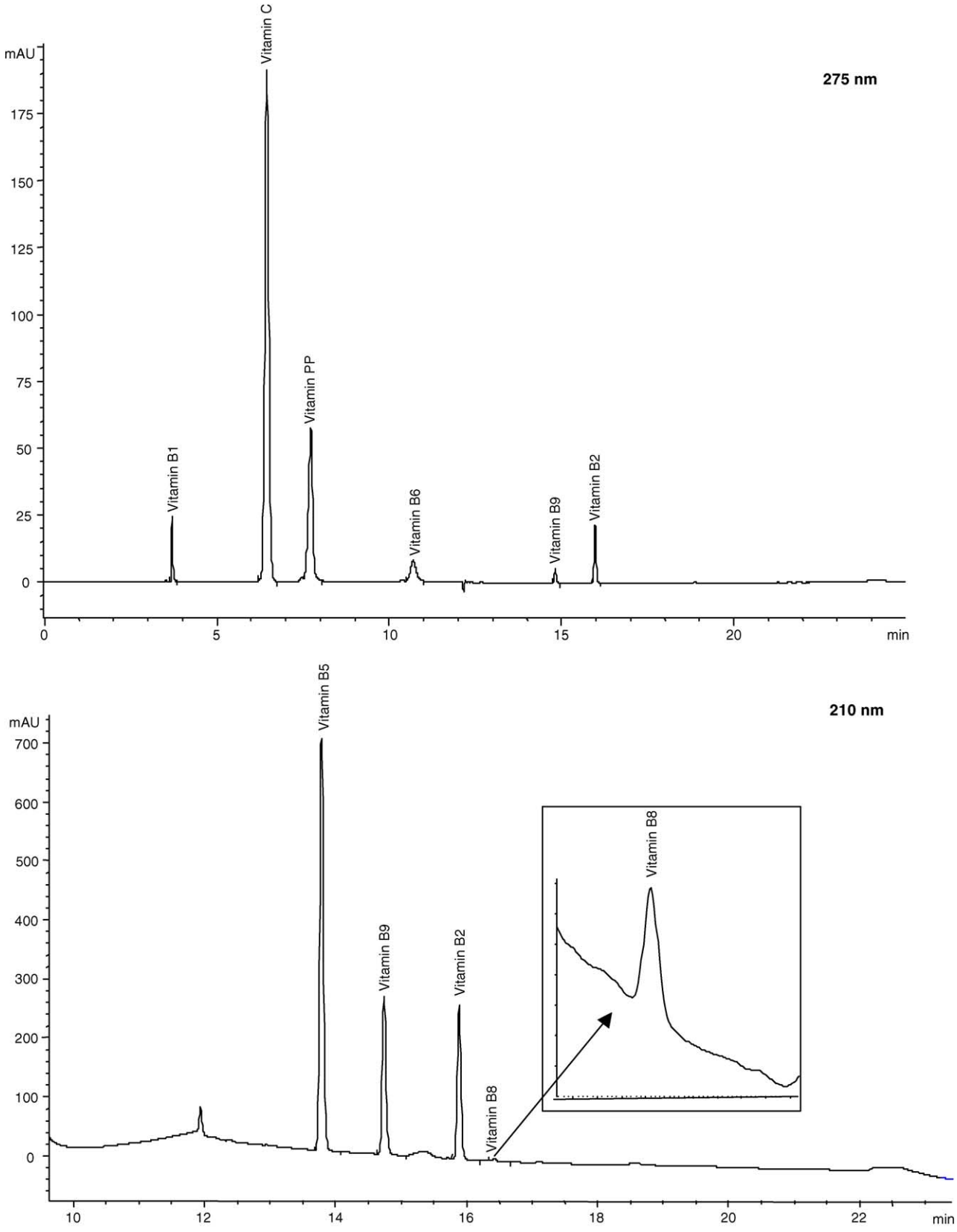

Fig. 3. LC-UV chromatogram of water-soluble vitamins obtained in premixes. The sample was prepared as mentioned in Section 2.3 and was diluted 40-fold with distilled water before the LC-analysis to obtain the chromatogram displayed at 275 nm, or was injected without any dilution to obtain the chromatogram displayed at 210 nm. The insert box represents a zoom of vitamin B_8 peak.

Table 4
Determination of water-soluble vitamins in premixes

Vitamins	Found[a] (declared) (g kg^{-1})											
	Premix 1	Premix 2	Premix 3	Premix 4	Premix 5	Premix 6	Premix 7	Premix 8	Premix 9	Premix 10	Premix 11	Premix 12
B_1	7.6 (7.9)	5.1 (5.2)	3.2 (3.6)	1.2 (1.5)	8.1 (8.7)	1.6 (1.8)	1.4 (1.5)	2.3 (2.9)	4.6 (4.5)	2.1 (2.5)	2.0 (2.2)	1.4 (1.7)
C	293 (304)	466 (488)	367 (402)	351 (368)	681 (797)	339 (372)	149 (190)	339 (386)	464 (517)	142 (156)	262 (315)	290 (340)
PP	60.7 (66.7)	66.7 (73.1)	56 (64.3)	21 (24.4)	107 (123)	22.2 (25.8)	9.2 (11)	35 (44.8)	59 (73)	37.1 (41.1)	33.7 (37.4)	26.6 (21.9)
B_6	10.4 (10.9)	7.4 (7.7)	4.2 (4.7)	1.3 (1.5)	11.5 (12.8)	1.7 (1.8)	10.4 (2.0)	3.6 (4.1)	7.2 (6.6)	3.0 (3.1)	3.3 (3.7)	3.2 (2.3)
B_5	30.1 (32.6)	18.3 (19.3)	17.7 (17.8)	7.5 (8.3)	nd	12.1 (11.9)	5.1 (5.3)	nd	12.6 (12)	8.2 (8.1)	nd	14.3 (14.8)
B_9	1.0 (1.0)	0.8 (0.8)	0.8 (0.8)	0.3 (0.3)	3.4 (3.4)	0.3 (0.3)	0.4 (0.4)	0.5 (0.5)	0.8 (0.8)	0.4 (0.4)	1.7 (1.6)	0.3 (0.3)
B_{12}	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
B_2	6.6 (7.2)	2.3 (2.4)	1.9 (2.1)	0.8 (0.8)	5.3 (5.9)	0.9 (0.9)	1.1 (1.2)	4.3 (4.9)	2.2 (2.5)	4.6 (4.5)	3.3 (3.9)	nd
B_8	0.7 (0.6)	0.14 (0.13)	0.09 (0.08)	0.07 (0.05)	ND	0.07 (0.05)	0.03 (0.02)	ND	0.14 (0.13)	0.06 (0.03)	0.38 (0.33)	nd

nd: not declared; ND: not found.
[a] Mean of two determinations.

Table 5
Precision data

Vitamin	Repeatability[a] CV_r (%)	Intermediate precision[b] CV_I (%)	Intermediate precision[b] CV_I (%)
B_1	3.1[c]	6.6[c]	9.6[c]
C	2.1[c]	5.0[c]	5.9[c]
PP	2.5[c]	5.9[c]	5.6[c]
B_6	6.2[c]	7.0[c]	4.2[c]
B_5	2.6[d]	4.4[d]	nd
B_9	5.3[c]	7.8[c]	7.0[c]
B_{12}	ND	ND	ND
B_2	2.3[c]	3.8[c]	5.3[c]
B_8	1.2[d]	4.1[d]	nd

nd: not declared; ND: not found.
[a] The repeatability data of each vitamin were obtained by analyzing in duplicate 12 premixes.
[b] Represents the intermediate precision that was obtained by analyzing each vitamin in two different premixes during 6 days.
[c] For vitamins B_1, C, PP, B_6, B_9 and B_2, the precision data were obtained on premixes 10 and 11.
[d] For vitamins B_5 and B_8, the precision data were obtained on premix 2.

B_6 (25 μg ml^{-1}), B_5 (100 μg ml^{-1}), B_9 (12.5 μg ml^{-1}), B_2 (12.5 μg ml^{-1}) and B_8 (10 μg ml^{-1}).

The applicability of the method was demonstrated by analyzing 12 premixes with different concentration of vitamins. The declared values and those found with our method are listed in Table 4. A *t*-test performed on the vitamins concentration obtained by the proposed method and the declared values demonstrated that the two sets values were comparable and do not show a bias at 95% confidence. Therefore, it was not relevant to assess the recovery of our method.

3.5. Repeatability and intermediate precision

The repeatability of the method was evaluated at different level of concentrations by analyzing in duplicate 12 premixes. The CV_r was below 6.5% (Table 5). The intermediate precision was determined at two levels of concentrations by analyzing two samples during 6 days and the CV_I values were below 9.6% (Table 5).

4. Conclusions

In the present study, a simple and rapid RP-LC method with endcapped column has been developed for the analysis of vitamins B_1, C, PP, B_6, B_5, B_9, B_2 and B_8. The successful application of the method to the simultaneous determination of water-soluble vitamins in premixes should make it highly desirable for the quality control of foodstuffs in the food industry. Moreover, the use of mobile phases consisting of water and acetonitrile that are fully compatible with MS technique will allow characterization of vitamin degradation products and therefore contribute to the better comprehension of the vitamin fate in food products.

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