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Quantitative analysis of water-soluble vitamins by ATR–FTIR spectroscopy

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HPLC and microbiology are the methods traditionally employed to control the vitamin content in food mixtures. However, considerations of cost, time of analysis per sample and complexities involved in the technique have hampered the acceptance of those methods for raw materials analysis. Fourier Transform Infrared (FTIR) spectroscopy has substantial potential as a quantitative quality control tool for the food industry. FTIR analysis methods are convenient, rapid, accurate, and in conjunction with Attenuated Total Reflectance (ATR) technology, simplify sample handling. The advantage of choosing FTIR as a quantitative technique lies in its ability to readily carry out multicomponent analysis in association with software such as Partial Least Squares (PLS) regression. Results presented here were obtained from water-soluble vitamins (B1, B2, B6 and Niacin) mixtures diluted into a glucose matrix without any chemical extraction.

INTRODUCTION

From a nutritional aspect, the vitamins are essential for health and especially for the growth of babies. So, the industry has to control the quantity and quality of vitamins in food such as baby food. Vitamin mixtures used as raw materials in agro-food are diluted in a glucose powder matrix. Depending on babyfood product, the concentration of each vitamin in the mixture is different and has to conform to strict specifications established by nutritionists. The potential financial rewards for substitution with a cheaper ingredient such as glucose are high. Before production, the level of vitamins must be precisely controlled. Regularly, each batch of sample has to be analysed. At present, to quantify four vitamins in a compound, two techniques are used: HPLC for vitamins B1 and B2 and microbiology for vitamins B6 and Niacin. It means that the analysis of one batch of compound is expensive and time consuming.

In recent years, much research was invested in trying to use mid-infrared spectroscopy for the analytical control of various matrices. This interest with vitamin analysis by FTIR is justified by specificity of the fundamental vibrational modes, widespread use of elaborate chemometrics methods for data processing and speed of quantitative determination.

The sampling method initially investigated was Diffuse Reflectance Infrared Fourier Transform Spectroscopy (DRIFTS) of powder, where light is scattered in all directions (Fuller and Griffiths, 1978), but it presents some disadvantages in sample preparation and in repeatability. Dry extract method (Dupuy *et al.*, 1992) cannot be used due to the sensitivity of vitamins to temperature (Le Corre *et al.*, 1987). Consequently, horizontal Attenuated Total Reflectance (ATR) infrared spectra of diluted samples is used. For this method, sample preparation is easy and rapid and then, quality control of the compound can be done without checking the making series in industry.

MATERIALS AND METHODS

Data analytical method

Complex mixtures used in this study contained a minimum of 90% glucose, vitamins B1: 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-5-(5-hydroxyethyl)-4-methyl thiazolium chloride monohydrochloride, B2: 7,8-dimethyl-10-(D-ribo-2,3,4,5-tetrahydroxypentyl) isoalloxazine and B6: 5-hydroxy-6-methyl-3,4-pyridinedimethanol hydrochloride, around 9 g kg^{-1} and Niacin:

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3–pyridinecarboxamide around 60 g kg^{-1}. They were prepared by ROCHE and used by DANONE. In the first stage of the study, 30 synthetic standards were prepared with the addition of pure ROCHE vitamins B1, B2, B6 and Niacin or Aldrich glucose in this sample. They are shacked with glass beads to improve the homogeneity. All samples were stored at 4°C and were allowed to warm to room temperature before analysis. In the second stage, polyvitamins mixtures were diluted with deionised water. For attenuated total reflectance (Harrick, 1979), the sample must be brought near the optical element where the light is totally internally reflected and where the sample interacts with the evanescent wave. The accessory used was a 6 reflection zinc selenide crystal. A reference spectrum of water left on the crystal was obtained and then, 1 ml of sample solution was deposited on the accessory. The crystal geometry was a 45° parallelogram with mirrored angles faces.

Standards spectra were recorded in triplicate on a Fourier Transform InfraRed Perkin Elmer Spectrometer (model PE2000g) run under the SPECTRUM operating system giving a total of 90 spectra. We co-added 100 scans of symmetrical interferograms and calculated the spectrum from 2000 to 800 cm^{-1} at 4 cm^{-1} of resolution. The application of interval data to food systems is limited because of the strong absorption of water across the mid-IR spectrum (Van de Voort, 1992). The mean of three spectra of the same sample is then calculated.

In parallel and depending on the type of vitamins, concentrations in the reference sample were confirmed by chromatography HPLC or by microbiology methods.

Vitamins B1 and B2 were analysed by HPLC (High Performance Liquid Chromatography) on a SHIMATZU CR-3A with a WATERS 600E pump. The method is adapted from 'Méthodes du Journal Officiel de la République Française, 25/11/1987'. It consists of extraction of vitamins B1 and B2 by acid and enzymatic hydrolysis, oxidation of vitamin B1 and then, separation of vitamins B1 and B2 by HPLC with fluorimetric measure. Vitamins B6 and Niacin are analysed by microbiological methods in the PASTEUR MERIEUX Laboratory in Lyon.

Spectral data treatment

The reproducibility of the signal at each wavelength is defined by the relative standard deviation according to the formulas (Dupuy *et al.*, 1994)

$$RSD = (\sigma/xm)x100$$

$$\sigma = \left(\sum_{i}^{N}(x_i - x_m)^2/(N-1)\right)^{1/2}$$

were xi represents the absorbance of one spectrum, xm is the average absorbance of all spectra of the same sample and N is the number of spectra.

For calibrations, the spectral data was first-derived or second-derived with the algorithm developed by Savitzky–Golay (Savitzky and Golay, 1964) in order to remove unwanted spectral variations as offsets.

Multivariate analysis method

The quantitative analysis was based on the additive nature of Beer's law. Mixtures of known concentrations are used as calibration standards and then the software directly calculates the concentrations of an unknown sample (Martens, 1979). In the case of mixtures, one of the major difficulties is the interference and overlapping of the absorption bands. This problem may be overcome by using powerful multicomponent quantitative analysis as Partial Least Squares regression. PLS (Fuller *et al.*, 1988; Haaland and Thomas, 1988) allows a sophisticated statistical approach using the full spectral regions rather than unique and isolated analytical bands. The algorithm is based on the ability to mathematically correlate spectral data to concentration matrix of interest while simultaneously accounting for all other significant spectral factors that perturb the spectrum. It is a multivariate regression method based on the use of latent variables. In PLS1 regression, each property is analysed individually with respect to spectral data, whereas PLS2 regression calibrates each property simultaneously. The evaluation of the calibration performance is estimated by computing the standard error of calibration after comparing the real concentration with the computed one for each component.

$$SEC = \left(\sum_{i=1}^{N}(Ci - C'i)^2/(N-1-p)\right)^{1/2}$$

where Ci is the known value, $C'i$ is the calculated value, N the number of samples and p is the number of independent variables in the regression.

The standard error of prediction gives the estimation of the prediction performance during the step of validation of the calibration equation:

$$SEP = \left(\sum_{i=1}^{M}(Ci - C'i)^2/(M-1)\right)^{1/2}$$

where Ci is the known value, $C'i$ is the value calculated by the calibration equation, and M is the number of prediction samples.

The relative error corresponds to this formula:

$$\text{error } \% = \frac{\text{SEP}}{\text{mean}}x100$$

The coefficient of variation (CV) is calculated on several spectra of the same sample in order to evaluate the reproducibility of the methods:

$$CV\% = (\sigma_c/Cm)x100$$

$$\sigma_c = \left(\sum_i^N (C_i - C_m)^2/(M-1)\right)^{1/2}$$

were *Ci* represents the concentration of one vitamin for one sample, *Cm* is the average concentration of the same vitamin for all samples and *M* is the number of samples.

RESULTS AND DISCUSSION

Sample preparation

The main interest of the method is to control water-soluble vitamins concentration in one powder mixture which is assumed to correspond to specified composition. It is necessary to prepare a range of synthetic standards close to the target mixture with addition of pure vitamins B1, B2, B6 and Niacin or glucose. The synthetic samples of vitamins must be representative of the compound under control, so, the concentration set should bracket the expected concentration of each component. The exact composition of each standard is calculated from the one of reference sample and corresponds to the expected value. Each standard must be unique to avoid overfitting. The concentration of B1, B2, B6 and Niacin in the mixtures varies between 6.9 and 9.4 g kg^{-1}, 6.2 and 9.8 g kg^{-1}, 8 and 11.8 g kg^{-1}, and 45 and 65 g kg^{-1}, respectively as seen in Table 2.

Data acquisition

Since the more convenient method for powder sample preparation is diffuse reflectance, it was investigated. The spectra of ten samples of the same compound diluted at 1% in KBr were recorded. The relative standard deviation calculated at different wavenumbers was about 10%. This poor result is in agreement with the sampling technique performances (Dupuy *et al.*, 1993) and probably emphasised by the inhomogeneity of vitamins in compounds especially when a weak amount of sample is used (only few milligrams). For this reason, a more important amount of sample to improve homogeneity was inevitable. Horizontal attenuated total reflectance was used for a long time as a qualitative analysis and more recently as a quantitative analysis of liquid solutions. One gram of mixture is weighed and water volume is adjusted to the required dilution. The solution is then deposed on the ATR accessory. The reproducibility (expressed in term of RSD) calculated at 6 wavenumbers of the spectrum of the mixture for a solution at 250 g l^{-1} (as seen in Table 1) is about 5% and justifies the choice of the ATR method. The poor result obtained near 1500 cm^{-1} (7.5%) can be explained by water but it is still an important region of the scale as the vitamins absorb.

Spectra of water solutions of vitamins B1, B2, B6, Niacin, polyvitamin compound and glucose are presented in Fig. 1. Pures vitamins were diluted in water in the same ratio as in the polyvitamin compound (2 g l^{-1} for B1 and B2, 2.5 g l^{-1} for B6 and 13.75 g l^{-1} for Niacin) in order to illustrate where characteristics absorption bands appear. Vitamins spectra are sufficiently

Table 1. Reproducibility of the method at different wavenumbers

Wavenumber (cm^{-1})	Reproducibility (%)
1729	5.5
1533	7.8
1393	5
1339	5.4
1221	3.8
955	4.6
	average: 5.3%

Table 2. Different component concentrations for polyvitamin compounds used in calibration and prediction

	Calibration set			
g kg^{-1}	B1	B2	B6	Niacin
Z	8.00	8.00	10.00	55.00
A	6.96	6.93	8.70	47.83
B	8.97	8.36	10.36	58.39
C	7.45	6.22	11.81	60.06
D	7.59	7.07	8.83	50.35
E	7.24	9.42	9.07	57.35
F	8.56	9.96	11.57	55.87
G	7.71	9.42	7.75	65.85
H	7.14	9.82	12.64	49.42
J	7.78	7.78	9.73	53.50
K	8.39	8.41	8.26	45.92
L	7.49	7.31	12.01	51.32
M	8.79	8.80	11.15	53.89
N	7.90	9.08	11.86	64.36
O	8.23	9.57	10.36	52.78
P	8.52	6.44	8.05	62.00
Q	8.16	6.41	10.58	63.50
R	8.31	7.84	10.47	61.47
S	9.43	6.78	8.47	46.61
Prediction set				
1	8.00	8.00	10.00	55.00
2	8.34	7.96	11.95	56.83
3	7.92	7.92	9.90	64.36
4	8.55	9.14	9.98	54.91
5	7.95	9.90	11.53	56.68
6	9.03	6.59	10.71	50.36
7	8.29	6.95	11.44	65.62
8	9.03	7.68	11.09	58.92
9	7.94	8.91	11.11	55.86
10	7.27	7.27	9.09	49.97
11	7.65	8.40	10.57	49.21
12	8.33	6.56	11.88	53.28

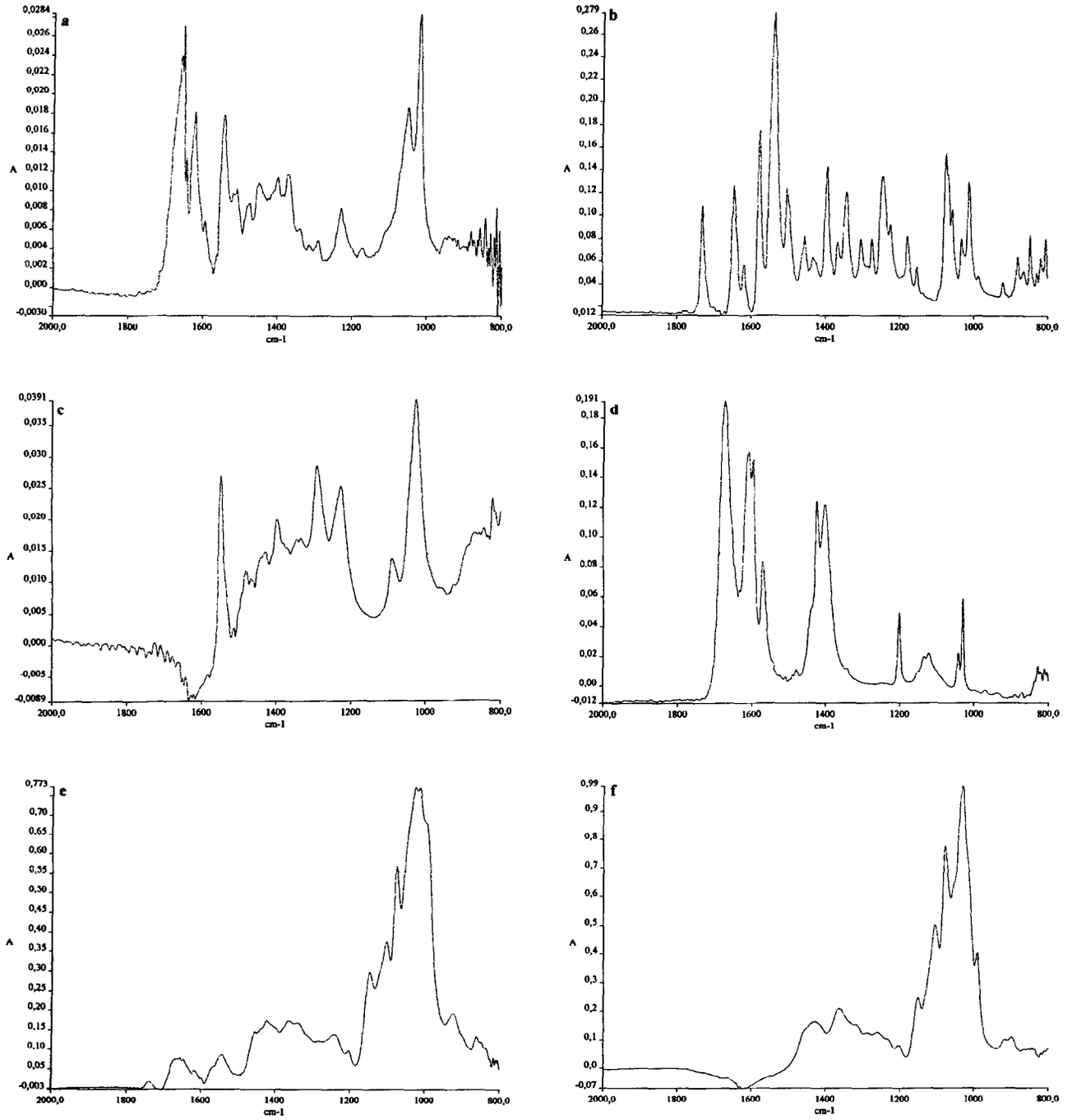

Fig. 1. Infrared spectra (a) of the vitamin B1; (b) of the vitamin B2; (c) of the vitamin B6; (d) of the Niacin; (e) of the glucose matrix; (f) of the vitamin compound.

specific and different to allow simultaneous determination of their concentration but we can see that specific intensities are very different and there are many overlapping bands. First, absorbance units for vitamins B1 (Fig. 1a) and B6 (Fig. 1c) are weak in comparison to vitamins B2 and Niacin. Since the molecular structure of vitamin B6 is composed of pyridine and alcohol functions which are also present in others vitamins, this product does not have very specific absorptions bands. In contrast, the vitamin B1 structure has a characteristic aromatic with a sulphur function. Although vitamin B2 (Fig. 1b) unit absorbance is high, the more intense band of riboflavin spectrum at 1540 cm^{-1} is common, with vitamins B1 (at 1544 cm^{-1}) and B6 (at 1547 cm^{-1}) and the other bands present only at medium intensity. The keton band at 1733 cm^{-1} is hidden by the strong absorption of the Niacin. Finally, the nicotinamide (Niacin) spectrum (Fig. d) is intense and the function amide is characteristic, with two bands between 1610 and 1600 cm^{-1}.

In the polyvitamin compound spectrum (Fig. 1e), the glucose (Fig. 1f) matrix is clearly recognised by a large

band between 1200 and 1000 cm^{-1} and characteristic of saccharides. In comparison to Niacin and vitamin B2, contribution of vitamins B1 and B6 in the polyvitamin spectrum is minor. The bands of each vitamin may be hidden by the glucose which is more intense. The absence of specific bands for vitamins B2 and B6 makes it difficult to quantify them, especially for vitamin B6 where the signal is weak.

The relationship between the signal on the first-derivative spectra and the concentration is studyied at several wavenumbers. Linearity of the ATR spectra is confirmed for concentrations between 50 and 400 $g\,l^{-1}$ of reference compounds in aqueous solution. Beer Lambert's law can be exploited directly without any mathematical correction for concentrations below 400 $g\,l^{-1}$.

Quantitative analysis

There are two steps in quantitative analysis: the calibration and the prediction that tests the calibration validity. In general, for n components to be analysed in the mid-IR, at least $(2n+2)$ standards are needed for the calibration (Beccard, 1987). Ten samples, at least are necessary, and, to improve the model, eighteen samples were used for calibration and twelve for prediction (Table 2). For vitamin B1, the important noise below 1000 cm^{-1}, as seen in Fig. 1 prevents a correct calibration curve. So, the interval data 1000–800 cm^{-1} is eliminated for this calibration. In order to eliminate regions contributing purely noise as water vapour, we used two regions, 1800–1700 cm^{-1} and 1450–850 cm^{-1} for the B2 vitamin calibration. During vitamin B6 analysis, the region 2000–1600 cm^{-1} is automatically suppressed from the calibration because there is no absorption band in this interval. The different interval data for each component justifies the choice of PLS1 regression.

After calibration, the program returns an error table showing the minimum prediction errors for the calibration samples (SEC) as a function of increasing factor number. The maximum number of factors used should be $n/3$ when n is the number of calibration samples. It means that the maximum factor number should be 6. Figure 2 represents the SEP calculated for each factor number in calibration and for each property. SEP decreases when the factor number increases until a local minimum at factor 6. Results are better on first-derivative for B1, B2 vitamins and Niacin and second-derivative for B6 vitamin, than absorbance spectra.

The concentration of 12 predicted samples is calculated with regard to the cross validation results (Lorber and Kowalski, 1990). In order to estimate the validity of the calibration, SEP and error are tabulated in Table 3. Errors in prediction vary from 4.27% for vitamin B1 to 7.46% for vitamin B2 (5.68.% for Niacin and 7.14% for vitamin B6). Figure 3 shows prediction results versus actual concentrations. The spectral loadings (or latent

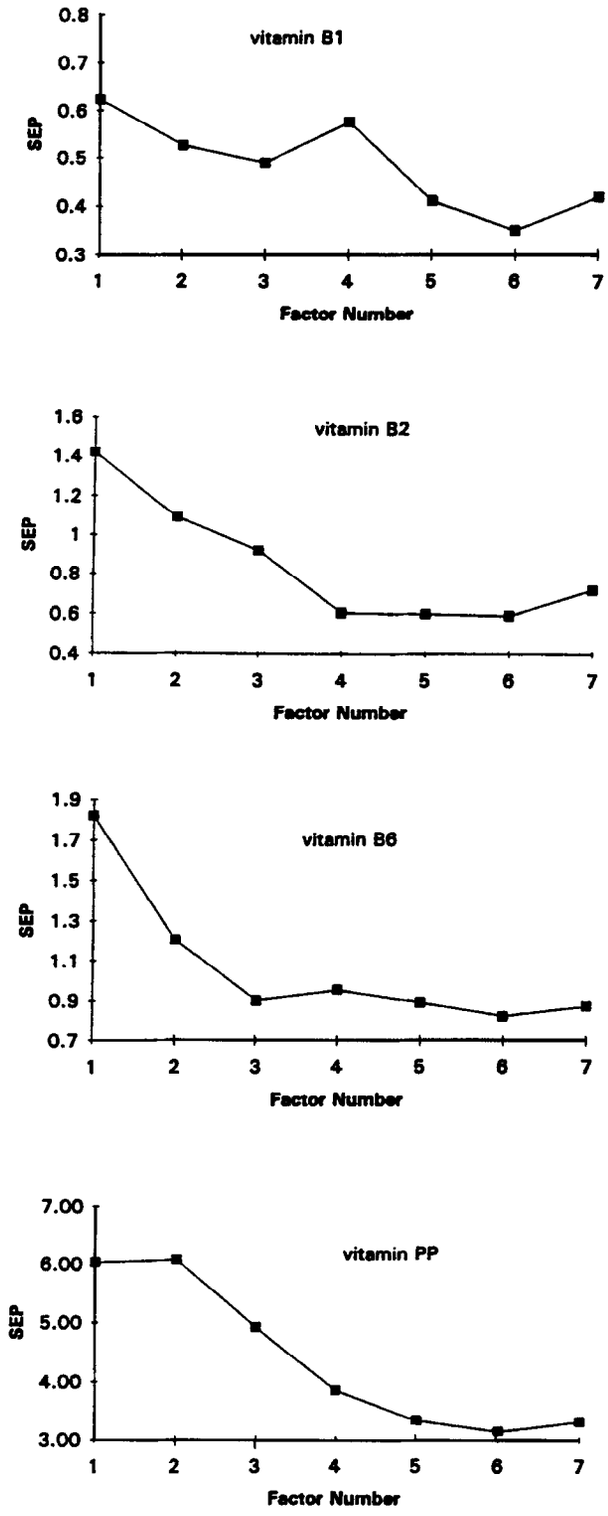

Fig. 2. SEP plot of calibration in function of number of factor and for each property.

variables) were studied to explain the poor prediction results for vitamins B2 (see Fig. 4) and B6 (see Fig. 5). On the second B2 loading, an important contribution can be seen to the 1720 cm^{-1} specific vibration band, but it seems insufficient to obtain a good prediction since the B2 analysis is more difficult. Moreover, there is no apparent significant information extracted in the

Table 3. Quantitative analysis results

Vitamin	Number of factor	SEC ($g\,kg^{-1}$)	SEP ($g\,kg^{-1}$)	Error (%)
B1	6 (first derived)	0.27	0.35	4.27
B2	6 (first derived)	0.14	0.59	7.46
B6	6 (second derived)	0.26	0.77	7.14
Niacin	6 (first derived)	1.23	3.18	5.68

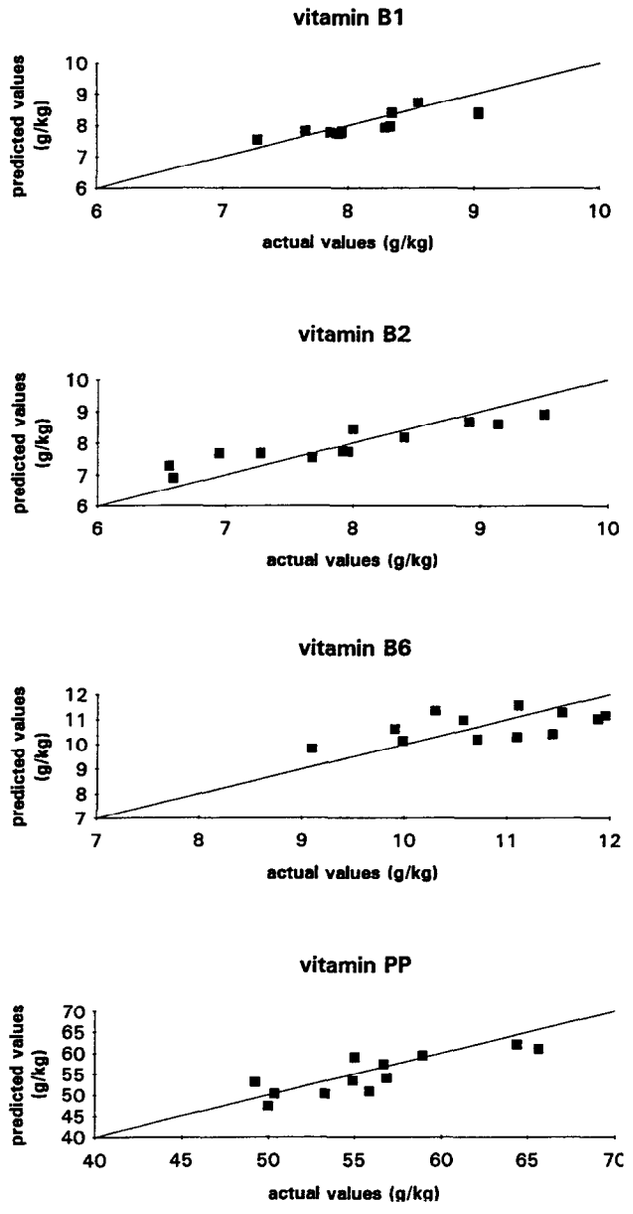

Fig. 3. Prediction results vs actual concentrations.

region 1400–1200 cm^{-1}. Nevertheless, this region was important because worse results were obtained when this spectral region was deleted.

So, we can deduce from these observations that the extraction of the information related to this vitamin was difficult and explained the poor prediction results. For vitamin B6, the intensity of each loading was low. So, it appears that the important overlapping with the matrix

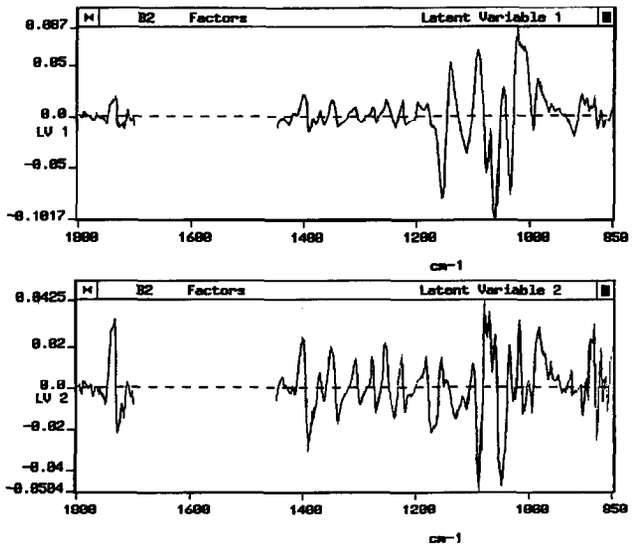

Fig. 4. First and second loading of vitamin B2.

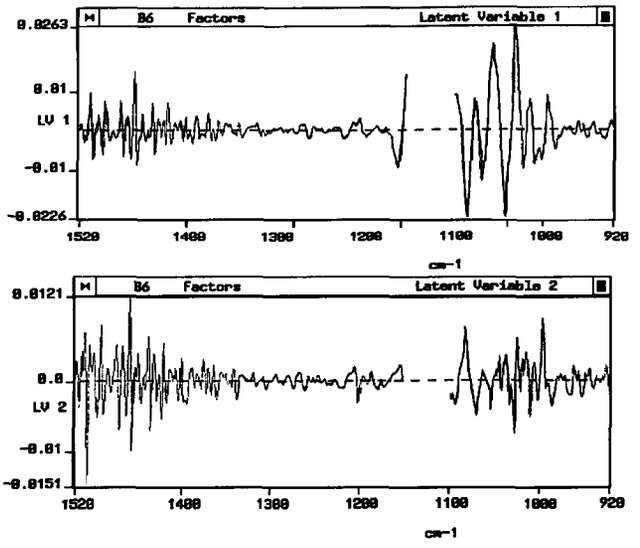

Fig. 5. First and second loading of vitamin B6.

spectrum dilutes the significant information through several loading. This result may also be as result of the fact that the best results are obtained after more mathematical treatment such as second derivative. The maximum of extracted information was in the 1100–950 cm^{-1} region under the glucose absorption.

Six samples were analysed by FTIR and microbiology to quantify vitamins B6 and Niacin and six samples were analysed in parallel by FTIR and HPLC to quantity vitamins B1 and B2. Table 4 represents results and SEP for those samples and for each method. FTIR method seems to be more precise than HPLC because standard errors of prediction are less important, even if for vitamin B2, the SEP is 0.59 $g\,kg^{-1}$. Microbiology values are near to expected ones. Errors calculated for FTIR are in within errors accepted for a quality control (between 5 and 10% depending on matrices).

Table 4. Comparison results of ATR–FTIR with HPLC and microbiology methods

$g\,kg^{-1}$	Vitamin B1			Vitamin B2		
Sample	Actual values	FTIR	HPLC	Actual values	FTIR	HPLC
1	8.00	7.75	7.85	8.00	8.43	7.44
4	8.55	8.71	7.94	9.14	8.59	8.6
5	7.95	7.81	7.76	9.50	8.90	8.24
8	9.03	8.41	8.6	6.59	6.90	6.01
7	8.29	7.92	7.66	6.95	7.68	6.41
11	7.65	7.81	7.06	8.40	8.17	7.84
SEP		0.36	0.52		0.55	0.79
$g\,kg^{-1}$	Vitamin B6			Niacin		
Sample	Actual values	FTIR	Microbiology	Actual values	FTIR	Microbiology
1	10	11.34	10.30	55.00	58.95	54.88
2	11.95	11.15	11.91	56.83	53.96	57.07
3	9.90	10.61	9.77	64.36	62.00	63.98
8	11.09	10.29	11.29	58.92	59.43	57.56
10	9.09	9.82	9.45	49.97	47.37	47.79
12	11.88	11.02	11.69	53.28	50.23	52.19
SEP		0.98	0.23		2.94	1.26

The repeatability of the infrared was then measured on the same compound prepared and tested ten times. The calculated coefficient of variation is between 1.93% for vitamin B1 and 7.79% for vitamin B6 (6.60% for vitamin B2 and 2.62 for Niacin). Errors correspond to sampling preparation and inhomogeneity of the mixture. The poor value of vitamin B6 certainly contributes to the difficulty to calibrate it. Otherwise, coefficients of variation are in the same order of error calculated in the model and the HPLC repeatability for two samples is accepted until 5%.

CONCLUSION

The analysis of water-soluble vitamins as B1, B2, B6 and Niacin in synthetic mixtures can be measured by ATR–FTIR with a precision of approximately 4–8%, whereas the time required to obtain the concentration of each component is approximately 10 min. The same method has also been adapted for another compound and gives a similar level of precision. For the quality control of the polyvitamin compound, the batch is accepted for production if each analysed vitamin concentration value corresponds to the target 10%. With regard to traditional methods, one analysis is about ten times less expensive and requires only 1 g of polyvitamin mixture. Considering that vitamin quantities are weak relating to glucose matrix (less than 1% of B1, B2 and B6) and that precision is limited in comparison to microbiology techniques, the ATR–FTIR seems to be suited for fast and low-cost analysis of each polyvitamins compounds mixture since it easily reveals when a new batch has defects in quality without a loss of time in production.

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