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Flow-injection solvent extraction without phase separation Fluorimetric determination of thiamine by the thiochrome method

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Abstract

Two modes of liquid–liquid extraction in flow-injection systems were compared and applied to the fluorimetric determination of thiamine (Vitamin B_1). The first included phase segmentation, but fluorescence was measured without phase separation. In this mode, thiamine was detected at concentrations higher than 8 μg/l with a linear application range of 30–2000 μg/l, an R.S.D. of 1.9% (150 μg/l, $n = 10$) and a sampling frequency of 60/h. In the second mode, a single segment of organic solution was injected into the aqueous stream and fluorescence was also measured without phase separation. Using this mode, concentrations of thiamine higher than 1 μg/l were detected, with a linear application range between 5 and 280 μg/l, an R.S.D. of 2.4% (150 μg/l, $n = 10$) and a sampling frequency of 60/h. The two forms were applied to the analysis of thiamine in pharmaceuticals.

Keywords: Liquid–liquid extraction; Flow-injection; Thiamine; Pharmaceuticals

1. Introduction

Since it was first reported in 1978, solvent extraction in flow-injection systems (FI-SE) has been used in a large number of analytical procedures [1–3]. Most such applications include segmentation and phase separation, but the drawbacks involved in these steps have led to new modes without segmentation [4,5], without separation [6–11] or with neither [1,12–15].

In the present work, two different modes of FI-SE were considered and compared on the basis of their application for the determination of thiamine (Vitamin B_1). In the first form (mode A), measurements were carried out with segmentation but without phase separation. In the second form (mode B), a single segment of organic solvent was injected into an aqueous stream, and measurements were also performed without phase separation.

Several analytical methods have been proposed for the determination of thiamine, e.g. spectrophotometry [16], high-performance liquid chromatography (HPLC) [17], micellar liquid chromatography [18], fluorimetry [19–25] and chemiluminescence methods [26–27], in some cases including FI methodology [20–34]. Determination based on measurement of the fluorescent product thiochrome, obtained by oxidation of the vitamin with $Fe(CN)_6^{3-}$ in basic media is an old batch method [19] used for the determination of this vitamin in products destined for animal nutrition, biological samples and pharmaceutical products. This procedure was the official USP method up to 2005 but has now been replaced by HPLC, although it still serves as an identification test [35]. The method has been automated by flow-injection (FI) with fluorimetric and chemiluminescence detection [28–30]. The oxidation of thiamine has been carried out with other different oxidizing agents, such as Hg(II) or Cu(II) or electrochemical oxidation [31].

The flow-injection method optimised by Karlberg and Thelander [28] involves extraction in chloroform and fluorimetric detection once the organic phase had been separated from the aqueous phase with a membrane.

The proposed method modifies the Karlberg system, avoiding the phase separator in order to improve the sensitivity of the process. Thus, the potential of the "flow-injection extrac-

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tion with phase segmentation and no separation" technique is demonstrated again [36]. With the same aim, and in order to achieve a reduction in the amount of organic solvent used, the monosegmented technique has been optimised. In this method, a small volume of organic phase is inserted in the aqueous phase where the thiochrome is formed and is extracted while travelling to the detector.

2. Experimental

2.1. Reagents

A 5 mg/l stock solution of Vitamin B_1 from thiamine hydrochloride in distilled–deionised water was prepared daily. Different concentrations (range: 10^{-2} to 10^{-5} M) of potassium ferricyanide were prepared by dissolving the solid salt in distilled–deionised water and adjusting pH with 1 M sodium hydroxide. These solutions were prepared when used and were protected from the light with aluminium foil. All chemicals were analytical-reagent grade and distilled–deionised water was used throughout.

Several types of tablets (Hidroxil, ALMIRALL, Benexol, ROCHE and Gammamida Complex LAPROQUIFAR) and solutions (Co Hepa B12, SMALLER and Antineurina MABO FARMA) were also used.

2.2. Apparatus and materials

Minipuls HP4 (Gilson, France) peristaltic pumps with silicone or vinyl pump tubes. Chloroform was propelled by means of a displacement bottle (Tecator, Sweden). PTFE sample-injection valve (Rheodyne). Detection was performed with a RF-5000 spectrofluorimeter (Shimadzu, Japan) fitted with a DR-15 data processor and a 25-μl flow-cell (Hellma, Germany). PTFE tubing of 0.5 mm i.d. with standard tube fittings and connectors (Upchurch Scientific, Inc.) was used.

2.3. Flow systems

The flow-injection systems used in the experiments are shown in Fig. 1.

In mode A (Fig. 1A), water is used as carrier stream (C_1) into which aqueous solutions of samples or standards can be injected with a rotary injection valve (I) Potassium ferricyanide at basic pH is pumped into channel C_2. These two streams merge and start the oxidative reaction from thiamine to the thiochrome in the reaction coil (E_1). Water is pumped through channel C_3 into a displacement bottle (DB) containing chloroform. The organic phase is displaced and merges with the aqueous stream containing the thiochrome at the phase segmentor (PS). Extraction of the thiochrome takes place in the extraction coil (E_2). The stream continues until it reaches the detector flow cell and the analytical signal is measured. After the detector, a restriction coil is added to the flow system to prevent the formation of bubbles in the flow cell.

In mode B (Fig. 1B), chloroform is injected into an aqueous stream with the thiochrome formed. The loop of the injection valve is filled with chloroform by means of a displacement bottle.

3. Results and discussion

3.1. Fluorescence spectra

In preliminary experiments, the emission and excitation spectra of the thiochrome in chloroform were recorded. Maximum fluorescence intensity was observed at $\lambda_{ex} = 375$ nm and $\lambda_{em} = 440$ nm.

It may be observed that under all conditions, the yield in the separation process was less than 100% [28]. This indicates that after the phase segmentation, the thiochrome is distributed in segments of the organic and aqueous phases, each with a different concentration of thiochrome. Thus, the analyte is fractionated into the aqueous/organic phases when it reaches the

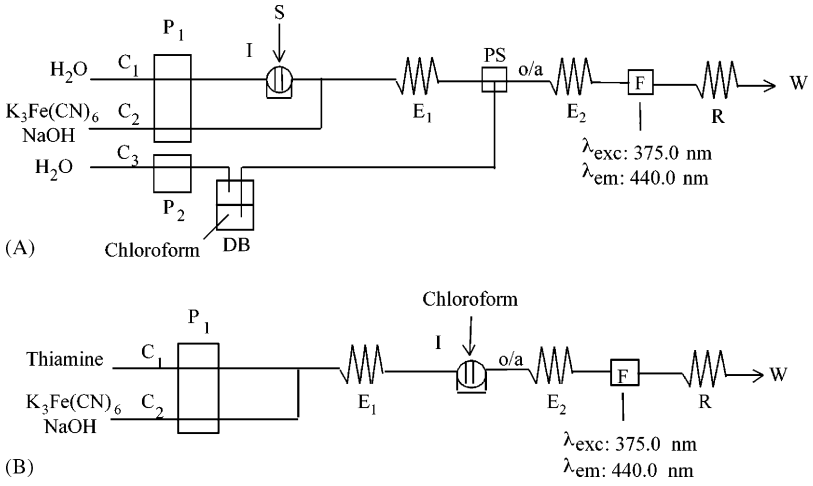

Fig. 1. Flow-injection systems: (A) mode A, with segmentation and no separation; (B) mode B, with a single segment of organic phase; P, peristaltic pumps; DB, displacement bottle; I, injection valve; S, sample; PS, PTFE phase segmentor; E_1, oxidation coil; E_2, extraction coil; F, fluorimeter; W, waste; o, organic phase; a, aqueous phase; R, restriction coil.

flow cell, but it behaves as though it were a single phase (the fluorimeter measures total fluorescence intensity).

In mode B, extraction occurs in only one segment of the organic phase. The thiochrome is extracted from each end of the organic segment and it diffuses towards the inside until a homogeneous segment is obtained (intrasegmental convection and diffusion process). Also, the aqueous phase containing the thiochrome emits fluorescence, which means that the baseline is not zero. However, this value is very low and it is possible to adjust the baseline to zero on the fluorimeter.

3.2. Preliminary studies

The oxidation of the thiamine in basic pH followed slow kinetics and the thiochrome formed was unstable. The rate of the decomposition reaction depended on the pH and the concentration of the oxidant.

In order to optimise the time taken to achieve the maximum oxidative reaction under different chemical conditions (oxidant concentration and alkaline pHs), in the proposed flow-system 1A E_1, E_2 and the whole of channel C_3 were removed and a minimum reaction coil (15 cm) was added to connect the merging point of C_1 and C_2 with the flow cell. Thiamine was injected and flow stops were carried out when the analyte reached the flow cell in the detector. Conditions: channel C_1, 1.5 ml/min, distilled–deionised water; channel C_2, 1.5 ml/min, ferricyanide solutions of different concentrations and pH (time between injection of the solution and this reaching flow cell = 1 s).

The reaction kinetics are shown in Fig. 2, where it may be observed that the analytical signal increased with increasing alkalinity (Fig. 2a and b). Upon increasing the concentration of oxidant for constant alkalinity (Fig. 2c), this signal decreased due to destruction of the thiochrome formed under these conditions.

In order to achieve the optimum oxidation time and as little decomposition as possible, it was necessary to work with flow rates and reaction coil lengths that would allow the injected volume to become merged with the organic phase in a minimum of 8 s.

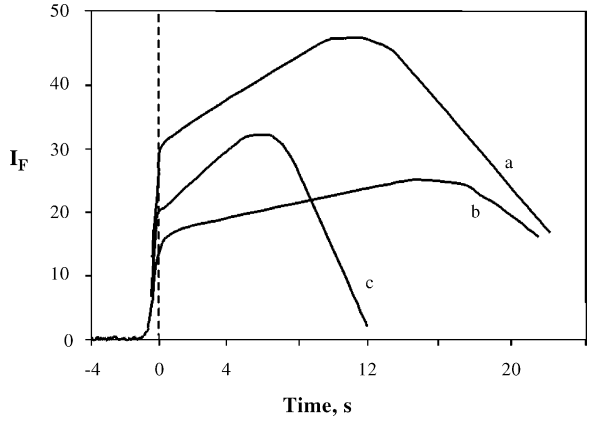

Fig. 2. Preliminary studies. (a) $[Fe(CN)_6^{3-}]$: 10^{-3} M, pH 13.5; (b) $[Fe(CN)_6^{3-}]$: 10^{-3} M, pH 10.9; (c) $[Fe(CN)_6^{3-}]10^{-2}$ M, pH 13.5; $t = 0$: time when pump is stopped.

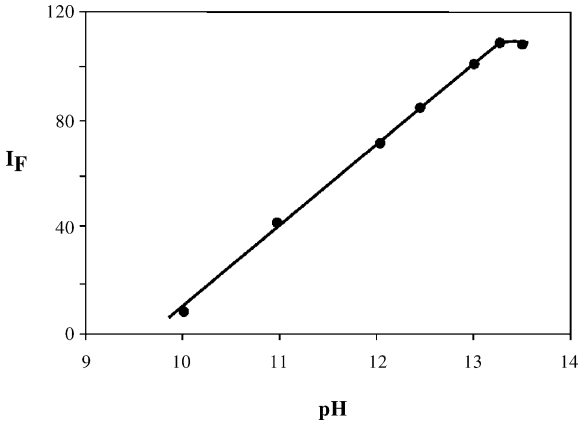

Fig. 3. Effect of pH of ferricyanide solution.

3.3. Optimisation of the experimental conditions

The main variables affecting the formation, extraction and detection of thiochrome in the proposed flow systems (Fig. 1) were considered. First, the effect of the chemical variables (pH of ferricyanide solution, concentration of ferricyanide) and the length of the reaction coil were investigated using mode A. Then the flow-rates, the injection volume and the length of the extraction coil were optimised for each mode.

Except for cases in which other values are specified, the following general working conditions were used. Channel C_1: 1.08 ml/min, distilled–deionised water. Channel C_2: 1.08 ml/min, ferricyanide solution, 10^{-3} M, pH 13.3. Channel C_3: 0.94 ml/min, distilled-deionised water that displaces chloroform. Reaction coil: 140 cm, 0.5 mm i.d. Extraction coil: 240 cm, 0.5 mm i.d. Injection volume: 65 μl. Standard solution: 320 μg/l of thiamine.

The reaction process depends on the alkalinity of stream C_2. Using the general conditions, pH was varied from 9.20 to 13.52.

The fluorescence intensity increased linearly with alkalinity, reaching a constant value from 13.3 (Fig. 3), indicating that the speed of the oxidation increased as pH attained more basic values. The extraction process was independent of alkalinity, as can be deduced from the product extracted. In all later experiments, including those employing mode B, a pH of 13.3 was chosen.

The concentration of ferricyanide was varied from 10^{-5} to 10^{-2} M. The fluorescence intensity was observed to increase with the concentration of ferricyanide, due to the increase in the oxidation rate, and to reach a maximum value for 10^{-3} M. The signal decreased for higher concentrations because the rate of the thiochrome decomposition reaction was increased (Fig. 4). For subsequent experiments, a value of 10^{-3} M in the concentration of ferricyanide was chosen.

Different reaction coil (E_1) lengths from 40 to 320 cm (oxidation reaction times: from 2 to 18 s) were assayed. The length of E_2 was 240 cm in all cases, such that the contact time between phases was 9 s.

The fluorescence intensity increased with the length of E_1, a constant value between 140 and 190 cm being reached (oxidation times: 8–11 s). For E_1 >190 cm values, intensity decreased because of the increase in thiochrome decomposed and dis-

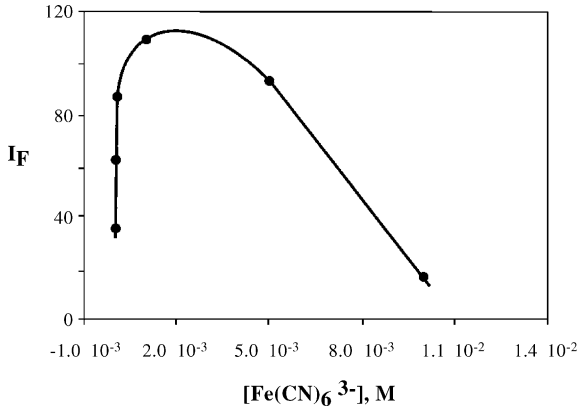

Fig. 4. Effect of concentration of ferricyanide on extraction efficiency.

persion of the analyte. A coil of 140 cm was chosen for later experiments.

The E_2 extraction coil lengths were varied from 10 to 600 cm (contact time between phases: 0.4–24 s) in mode A. The fluorescence intensity was found to increase with the length of E_2, although the slope was smaller from 240–250 cm, a constant value being achieved as from 500 cm, hence showing that the extraction process has slow kinetics. An extraction coil of 240 cm was therefore chosen for subsequent experiments.

In mode B, two experiments were carried out for two aqueous flow rates: (a) 0.20 ml/min (E_1: 15 cm, to keep the oxidation time to 8 s) and (b) 0.51 ml/min (E_1: 35 cm, for the same reason). The extraction coil length was varied from 45 to 300 cm. The injected volume was 30 μl in this case and the solution injected was 200 μg/l.

In both experiments, fluorescence intensity was found to increase initially with the length of E_2 because of the increase in contact time between phases.

At 0.51 ml/min, a constant value between 155 and 200 cm was attained (extraction times: 37 and 48 s, respectively). For E_2 >200 cm values, the analytical signal decreased because of the alteration of the organic phase segment when travelling to the detector (Fig. 5). This effect can be seen better at 0.20 ml/min, because the organic segment is present in the extraction coil for longer. Thus, in this case the analytical signal never reached the maximum value attained for 0.51 ml/min. Also, reproducibility in the signal was worse with lower aqueous flow rates and higher lengths of E_2. Accordingly, a length of 155 cm for E_2 and an aqueous flow rate of 0.51 ml/min were considered optimum for later experiments in this mode.

The volume of standard solution injected in mode A was varied from 41 to 400 μl. Fluorescence was found to increase with the volume injected up to 65 μl, and then to decrease, possibly due to the close relationship between the segments of the organic and aqueous phases. This relationship is governed by the optimum time of oxidation and the extraction process. A compromise between sensitivity and sampling frequency was required, and hence an injection volume of 65 μl was chosen as optimum for subsequent experiments with mode A.

In mode B, the volume of organic solution injected was varied from 30 to 280 μl, using E_1: 35 cm, E_2: 155 cm and an aqueous flow rate of 0.51 ml/min. The fluorescence measured was found to decrease as the volume was increased. This can be explained in terms of the notion that the preconcentration factor is decreased with increasing volume. For later experiments in mode B, a volume of 30 μl was used.

The flow rates of streams C_1, C_2 and C_3 (F_1, F_2 and F_3, respectively) are critical variables to be taken into account. Their effect was studied through the ratio of the aqueous to organic flow rates at the phase segmentor ($F_{wo} = (F_1 + F_2)/F_3$) and through the total flow rate in the extraction coil ($F_t = F_1 + F_2 + F_3$). The aqueous flow rate ($F_w = F_1 + F_2$) affects the oxidation; F_{wo} determines the relative size of the aqueous and organic segments, and therefore affects the extraction process and governs the concentration of thiochrome in the organic phase. The total flow rate (F_t) affects extraction efficiency since it determines the residence time in the extraction coil; i.e., the time of contact between phases.

In mode A, two series of experiments were performed. In the first one, F_1 and F_2 were fixed at 1.08 ml/min while F_3 was varied from 0.14 to 1.55 ml/min. Hence, F_t varied from 2.30 to 3.71 ml/min and F_{wo} from 15 to 1.4. In the second series, F_3 was fixed at 0.94 ml/min while F_w was varied from 0.35 to 2.50 ml/min, always keeping $F_1 = F_2$. In this second series, therefore, F_t was varied from 1.29 to 3.44 ml/min and F_{wo} from 0.4 to 2.6. The results of the series are shown in Fig. 6.

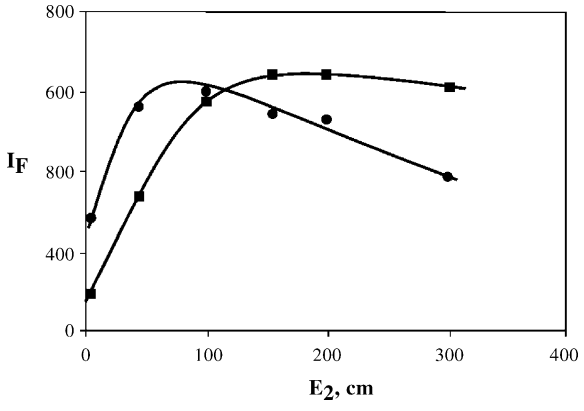

Fig. 5. Effect of the length of the extraction coil (E_2) in mode B. (●) $F_w = 0.20$ ml/min and (■) $F_w = 0.51$ ml/min.

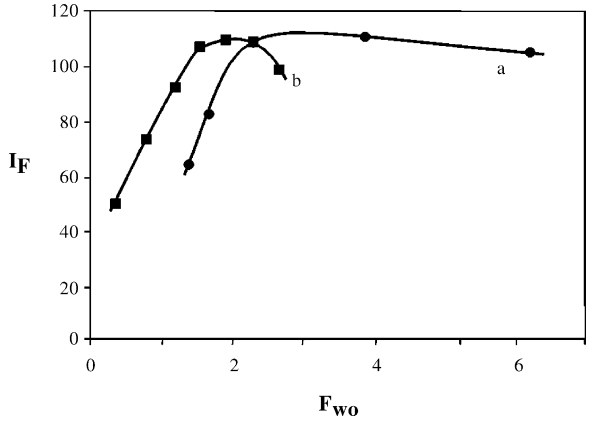

Fig. 6. Effect of the flow rates in mode A: (a) (●) keeping constant F_w in 2.16 ml/min and (b) (■) keeping constant F_o in 0.94 ml/min.

Fluorescence was found to increase with the flow rate, reaching an almost constant value and thereafter decreasing. At those constant values, the oxidation time, time of contact between phases and the ratio between segments of organic and aqueous phases are optimum.

In Fig. 6a, the flow rates that reached a constant value in fluorescence had an F_{wo} value between 2.3 and 3.9. When $F_{wo} > 3.9$, even with a longer time of contact between phases, they had slightly lower extraction efficiencies because the segments of the aqueous phase were very large as compared with the organic segments, limiting the extraction process. For $F_{wo} < 2.3$, the fluorescence intensity decreased because the time of contact between phases decreased, and hence so did the extraction efficiency.

In the second experiment, Fig. 6b, F_w was varied, keeping F_3 constant. For low F_{wo} values, the time of contact between the phases was high, as was the oxidation time, and hence a large amount of the thiochrome formed was destroyed, and the amount of compound extracted was very low. For higher values, optimum oxidation times were reached with maximum extraction efficiency at F_{wo} values between 1.5 and 2.3. For $F_{wo} > 2.3$, the analytical signal decreased, as did the oxidation and extraction times. $F_{wo} = 2.3$ ($F_3 = 0.94$ ml/min and $F_w = 2.16$ ml/min) was considered to be optimum for subsequent experiments.

In mode B, the aqueous flow rate F_w and the length of E_1 control the oxidation time; F_w and the length of E_2 control the extraction time and the physical stability of the organic segment injected. An experiment was carried out varying F_w from 0.12 to 0.70 ml/min (always keeping $F_1 = F_2$) with three different extraction coil lengths (E_2): 45, 100 and 155 cm. In order to ensure that F_w would affect only the extraction process, E_1 was varied according to the F_w value to obtain an oxidation time of 8 s. The volume of organic phase injected was 30 μl and the concentration of thiamine was 200 μg/l.

Fluorescence intensity reached a different maximum, depending on the value of E_2 (Fig. 7a), but in all cases the maximum extraction efficiency was reached when the organic segment was in contact with the aqueous phase for 35–45 s (Fig. 7b). This extraction time was achieved by controlling F_w. However, bearing in mind that reproducibility was poorer when F_w decreased, this was fixed at 0.51 ml/min, with $E_2 = 155$ cm (extraction time of 36 s).

Table 1
Interferences in thiamine determination[a]

Substance	Maximum tolerable [Substance]/[Thiamine]
Retinol	0.06
Riboflavin	0.06
Pyridoxine	20
Cyanocobalamin	20
Ascorbic Acid	10
Cholecalciferol	25
Biotin	0.1
Citrate	20
Tocopherol	50
Nicotinamide	50

[a] Thiamine concentration = 100 μg/l.

3.4. Interferences

Interferences caused by some products commonly used in commercially available pharmaceutical preparations were studied by injecting into the flow system solutions all containing 100 μg/l of thiamine and varying amounts of the substance studied as an interference. The results obtained are shown in Table 1. The tolerance limit was taken as the concentration causing an error of no more than 3% in thiamine determination. No interferences were found for glucose, saccharose fructose and amino acids.

3.5. Application to pharmaceutical products

Several types of tablets (Hidroxil, ALMIRALL, Benexol, ROCHE and Gammamida Complex LAPROQUIFAR) and solutions (Co Hepa B12, SMALLER and Antineurina MABO FARMA) were analysed with both techniques. One tablet was used for each assay. Each tablet was weighed, ground and mixed with a minimum amount of 6 M hydrochloric at 60 °C. Once cold, the solution was filtered and its pH adjusted to 5–5.5, bringing volume up to 1 l. This sample stock was used to prepare more dilute sample solutions. The first solution was a Vitamin B complex and the second one was an injectable solution. Both were diluted with distilled–deionised water and analysed in triplicate. The results obtained (Table 2) are in reasonably good agreement with those obtained by the reference method [37] and with the information provided by the manufacturers.

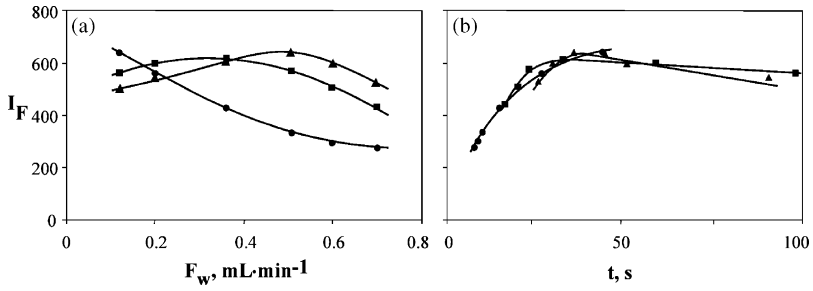

Fig. 7. Effect of the aqueous flow rate and the extraction time in the extraction efficiency in mode B: (●) E_2, 45 cm; (■) E_2, 100 cm; (▲) E_2, 155 cm.

Table 2
Thiamine contents found in the samples analysed

Product	Thiamine content[a,b]			
	Found		Provided by the manufacturer	Reference method
	Mode A	Mode B		
Hidroxil (Almiral Prodesfarma) (mg/table)	246 ± 6	248 ± 10	250	244 ± 5
Benexol (Roche Nicholas) (mg/tablet)	245 ± 6	251 ± 10	250	253 ± 5
Gammamida Complex (Laproquifar) (mg/tablet)	106 ± 5	99 ± 8	100	110 ± 4
CoHepa B12 (Smaller) (mg/5 ml)	4.8 ± 0.3	4.9 ± 0.4	5	5.0 ± 0.2
Antineurina (Mabo Farma) (mg/2 ml)	130 ± 5	126 ± 8	125	128 ± 4

Mode A, with segmentation and no separation; mode B, no segmentation and no separation.
[a] Average of three determinations.
[b] With 95% confidence limits.

Table 3
Working conditions and analytical characteristics for the modes compared

	Mode A	Mode B
Proposed working conditions		
Channel C_1	Water, 1.08 ml/min	B_1, 0.255 ml/min
Channel C_2	$K_3Fe(CN)_6$, 10^{-3} M, pH 13.3, 1.08 ml/min	$K_3Fe(CN)_6$, 10^{-3} M, pH 13.3, 0.255 ml/min
Channel C_3	Water displacing chloroform, 0.94 ml/min	–
Oxidation coil	PTFE, 140 cm, 0.5 mm d.i.	PTFE, 35 cm, 0.5 mm d.i.
Extraction coil	PTFE, 240 cm, 0.5 mm d.i.	PTFE, 155 cm, 0.5 mm d.i.
Injected solution (μl)	B_1 (65)	$CHCl_3$, (30)
Analytical characteristics		
Detection limit (μg/l)	8	1
Linear range (μg/l)	30–2000	5–280
Calibration equation (F: fluorescence intensity, C: concentration in μg/l)	$F = -3.9 + 0.341$ C ($r^2 = 0.998$; $n = 14$)	$F = -2.3 + 3.208$ C ($r^2 = 1.000$; $n = 12$)
R.S.D. (%), $n = 10$ (μg/l)	1.9 (150)	2.4 (150)
Sampling frequency (h^{-1})	60	60

Mode A, extraction with segmentation and no separation; mode B, extraction with no segmentation and no separation.

3.6. Comparison of modes

The proposed working conditions and the analytical characteristics found for modes A and B are summarised in Table 3. The slopes of the calibration straight lines indicate that B was more sensitive than A, because a higher preconcentration ratio was achieved when thiamine was extracted into a single small organic segment. As can be observed, the same sampling frequency was achieved, although higher R.S.Ds. and a narrower range of application were found for mode B. This mode consumes larger amounts of sample solution because the sample is pumped instead of injected, although, by contrast, a drastic saving in organic phase can be achieved since only a small amount of it is injected into the system.

4. Conclusions

From the results shown, it may be concluded that either of the modes – A or B – can be successfully applied for the determination of thiamine in pharmaceutical products. If accuracy or consumption of the sample solution were the main concern, then mode A would be better than B. For sensitivity, however, mode B seems to be the one of choice. Removal of the phase separator (mode A) and of the segmentor and separator (mode B) improves the sensitivity of the continuous mode extraction methods to a considerable extent, implying a considerable advance over the methods described to date. Additionally, in mode B, since only a small amount of organic phase is injected, the preconcentration factor is high, contributing notably to the improvement in sensitivity This mode also offers considerable saving as regards the volume of organic phase necessary (the amount is extremely small) and is simpler than mode A.

These conclusions do not refer mainly to this particular reaction but to the flow systems used. Thus, they can be extended to other chemical reactions based on solvent extraction in similar systems.

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