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Liquid chromatographic determination of vitamins B_1 and B_2 in foods. A collaborative study

F. Arella,[a] S. Lahély,[b] J. B. Bourguignon[c] & C. Hasselmann[ab]*

[a]*Laboratoire Interrégional de la Direction Générale de la Concurrence, de la Consommation et de la Répression des Fraudes, Chemin du Routoir, 67400 Illkirch, France*

[b]*Département des Sciences de l'Aliment, Faculté de Pharmacie, 74 route du Rhin, 67400 Illkirch, France*

[c]*Commission Générale d'Unification des Méthodes d'Analyse, DGCCRF, Ministère de l'Economie, 59 boulevard Vincent Auriol, 75703 Paris Cedex 13, France*

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A collaborative study was conducted to re-evaluate the chromatographic method proposed as the official French method in 1987 for the determination of vitamins B_1 and B_2 in foods. Twelve participants analysed nine samples containing various amounts of vitamin B_1 (from 1.1 to 4860 $\mu g\ g^{-1}$) and vitamin B_2 (from 2.1 to 871 $\mu g\ g^{-1}$). Reproducibility relative standard deviations (RSD_R) were generally between 13 and 21% for vitamin B_1 determination, between 5 and 13% for vitamin B_2 determination, and did not appear to depend on the vitamin concentration.

With all the foodstuffs studied, the recovery rate of the method was always superior to 89%, except with the chocolate powder, for which it reduced to approximately 50% for vitamin B_1 and 75% for vitamin B_2. In spite of this drawback, the chromatographic method proposed was confirmed as the official French method for vitamin B_1 and B_2 determination in foodstuffs for nutritional purposes. Copyright © 1996 Elsevier Science Ltd.

INTRODUCTION

Since 1987, an official method for the determination of vitamins B_1 and B_2 by reversed-phase liquid chromatography, including precolumn derivatization of thiamin to thiochrome and subsequent fluorescence detection, is widely used in French food control laboratories (Anon. (1987)). With time, it appeared that the recovery rate of the method [validated by testing a limited number of foodstuffs (yeast, cereal flour, biscuit, baby food)] was not always satisfactory, especially for the determination of vitamin B_1 in foodstuffs containing chocolate. For this reason, it seemed necessary to revalidate the method by testing a wider variety of foodstuffs through collaborative studies involving a large number of participants.

In the course of the elaboration of this method, through several collaborative trials, special attention had been given to the choice of enzymes used to release the vitamins from their phosphate esters (Hasselmann *et al.*, 1989). By using takadiastase alone, poor reproducibilities were obtained for the determination of vitamins B_1 and B_2 in samples containing large amounts of natural phosphorylated vitamins. The observed variability was most probably due to differences in phosphorylytic activity of this enzyme, according to its commercial origin. Finally, after testing various enzyme preparations, it appeared that a mixture of takadiastase (500 mg)–β-amylase (50 mg), in an aliquot containing *ca* 40 μg of vitamin, led to a total dephosphorylation in the foodstuffs studied (absence of thiamin or riboflavin phosphate peak in the chromatogram). In other respects, satisfactory reproducibilities (coefficients of variation $< 16\%$) were noted for both vitamin determinations. On account of the results obtained, this enzyme mixture was retained for the present study. Other extraction and hydrolysis protocols, slightly different of the previous one, have been proposed for the determination of vitamins B_1 and B_2 (see the relevant study of Hollman *et al.*, 1993), but none of them, at the moment, appears clearly more effective than the others.

MATERIALS AND METHODS

Participants

Twelve participants were analysts in food industries, commercial laboratories, universities and government laboratories.

*To whom correspondence should be addressed.

In a first collaborative study, they received nine different test samples: yeast, baby food (with vegetables), cereal (A) and solution for tube-feeding (obtained from Créalis-BSN, Brive, France), powdered milk, cereal (B), meal with fruit and chocolate powder (obtained from SOPAD-Nestlé, Cergy, France) and food complement (obtained from BIPEA, Gennevilliers, France).

In a second collaborative study, they received four chocolate powders, obtained from SOPAD-Nestlé, Cergy, France (samples 1–3) and CPC-France, Ludres, France (sample 4).

For each foodstuff tested, the laboratories taking part were asked to perform the determination in duplicate.

Reagents

Acetic acid solution
For a 0.02 M solution, 0.6 ml of glacial acetic acid (Carlo Erba, or equivalent) was dissolved in 500 ml distilled water.

Sodium acetate solution
To produce a 2.5 M solution, 170.10 g of sodium acetate trihydrate (Prolabo normapur, or equivalent) was dissolved in 500 ml distilled water.

Stock standard solutions
Stock standards of vitamin B_1 (thiamin hydrochloride, Merck, or equivalent) (1 g litre^{-1}) were prepared in distilled water. Stock standards of vitamin B_2 (Merck, or equivalent) (0.1 g litre^{-1}) were prepared as follows: 50 mg of vitamin B_2 were weighed and put in a 1000 ml conical flask. 0.02 M acetic acid (400 ml) was added; complete dissolution was obtained by moderate heating and shaking (1 h). After cooling, the solution was adjusted to pH 4.5 with 2.5 M sodium acetate, then put in a 500 ml volumetric flask and made up to 500 ml with distilled water.

Hydrochloric acid solution
For a 0.1 M solution, 8.5 ml of hydrochloric acid 36% min (Prolabo normapur, or equivalent) was dissolved in 1000 ml distilled water.

β-Amylase from barley
28 U mg^{-1} (Merck, or equivalent).

Takadiastase from Aspergillus orizae
Serva, or equivalent.

Sodium hydroxide solution
For a 3.75 M solution, 15 g of sodium hydroxide (Carlo Erba, RPE ACS, or equivalent) was dissolved in 100 ml distilled water.

Potassium ferricyanide solution
For a concentration of 10 g litre^{-1}, 100 mg of potassium ferricyanide (Prolabo, or equivalent) was dissolved in 10 ml distilled water (stable for 1 week at 4°C).

Sodium acetate solution
To produce a 0.05 M solution, 6.80 g of sodium acetate trihydrate (Prolabo normapur, or equivalent) was dissolved to 1000 ml with distilled water in a volumetric flask.

Methanol
Prolabo, chromanorm for HPLC, or equivalent.

Sample preparation

A finely ground sample (5 g for food sample containing about 8.0 μg g^{-1}) was weighed in a 250 ml conical flask; 0.1 M hydrochloric acid (65 ml) was added. The conical flask was put in a water bath at 100°C for 30 min. After cooling, the solution was adjusted to pH 4.5 with 2.5 M sodium acetate. β-Amylase (50 mg) and takadiastase (500 mg) were added with a small quantity of water. The solution was incubated 18 h in an oven at 37°C, then diluted to 125 ml with distilled water in a volumetric flask. The supernatant was filtered through a filter paper. The filtrate obtained after a second filtration through a cellulose acetate filter (0.2 μm) was used for the chromatographic determination of vitamin B_2.

An aliquot of the first filtrate (1 ml) was added to a 15 ml flask with an alkaline solution (3 ml) of potassium ferricyanide (1 ml of 1% potassium ferricyanide solution and 24 ml of 3.75 M sodium hydroxide solution). The solution was shaken, left to stand for exactly 1 min, then passed through a Sep Pak C_{18} cartridge (Waters). The cartridge was washed with 0.05 M sodium acetate (10 ml), then eluted with methanol–water (70:30 v/v) (8 ml). The elute was made up to 10 ml with the same solution and filtered through a cellulose acetate filter (0.2 μm). This filtrate was used for the chromatographic determination of vitamin B_1 (as thiochrome).

Chromatographic determination

Separation by reversed-phase HPLC was accomplished with an octadecylsilyl stationary phase (4 mm i.d. × 250 mm; 10 μm particle size) isocratically with a mobile phase consisting of methanol–0.05 M sodium acetate (30:70 v/v). The separation was performed at ambient temperature at a flow rate of 1 ml min^{-1}. The fluorometric detector operated at an excitation wavelength of 366 nm and at an emission wavelength of 435 nm for thiochrome and at an excitation wavelength of 422 nm and at an emission wavelength of 522 nm for vitamin B_2. Separate chromatographic runs were carried out for the determination of each vitamin. The injection volume was 20 μl. Data were quantified using external calibration.

Standard solutions for external calibration

Stock standard solution of vitamin B_1 was firstly diluted to 1/100, then again to 1/10, 1/25 and 1/50 with distilled water. An aliquot of each solution (1 ml) was treated as above (see section on Sample Preparation) in order to

transform vitamin B_1 into thiochrome. Concentrations of calibrated solutions obtained (expressed as thiamin hydrochloride) were, respectively, 1.00, 0.40 and 0.20 μg ml^{-1}.

Stock standard solution of vitamin B_2 was firstly diluted to 1/20, then again to 1/5, 1/10 and 1/25 with distilled water in order to obtain calibrated solutions containing, respectively, 1.00, 0.50 and 0.20 $\mu g\ ml^{-1}$ of vitamin B_2. These solutions were filtered through a cellulose acetate filter (0.2 μm) and the filtrates were used for external calibration.

Recovery rates

For each foodstuff, two series of two samples were weighed exactly. The two samples of the first series (samples 1 and 2) were analysed as described above. Concerning the samples of the second series (samples 3 and 4), an addition of vitamin was carried out before the acid hydrolysis step (see section on Sample Preparation).

The mean recovery rate for the method is given by:

$$\tau \equiv \frac{2(x_3 + x_4) - (x_1/m_1 + x_2/m_2)(m_3 + m_4)}{4x_0}$$

in which: m_1, m_2, m_3 and m_4 represent the weight (in g) of the different samples; x_1, x_2, x_3 and x_4 represent the amount of vitamin (thiamin hydrochloride or vitamin B_2, in $\mu g\ ml^{-1}$) in the solution injected for the sample studied; and x_0 represents the amount of vitamin added (thiamin hydrochloride or vitamin B_2, in $\mu g\ ml^{-1}$) present in the solution injected for samples 3 and 4 [in order to obtain a x_0 value of 0.40 $\mu g\ ml^{-1}$, 500 μl of standard solution of vitamin (0.1 g $litre^{-1}$) must be added to the extractive solution before the acid hydrolysis step].

Calculations

The amount X of vitamin in a sample analysed (expressed as thiamin hydrochloride or vitamin B_2, in $\mu g\ g^{-1}$) is given by the formula:

$$X = \frac{125x}{m.\bar{\tau}}$$

in which: x represents the amount of vitamin ($\mu g\ ml^{-1}$) in the solution injected for the sample studied; m represents the weight (in g) of the sample; and $\bar{\tau}$ represents the mean recovery rate of the method.

Statistical methods

Statistical interpretation of the results was carried out according to the ISO 5725 standard. Cochran test was performed to remove data showing significantly greater variability among replicate (within laboratory) analyses than other laboratories for a given sample. The Dixon test was performed to remove laboratories with extreme averages.

Precision of the method was estimated by calculating the following parameters: repeatability and reproducibility standard deviations (S_r and S_R), relative standard deviations (RSD = 100 SD/X) for repeatability (RSD_r) and for reproducibility (RSD_R), repeatability ($r = 2.8\ S_r$) and reproducibility ($R = 2.8\ S_R$). S_r represents the standard deviation (SD) within laboratory and S_R the standard deviation amoung laboratories.

Table 1. Collaborative results (in duplicate) on LC determination of thiamin hydrochloride ($\mu g\ g^{-1}$) in various foodstuffs

Laboratory	Tube-feeding solution	Baby food	Powdered milk	Meal with fruits	Yeast	Cereal A	Chocolate powder	Cereal B	Food complement
1	0.8	1.8	5.2	11.4	13.3	3.7[a]	17.0	36.1	4270
	0.8	1.7	5.2	11.3	13.1	3.9[a]	17.8	34.8	4260
2	0.8	3.0	4.9	11.9	14.0	13.7	15.2	25.0	5460
	0.8	3.0	4.4	11.9	14.0	13.7	13.0	26.2	5430
3	1.1	1.8	5.3	12.0	12.6	13.5	12.4	33.7	4190
	1.2	1.9	5.3	11.0	12.6	13.0	13.0	31.6	3930
4	—	1.9	5.4	9.0	10.8	11.1	16.3	32.6	4520
	—	2.2	5.1	7.4	11.3	11.0	16.1	33.2	5240
5	1.1	1.9	6.3	12.8	15.3	13.8	20.0	62.1[b]	4390
	1.1	2.1	6.6	12.8	15.3	14.3	22.6	31.6[b]	4400
6	—	1.9	5.7	7.9	13.8	12.2	15.8	29.7	—
	—	1.7	6.0	7.9	15.4	12.0	16.2	30.4	—
7	0.7	1.8	6.4	9.0	12.9	19.8	13.3	28.5	4780
	0.7	1.8	6.7	9.3	13.3	20.7	14.0	26.6	4730
8	1.2	2.2	7.1	9.7	15.1	13.7	37.9[a]	32.5	5360
	1.2	1.9	6.6	10.1	12.9	13.1	38.4[a]	32.1	5100
10	1.2	1.5	4.0	8.9	11.9	12.2	13.3	28.1	3890
	1.2	1.6	4.6	8.0	11.9	12.5	13.5	24.6	4070
11	1.7	2.4	6.0	12.0	9.5	15.3	15.0	24.1	5360
	1.9	2.4	6.0	12.5	12.1	16.2	13.2	25.5	6220
Mean ($\bar{X}$)	1.1	2.0	5.6	10.4	13.1	14.2	15.5	29.5	4860

[a]Dixon test outlier.
[b]Cochran test outlier.

Table 2. Collaborative results (in duplicate) on LC determination of riboflavin ($\mu g\ g^{-1}$) in various foodstuffs

Laboratory	Tube-feeding solution	Baby food	Powdered milk	Meal with fruits	Yeast	Cereal A	Chocolate powder	Cereal B	Food complement
1	2.0	3.6	6.6[a]	5.8	50.9	2.8	12.6	23.7	884
	2.0	3.5	6.5[a]	5.7	51.0	2.7	12.4	23.3	859
2	2.1	2.9	11.4	5.7	48.8	4.9	14.2	23.5	826
	2.1	2.9	11.1	6.0	49.6	4.9	14.4	24.8	787
3	2.0	2.9	11.1	6.1	46.7	4.9	13.6	26.7	889
	2.0	2.9	11.1	5.9	44.4	4.9	14.1	25.6	933
4	—	4.2[b]	11.5	7.5[a]	46.6	11.3[b]	13.5	27.2	768
	—	3.8[b]	12.1	7.2[a]	45.1	7.5[b]	12.9	26.9	793
5	2.2	3.0	10.5	6.2	39.4	4.7	12.7	25.3	866
	2.1	3.1	10.5	6.1	38.7	4.8	12.1	24.9	843
6	—	2.8	11.4	6.3	40.5	4.3	11.1	23.6	—
	—	2.8	11.2	6.4	40.3	4.8	10.9	24.0	—
7	2.1	2.8	11.5	6.0	42.0	4.6	10.8	24.3	894
	2.1	2.8	11.3	5.7	43.4	4.5	11.1	24.4	844
8	2.0	3.0	9.7	5.4	43.6	4.3	10.3	24.9	885
	2.0	3.0	9.5	5.4	43.4	4.3	10.9	25.4	873
9	—	2.6	11.9	6.1	37.0	4.0	13.9	27.2[b]	—
	—	2.5	11.8	6.5	39.7	3.6	13.0	29.9[b]	—
10	2.2	3.4	12.5	6.1	42.8	4.3	13.3	21.4	945
	2.2	3.4	12.7	5.9	42.3	4.3	13.0	21.3	969
11	2.6[a]	3.1	11.0	6.1	39.1	4.5	13.3	27.6	887
	2.6[a]	3.1	11.6	6.2	39.7	4.7	13.1	27.7	931
Mean ($\bar{X}$)	2.1	3.0	11.3	6.0	43.4	4.3	12.6	24.8	871

[a]Dixon test outlier.
[b]Cochran test outlier.

RESULTS AND DISCUSSION

The method proposed in the collaborative studies for vitamin B_1 and B_2 determination was the method described by Hasselmann *et al.* (1989) with only minor modifications [filtration of the solution obtained after the incubation step through a filter paper and slight modification of the methanol–0.05 M sodium acetate proportion of the mobile phase (30:70 v/v instead of 40:60 v/v) without adjusting to pH 4.5).

All foodstuffs studied in the first collaborative study, with the exception of the tube-feeding solution and food complement, were complex matrix foodstuffs.

The amounts of vitamins B_1 and B_2, measured during this collaborative study, are indicated in Tables 1 and 2.

Certain laboratory results were rejected, either because they showed significantly greater variability among replicate (within laboratory) analyses than did other laboratories for a given sample (Cochran test, one case for vitamin B_1, three cases for vitamin B_2) or because they showed extreme averages (Dixon test, two cases for vitamin B_1, three cases for vitamin B_2). The rejection rate (three out of 87 for vitamin B_1, six out of 94 for vitamin B_2) is not excessive. No more than one result was rejected for a vitamin determination in a given foodstuff.

Statistical analysis of the results are presented in Table 3 (vitamin B_1) and Table 4 (vitamin B_2).

For vitamin B_1 determination, the relative standard deviation for repeatability (RSD_r) was always between

Table 3. Summary statistics[a] for collaborative data on LC determination of thiamin hydrochloride ($\mu g\ g^{-1}$) in various foodstuffs

Material	Tube-feeding solution	Baby food	Powdered milk	Meal with fruits	Yeast	Cereal A	Chocolate powder	Cereal B	Food complement
n	8	10	10	10	10	10	10	10	9
n'	8	10	10	10	10	9	9	9	9
N	16	20	20	20	20	18	18	18	18
($\bar{X}$)	1.1	2.0	5.6	10.4	13.1	14.2	15.5	29.5	4860
S_r	0.1	0.2	0.4	0.7	1.2	0.6	1.3	1.8	390
RSD_r (%)	7	8	7	7	9	4	8	6	8
r	0.2	0.5	1.0	2.0	3.4	1.6	3.6	4.9	1110
S_R	0.4	0.4	0.8	1.9	1.7	2.7	2.8	4.1	750
RSD_R (%)	32	21	16	19	13	19	19	14	15
R	1.0	1.2	2.5	5.5	4.8	7.5	8.0	11.6	2120

[a]Symbols used: n, number of participants; n', number of participants retained; N, number of results; ($\bar{X}$) ($\mu g\ g^{-1}$), material mean; for others symbols, see section on Statistical Methods.

4 and 9% and did not appear to depend on the vitamin concentration. As far as the reproducibility of the measurements was concerned, a similar result was observed. The relationship between the reproducibility (R) and the vitamin concentration was roughly linear ($R=0.392$ [vitamin B_1] + 0.080, with a correlation factor of 0.972). For all foodstuffs, except for tube-feeding solution which contained very low level of vitamin B_1, the relative standard deviations were always between 13 and 21%.

For vitamin B_2 determination, the relative standard deviations for repeatability and reproducibility were always superior to the values obtained for the determination of vitamin B_1, most likely because the analytical protocol for vitamin B_1 is more complex (need to carry out a precolumn derivatization). The mean value for RSD_R went down to (9 ± 4)% and, as for vitamin B_1, the relationship between the reproducibility R and the vitamin B_2 concentration was roughly linear ($R=0.265$ [vitamin B_2]-0.008, with a correlation factor of 0.984).

Table 4. Summary statistics[a] for collaborative data on LC determination of riboflavin ($\mu g\ g^{-1}$) in various foodstuffs

Material	Tube-feeding solution	Baby food	Powdered milk	Meal with fruits	Yeast	Cereal A	Chocolate powder	Cereal B	Food complement
n	8	11	11	11	11	11	11	11	9
n'	7	10	10	10	11	10	11	10	9
N	14	20	20	20	22	20	22	20	18
$(\bar{X})$	2.1	3.0	11.3	6.0	43.4	4.3	12.6	24.8	871
S_r	0.0	0.1	0.3	0.2	1.3	0.2	0.5	0.7	34
RSD_r (%)	<1	3	3	4	3	5	4	3	4
r	0.0	0.2	0.8	0.6	3.7	0.6	1.4	1.8	95
S_R	0.1	0.3	1.0	0.3	4.3	0.7	1.3	1.9	59
RSD_R (%)	4	10	9	6	10	16	11	8	7
R	0.2	0.9	2.7	0.9	12.0	1.9	3.7	5.3	166

[a]Symbols used: n, number of participants; n', number of participants retained; N, number of results; $(\bar{X})$ ($\mu g\ g^{-1}$), material mean; for others symbols, see section on Statistical Methods.

Table 5. Summary statistics[a] for collaborative data on LC determination of thiamin hydrochloride ($\mu g\ g^{-1}$) in various chocolate powders (A, hydrolysis with 0.1 M HCl; B, hydrolysis with 0.5 M HCl)

Material	Chocolate powder (1)		Chocolate powder (2)		Chocolate powder (3)		Chocolate powder (4)	
	A	B	A	B	A	B	A	B
n	11	11	11	11	11	11	11	11
n'	9	11	11	11	11	11	11	11
N	18	22	22	22	22	22	22	20
$(\bar{X})$	8.5	12.1	6.7	10.1	5.5	6.0	2.0	4.1
S_R	2.0	2.0	1.0	1.8	1.3	1.0	0.5	0.9
RSD_R (%)	24	17	15	15	24	17	25	22
R	5.6	5.6	2.8	5.1	3.6	2.8	1.4	2.5

[a]Symbols used: n, number of participants: n', number of participants retained; N, number of results; $(\bar{X})$ ($\mu g\ g^{-1}$), material mean; for others symbols, see section on Statistical Methods.

Table 6. Summary statistics[a] for collaborative data on LC determination of riboflavin ($\mu g\ g^{-1}$) in various chocolate powders[b] (A, hydrolysis with 0.1 M HCl; B, hydrolysis with 0.5 M HCl)

Material	Chocolate powder (1)		Chocolate powder (2)		Chocolate powder (3)	
	A	B	A	B	A	B
n	10	10	10	10	10	10
n'	8	9	10	10	10	10
N	16	18	20	20	20	20
$(\bar{X})$	17.6	16.2	10.2	9.8	3.5	3.3
S_R	1.0	1.5	0.6	0.7	0.4	0.3
RSD_R (%)	6	10	6	7	12	9
R	2.8	4.2	1.7	2.0	1.1	0.8

[a]Symbols used: n, number of participants: n', number of participants retained; N, number of results; $(\bar{X})$ ($\mu g\ g^{-1}$), material mean; for others symbols, see section on Statistical Methods.[b]The amount of vitamin B_2 in chocolate powder (4) was negligible.

For both vitamins B_1 and B_2, the recovery rates of the method were always satisfactory, except for the determination of vitamins in the chocolate powder. The mean values ranged from 89% (food complement) to 100% (yeast and cereal A) for vitamin B_1 determination, and from 89% (powdered milk) to 94% (yeast) for vitamin B_2 determination. For determination of vitamins B_1 and B_2 in the chocolate powder, the values reduced, respectively, to (49 ± 6) and (75 ± 9)%. These results were not surprising. Such recovery rates had already been mentioned to the Commission Générale d'Unification des Méthodes d'Analyse (Paris, France) by analysts in food industries, who never found in chocolate powders more than half of the vitamin B_1 added, and were confirmed by routine analysis carried out in French official laboratories of food control. Most probably, each vitamin and a component of the chocolate form a molecular complex, only partially released by hydrochloric hydrolysis.

During previous routine laboratory analyses, it had been noted that an increase in hydrochloric acid concentration did not modify experimental levels of vitamin B_1 in foodstuffs containing little (as in chocolate cereals) or no chocolate. However, this protocol modification gave rise to significantly higher (30–50% increase) vitamin B_1 levels in chocolate powders and thus to a significant increase in the recovery rate of the method. A higher hydrochloric acid level (1 M) did not however improve the results, but in fact gave rise to numerous disturbing peaks during chromatographic elution. An interlaboratory study concerning the analysis of vitamins B_1 and B_2 in four different chocolate powders was therefore carried out in order to confirm or refute these preliminary indications. The results obtained (Tables 5 and 6) only partially confirmed them. In the case of vitamin B_1, an increase of the experimental levels was observed in all the samples analysed with an increase in hydrochloric acid concentration from 0.1 to 0.5 M, but there was considerable sample variation [weak ($< 10\%$) in sample 3, very high (near 100%) in sample 4, about 40–50% in the two other samples]. On the other hand, in the case of vitamin B_2, no effect of hydrochloric acid concentration could be detected in the samples analysed. This change in experimental protocol for the proposed method did not therefore guarantee a satisfactory recovery rate for vitamin B_1 and B_2 determination in chocolate powders.

As a result, the Commission has decided not to modify the official French method for the determination of the two vitamins, although aware that the analysis of chocolate powders presents a problem. In fact it is not certain that the important fraction of the vitamins non-released by acid hydrolysis is biologically available. Under these conditions it could be queried whether the vitamin B_1 and B_2 determination in chocolate powders should take into account the recovery rate for this method, or not.

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