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Coupling on-line of a dialyser with a flow-continuous system to separate Vitamin B_{12} from milk

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Abstract

The use of membranes for on-line separations in flow-through dialyser as a part of a flow system is extremely useful for automated samples preparation. In this paper a method to couple the dialysis and the UV detection on-line of Vitamin B_{12} from milk is proposed. Firstly, the milk samples were pre-treated with trichloroacetic and centrifuged (to eliminate proteins and fats) and later, using a dialyser coupled a flow-continuous manifold was possible dialyse the Vitamin B_{12}, which was monitored spectrophotometrically at 361 nm. On the other hand, the milk samples were also dialysed on-line but without the acid treatment and the results were compared. The influence of various parameters, including the pump speed for both the donor and acceptor stream, dialysis time, donor and acceptor loop volume on dialysis efficiency was studied. The method was applied to different kinds of milk (skimmed and semi-skimmed milk, evaporated milk, lactose free milk and liquid and powder whole milk). The relative standard deviation (R.S.D.) of the proposed method was of 0.45% and the obtained dialysis percentage was of 5.8%. The proposed method very easy permit a pre-treatment of the sample, quick and on-line with the detection. The dialysis process permitted the pass of vitamin and avoided the pass of other analytes as proteins in the case of the milk samples without acid treatment.

Keywords: Vitamin B_{12}; Dialysis on-line; Continuous-flow system; Milk samples

1. Introduction

Vitamin B_{12} (cyanocobalamin) has an important function in human physiology. Vitamin B_{12} is important in the proteins, carbohydrates and fats metabolism and avoid the pernicious anemia. The human body produces this vitamin in the colon but it is not absorbed, so it is necessary to consume it from animal products as milk, egg, chicken, sardine, etc. Several methods to determinate Vitamin B_{12} have been reported including HPLC [1–3], (inmuno)afinity chromatography [4], spectrophotometry [5,6], capillary electrophoresis [7] and microbilogical assays [8]. All these methods have an excellent precision but the treatment of complex matrices (milk, liver, etc.) is very tedious and time-consuming, for example, in the case of microbiological methods it is necessary that the tissue is cultured and preserved. Generally, the analyzed samples are vitamin tablets that do not require a laborious treatment. Vitamin B_{12} could be indirectly determined by chemiluminescence and atomic absorption spectrometry by determining of the cobalt, but the interference could be arisen from cobalt out of Vitamin B_{12} [9].

Dialysis is a valuable tool that fulfills a very important role for on-line separations as part of continuous-flow systems [10] because it is simple, repeatable, reliable, requires little or no pre-treatment of a sample and shows no interferences from samples colour and turbidity.

The dialysis process allows the analysis of different analytes from complex matrices especially in clinical [11,12], food [13–15] and environmental [16]. Kuban and Karlberg [17] reported a Flow Injection-Capillary Electrophoresis system with on-line dialysis applied to the determination of small anions (chloride, sulfate, citrate, etc.) in milk and juice samples. Staden and Mulaudzi used a dialyser coupling a flow injection system to monitore chloride in milk [18] and for the simultaneous determination of free calcium, total calcium and total chloride [19]. Others authors have coupled the dialysis process to manifolds of flow injection to determinate different analytes in milk

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as lactulose [20] or lactose [21] where the determinations are enzymatic.

The official colorimetric methods to determinate vitamins have several disadvantages related to sample preparation because it is necessary an alkaline digestion where the sample is refluxed during 30 min and later a extraction with hexane. Finally, the extract is centrifuged and diluted to suitable volume. The method is very long and with many steps causing a poor precision. To separate the Vitamin B_{12} of other components as decomposition compounds or interference substances in complex samples, ion exchanges resins are used. As Vitamin B_{12} is a neutral substance it is not retained by the anion or cation exchange resins while that other hydrosoluble compounds are. The cellulose exchange are essentially adequate for the purification of the vitamin. The principal disadvantage of this procedure is that the columns must be washed with high volumes of water with the main objective to elute quantitatively the vitamin. The obtained vitamin concentrations are very small, which cannot be measured spectrophotometrically so it should be necessary a extraction with phenols [8]. Actually do not exist official methods for the treatment of milk samples to determine Vitamin B_{12} therefore we propose a simple purification methodology coupling a dialiser on-line to a flow-continuous manifold.

Firstly, the samples were treated with trichloroacetic acid (TCA) (to precipitate proteins and eliminate fats) and later they passed through the dialysis chamber coupled to a flow-continuous manifold. The results were compared with the obtained from milk samples without using trichloroacetic acid as a pre-treatment. The dialysed Vitamin B_{12} was measured at 361 nm for both analysis. The method was applied to different kinds of milk: skimmed, whole, lactose free, evaporated and powder milk.

2. Experimental

2.1. Reagents and solutions

All solutions from analytical reagent grade chemicals were prepared.

A 1.16×10^{-3} M stock solution of Vitamin B_{12} was prepared dissolving 0.1574 g of Vitamin B_{12} (Sigma) in 100 ml of water. Working standard solutions were prepared by suitable dilution of the stock solution with distilled water.

A 4% trichloroacetic acid, (Baker) solution was also prepared.

2.2. Instrumentation

To carry the dialysis process of the Vitamin B_{12} from milk treated with TCA and milk samples without acid treatment out, the manifold depicted in Fig. 1 was used. The system was constructed with the following components: a Gilson Minipulps (France) peristaltic pump, two Rheodyne injection valves as selection valves to obtain a open–closed system and a Varian Cary 1 UV–vis spectrophotometer (Australia) equipped with a 10 mm Hellma flow-through cell (18 μl) for absorbance measurement.

Data acquisition using the Cary software were achieved. Tygon and Teflon tubing for the acceptor and donor loops and reaction coil were used. The diameter of the Teflon tubing were 0.8 mm i.d.

The used dialyser unit was made in our laboratory and consisted of a 45 mm × 20 mm × 25 mm single dialyser. The path length of both the donor and acceptor streams was 30 mm. The grooves of the dialyser had an i.d. of 0.2 mm. A cellulose Science Ware membrane was used. Membrane thickness and molecular weight cutoff was 0.073 mm and 6000 Da, respectability. This membrane allows the pass of the Vitamin B_{12} and is very resistant to acid mediums.

2.3. Sample preparation

Different kinds of milk (skimmed milk, whole milk, lactose free milk, powder milk, evaporated milk and semi-skimmed milk) were used.

The milk samples without any acid treatment and treated with trichloroacetic acid in accordance to the proposed method by Albalá et al. [22] were dialysed.

Treatment with trichloroacetic acid:

(a) Liquid milk: 50.5 g of sample were accurately weighed into a 100 ml centrifuge tube (60 mm diameter). Then 3 g TCA solid and a magnetic stirring bar were added. The mixture was thoroughly shaken for 10 min over a magnetic stirring plate and centrifuged for 10 min at 1250 g to separate the two phases. Later, 3 ml 4% TCA were added to the obtained solid residue, mixed thoroughly for 10 min and centrifuged. Solid phase was rejected. The two acid extract were combined in a 10 ml volumetric flask and it was filled with 4% TCA. Samples must always be protected from light by covering tubes and flasks with aluminum foil and working under subdued lighting conditions.
(b) Powder milk: 30.0 g were accurately weighed and 15 ml of distilled water were added. The process with trichloroacetic acid was the same.

2.4. Procedure

Fig. 1 shows the manifold of flow injection analysis used to measure the vitamin from milk after a suitable dialysis time.

The dialyser has two parts, named: (a) the upper chamber, where the donor stream (sample with the Vitamin B_{12}) is passed and (b) the lower chamber, where the acceptor stream (distilled water) is located. Between the two chambers a cellulose membrane that allows the pass of the vitamin is located: in the upper chamber the Vitamin B_{12} sample is aspirated through the injection valve IV_1 using the peristaltic pump P_1. When the donor loop is full up with the donor solution (3 ml) the injection valve IV_1 is changed of position and then the system is closed during a suitable time. At the same time, the acceptor stream is aspired by the peristaltic pump P_4 and passed by the lower chamber of the dialyser. When the acceptor loop is filled (3 ml) the system is closed switching the position of the injection valve IV_2. The direction of the donor stream is opposite

to the acceptor one to avoid over pressure in the closed system and to have a better mass transference. After a suitable dialysis time, the injection valve, IV_2, is switched of position and the acceptor solution is brought to injection valve, IV_3, where the injection loop is filled (100 μl) with this solution. Subsequently, this is injected into a water stream leading the plug to the flow-cell where the vitamin is measured at 361 nm.

3. Results and discussion

The initial calibration curve of Vitamin B_{12} was constructed from Vitamin B_{12} standards which were directly injected (without dialysis process) in the FI-manifold shown in Fig. 1. The injection volume was of 100 μl and the flow-rate of 1 ml min^{-1}. The linear range was of 0.2×10^{-1} to 40.00 mg l^{-1} with a regression coefficient of 0.9992, a precision, expressed as relative standard deviation of 0.2% and a detection limit of 0.01 mg l^{-1}. The linear equation was $Y = 0.0095X + 0.0043$.

Once that the UV determination of the vitamin was optimized, the milk samples were treated with trichloroacetic acid. The obtained extracts (also without dialysis process) were injected in the same system that the standards. In Fig. 2 are shown the obtained signals for a 24 mg l^{-1} Vitamin B_{12} standard (A), an extract from milk treated with trichloroacetic acid (B) and the same extract added with the concentration of the standard of Vitamin B_{12} (24 mg l^{-1}) (C).

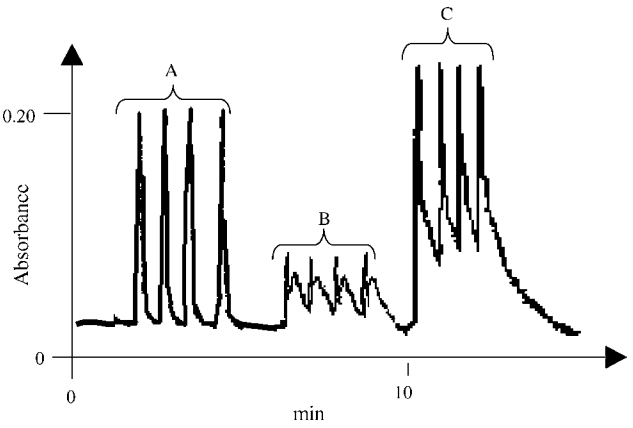

Fig. 2. FI-signals obtained from direct injection of a 24 mg l^{-1} Vitamin B_{12} (A); from milk trichloroacetic acid extract (B) and from milk trichloroacetic acid extract added with 24 mg l^{-1} Vitamin B_{12} (C).

The shape of the signals is different to those of the standards because the extracts were cloudy. This cloudy could be caused for the Schlieren effect [23] because of different densities between the carrier and the sample. Various tests were done including more time of mixed and TCA added to the carrier. Even so the cloudiness in the sample continued interfering with the shape of the pick. So, a dialysis microcell with a cellulose membrane coupled to a flow-continuous system to eliminate the cloudy, was used. The selected membrane allowed the pass of the Vitamin B_{12} from the donor solution to the acceptor

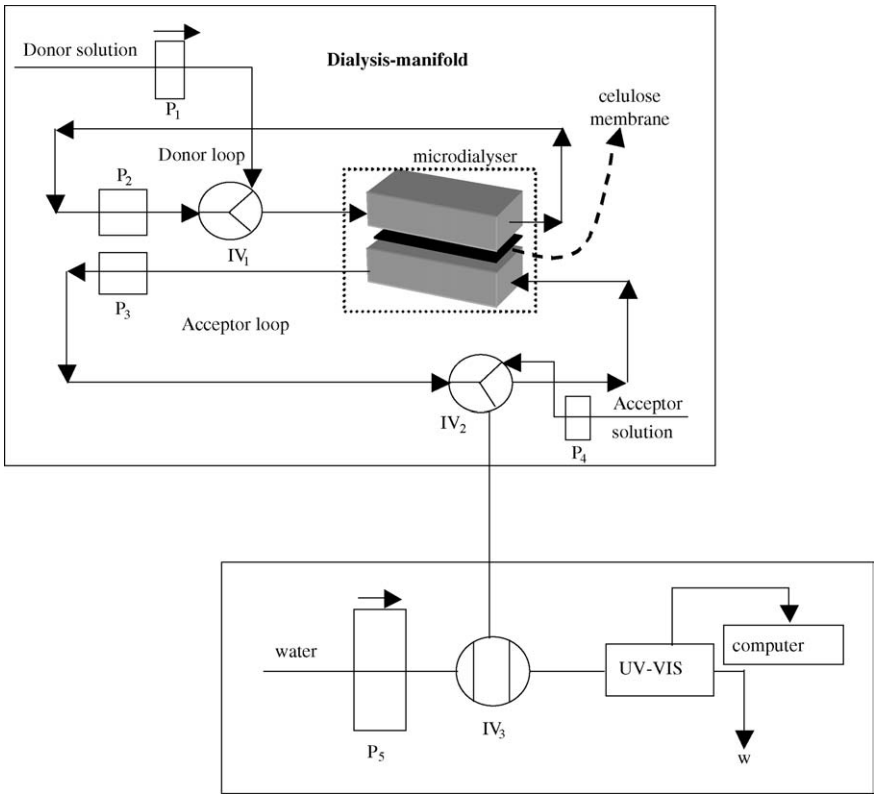

Fig. 1. Used flow-continuous manifold to carry out the Vitamin B_{12} dialysis process on-line coupling a microdialyser with the UV detection. P, peristaltic pump; IV, injection valve; w, waste. To fill the donor and acceptor loops peristaltic pumps P_1 and P_4 were used. To open–close system, the peristaltic pumps P_2 and P_3, injection valves IV_1 and IV_2 were used. The arrows show the flow direction.

solution and the shape of signals were the same that than standards.

3.1. Optimization of the dialysis process in continuous

A microdialyser coupled to a flow-continuous manifold to dialyse the Vitamin B_{12} from TCA extracts was used. The loop volume of the acceptor and the donor solutions, flow-rate of donor and acceptor streams and Schlieren the dialysis time were evaluated.

Study of the dialysis time. The contact time of the sample zone with the membrane is very important on the quantity of vitamin that was dialysed through the membrane.

Using the manifold shown in Fig. 1 the study of the dialysis time was carried out. Firstly, the donor stream was passed through the upper chamber of the dialyser, the acceptor stream through the lower and the measurement was carried out in continuous during 30 min placed the UV-spectrophotometer between dialysis cell and the SV_2. The flow-rate was of 0.1 ml min^{-1} in both streams. The obtained signal was very small, increasing only a 2% after 30 min.

So, two tests with the aim of increase the dialysis of the vitamin were carried out. In both tests a 5.4 mg l^{-1} Vitamin B_{12} solution was used in the donor stream.

1) *Donor-stream continuous system and acceptor-stream open–closed system*: the donor stream was continually passed by upper chamber without closing the system and the acceptor system was closed. The flow-rate of the donor stream was the lowest: 0.1 ml min^{-1}, and the acceptor stream was studied in a range between 0.1 and 1 ml min^{-1} with a loop volume of 6 ml. The optimum acceptor flow-rate was also the minimum, 0.1 ml min^{-1}, because it flow-rate increases the contact time between the donor and the acceptor solutions therefore the dialysis time, which was demonstrated because when the acceptor flow-rate increased, the vitamin signal decreased. The results were compared with the obtained using a open–close system for the donor stream.
2) *Donor-stream and acceptor-stream open–closed systems*: the donor loop was filled with the donor stream and the system was closed. The donor solution was passed through the upper chamber during a suitable time and the loop of the acceptor stream was filled with distilled water. The initial loop in both chambers of the dialyser was 6 ml. The signal was measured during 30 min. The obtained signal was very small because the acceptor loop volume was too large. So, the length of the acceptor loop was changed to the half 3 ml, to increase it. Smaller lengths were not studied because this volume was the minimum permissible. In those conditions the signal increased up to 60%. The donor loop was also optimized. A range between 3 and 15 ml was studied. After 6 ml, the obtained signal was almost the same, therefore the selected optimum value was 6 ml. The results showed that using a loop in both chambers the dialysis is higher than when it is not used in the donor chamber. So, for a better dialysis of Vitamin B_{12} from trichloroacetic acid extracts, the optimum values for donor and acceptor loops were 6 and 3 ml, respectability, and the flow-rate selected was 0.1 ml min^{-1} for both streams.

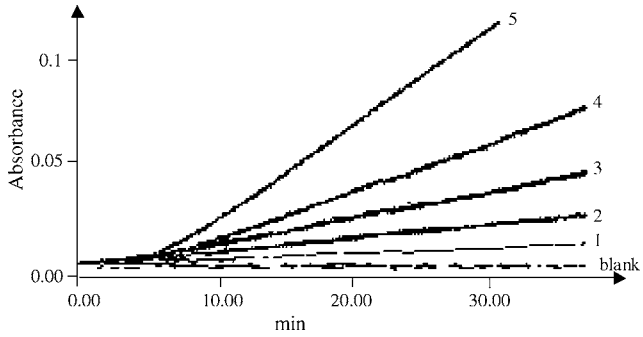

Fig. 3. Plot absorbance vs. time for eight Vitamin B_{12} standards using the manifold of the Fig. 1. (1) 5.4 mg l^{-1}; (2) 27.1 mg l^{-1}; (3) 54 mg l^{-1}; (4) 135.13 mg l^{-1}; (5) 269.72 mg l^{-1}.

Fig. 3 shows the obtained curves for different dialysed Vitamin B_{12} standards. The curves demonstrated that the amount of dialysed vitamin in the acceptor stream increased with the time and besides was proportional to the concentration of vitamin in the donor solution. The dialysis time was selected as an agreement between analysis time and percentage of dialysis. Two standards of Vitamin B_{12} (60 and 5 mg l^{-1}) were used to choose the optimum dialysis time. The results shown that the dialysis increased with the time but after 25 min the signal was almost the same, this is probably because the equilibrium is reached and the concentration of Vitamin B_{12} in the acceptor channel could not be increased, so the dialysis time was larger and the analysis time too. For this reason the selected dialysis time was 25 min.

Study of the dialysis percentage. With the optimized dialysis time, Vitamin B_{12} standards in the range of 0.15–540 mg l^{-1} were dialysed and their absorbances measured.

The linear range was from 0.30 to 250 mg l^{-1} with a regression coefficient of 0.9960. The equation of the curve was: $Y = 0.003X + 0.00029$. The limit of detection (L.D.) was 0.025 mg l^{-1} and the limit of quantification (L.Q.) was 0.32 mg l^{-1}. Both limits were calculated using the calibration curve values and the standard deviation: L.D. = $y_B + 3s$ and L.Q. = $y_B + 10s$ [24].

To calculate the dialysis percentage, the built calibration curves from vitamin standards without dialysis and with dialysis process were related. So, it was possible to calculate how many vitamin had passed through the membrane. The general equation to calculate the % dialysis was:

$$\%\,\text{dialysis} = \left(\frac{X_1}{X_2}\right) \times 100 \quad (1)$$

where X_1 and X_2 are the obtained Vitamin B_{12} concentrations after the dialysis process and without dialysis, respectively.

Using the obtained calibration graphs with dialysis and without dialysis process and substituting into Eq. (1), the percentage

Table 1
Tests results of the determination of within-laboratory reproducibility and repeatability (recoveries expressed in $mg\,l^{-1}$)

Day	Vitamin B_{12} measured ($0.5\,mg\,l^{-1}$)		Vitamin B_{12} measured ($10\,mg\,l^{-1}$)	
	Replicate 1	Replicate 2	Replicate 1	Replicate 2
1	0.489	0.510	10.20	10.1
2	0.501	0.486	9.50	9.80
3	0.515	0.532	9.98	10.2
4	0.480	0.490	9.99	9.70
5	0.519	0.532	10.10	10.50
6	0.482	0.478	10.12	9.95
7	0.521	0.533	10.15	10.20

of dialysis was calculated using the next equation:

$$\%\,\text{dialysis} = (0.0095\,Ab_1 - 0.003\,Ab_2 + 1.02 \times 10^{-5}) \times 100 \quad (2)$$

where Ab_1 is the absorbance of the dialysed vitamin and Ab_2 is the absorbance of the vitamin without dialyse. A maximum dialysis of 5.8% was obtained and the precision was calculated dialysing seven standards with a concentration of $10\,mg\,l^{-1}$. The precision obtained, expressed as relative standard deviation, was of 0.45%. During all the tests the same dialysis membrane was used and the percentage of dialysis did not change.

Reproducibility and repeatability of the proposed method. In order to evaluate the precision of the method, within-laboratory reproducibility and repeatability were evaluated in a single experimental set-up with duplicates. The experiments were carried out with two standards of Vitamin B_{12} (0.5 and $10\,mg\,l^{-1}$) using reagent with purity of 99.0% because do not exist certificated standards. Two measurements of each standard per day were carried out on 7 days. The results obtained are listed in Tables 1 and 2. The repeatability, expressed as relative standard deviation (R.S.D.) was 1.97 and 1.73, respectively; while the within laboratory reproducibility expressed as relative standard deviation was 4.22 and 2.51, respectively.

Table 2
ANOVA table

Concentration Vitamin B_{12} ($mg\,l^{-1}$)	Source	SS[a]	d.f.	MS[b]
0.5	Between days	4.867×10^{-3}	6	8.113×10^{-4}
	Within days	6.92×10^{-4}	7	9.886×10^{-5}
	Total	5.559×10^{-3}	13	–
10	Between days	0.5808	6	9.68×10^{-3}
	Within days	0.21195	7	3.027×10^{-2}
	Total	0.79275	13	–

[a] Sum of squares.
[b] Mean square.

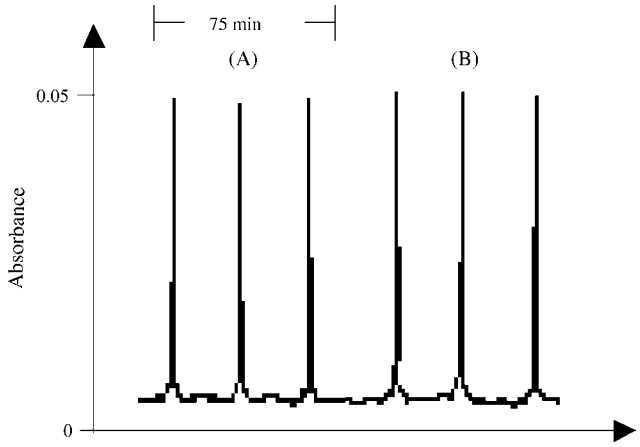

Fig. 4. Obtained signals from milk added with 54 mg of Vitamin B_{12} per 100 g of milk sample after the dialysis process using the proposed method: (A) signals from milk extract using trichloroacetic acid and (B) signals from milk sample without using trichloroacetic acid.

3.2. Applying in milk samples

A study between the obtained signals from the milk TCA extracts without dialysis and injected in the proposed manifold and the signals achieved after the dialysis and injection in the same manifold was carried out. For both studies a skimmed milk sample added with 54 mg of Vitamin B_{12} per 100 g of sample was used to make sure that the vitamin was dialysed. Fig. 4 shows the obtained signals. The results demonstrate that when a microdialyser coupled to the proposed flow-continuous manifold is used it is possible to obtain signals of Vitamin B_{12} at 361 nm without interferences, both the milk sample without acid treatment (Fig. 4A) and the milk sample treated with trichloroacetic acid (Fig. 4B). So, another kind of milk samples were also analyzed. Skimmed and semi-skimmed milk samples were dialysed without treatment acid but the whole and powder milk samples were treated with trichloroacetic acid before they passed through the dialyser because the whole milk has many fatty and the used powder milk was elaborated with vegetal fatty, which blocked the dialysis membrane.

To calculate the concentration in different kinds of milk the system shown in Fig. 1 was used. The milk samples were also added with two amounts of Vitamin B_{12} (0.5 and 10 mg per 100 g of sample) to assure that do not exist interferences by matrix effect and the recovery was calculated. In Table 3 the obtained results are shown. The whole and powder milk samples were always treated with trichloroacetic acid before passing through the dialysis system. The results show that the Vitamin B_{12} added to the milk is recovered with an excellent precision. The results of Table 3 shown that the values of two milk samples (whole power and evaporated) were by low of the limit of quantification; these results were possibly caused because the concentration in these samples is lower than the other samples and with the proposed method is not possible to measure with precision concentrations lower than $0.32\,mg\,l^{-1}$ (equivalent approximately at 0.025 mg per 100 g of milk sample, bearing in mind that 1 ml of milk weights approximately 1.26 g, although each milk sample has different density).

Table 3
Application of the method to the determination of Vitamin B_{12} in milk

Milk sample	Vitamin B_{12}	Addition (Vitamin B_{12}), recovery		Vitamin B_{12}	Addition (Vitamin B_{12}), recovery	
	Acid treatment (mg 100 g^{-1})	0.5 mg 100 g^{-1}	10.0 mg 100 g^{-1}	Without acid treatment (mg 100 g^{-1})	0.5 mg 100 g^{-1}	10.0 mg 100 g^{-1}
Liquid whole	0.042	97.00	97.3	–[a]	–[a]	–[a]
Skimmed	0.035	98.00	101.00	0.038	98.35	96.50
Semi-skimmed	0.045	96.50	100.25	0.044	98.50	99.60
Whole powder	<L.Q.	102.00	99.50	–[a]	–[a]	–[a]
Evaporate	<L.Q.	100.95	99.00	<L.Q.	98.50	99.00
Lactose free	0.040	102.50	100.30	0.042	102.00	101.00

L.Q., limit of quantification.
[a] The test did not carry out (see text).

4. Conclusions

The acid treatment (trichloroacetic acid) applied to the milk samples is not good enough to measure directly at 361 nm Vitamin B_{12}, so it is necessary to carry other treatment out. The proposed on-line dialysis system is a fast and economic method to measure the Vitamin B_{12} after the acid treatment. Another advantage of this system is that it is possible to use it in some milk samples without carry an acid pre-treatment out, only the whole and powder milk require this treatment before to be dialysed.

Coupling a on-line microdialyser with a continuous-flow manifold is a good alternative for the pre-treatment of complex samples as the milk to the UV determination of Vitamin B_{12}.

The dialysis-continuous-flow system could be used in other kind of samples as liver after to eliminate the fatty with trichloroacetic acid.

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References

[1] C.K. Markopoulou, K.A. Kagkadis, J. Pharm. Biom. Anal. 30 (2002) 1403.
[2] H.-B. Li, F. Chen, Y. Jiang, J. Chromatogr. A 891 (2000) 243.
[3] P. Moreno, V.J. Salvado, J. Chromatogr. A 870 (2000) 207.
[4] M. Rhemrev-Boom, M. Yates, M. Rudolf, J. Pharm. Biom. Anal. 24 (2001) 825.
[5] B.J. Morelli, Pharm. Sci. 84 (1995) 34.
[6] A.J. Nepote, P.C. Damiani, J. Pharm. Biom. Anal. 31 (2003) 621.
[7] S.A. Baker, N.J. Miller-Inli, Spectrochim. Acta Part B 55 (2000) 823.
[8] P. Cunnif, Official Methods of Analysis of AOAC International, 16th ed., 1995, vol. II, chapter 45, pp. 1–46.
[9] R.I. Barbara Saez, R. Bosque Peralta, F. Rovira, Ann. Bromatol. 37 (1986) 253.
[10] J.F. van Staden, Fresenius J. Anal. Chem. 352 (1995) 271.
[11] Y. Huang, Z. Zhang, J. Lv, H. Cheng, Anal. Chim. Acta 419 (2000) 175.
[12] K. Dai, A.G. Vlessidis, N.P. Evmiridis, Talanta 59 (2003) 55–65.
[13] E. Mataix, M.D. Luque de Castro, Anal. Chim. Acta 428 (2001) 7.
[14] R.A.S. Lapa, J.L.F.C. Lima, I.V.O.S. Pinto, Food Chem. 81 (2003) 141.
[15] S. Morais, M.I. Alcaina-Miranda, F. Lázaro, M. Planta, A. Maqueira, R. Puchades, Anal. Chim. Acta. 353 (1997) 245.
[16] O. Hernández, F. Jiménez, A.I. Jiménez, J.J. Arias, J. Havel, Anal. Chim. Acta 320 (1996) 177.
[17] P. Kuban, B. Karlberg, Anal. Chem. 69 (1997) 1169.
[18] J.F. Van Staden, L.V. Mulaudzi, South Afr. J. Chem. 52 (4) (1999) 145.
[19] J.F. Van Staden, A. Vanrensburg, Fresenius J. Anal. Chem. 37 (1990) 393.
[20] M. Mayer, M. Genrich, W. Kunnecke, U. Bilitewski, Anal. Chim. Acta 324 (1) (1996) 37.
[21] R. Puchades, A. Maqueira, L. Torro, Analyst 118 (7) (1993) 855.
[22] S. Albalá, T. Veciana, M. Izquierdo, J. Chromatogr. A 778 (1997) 247.
[23] E.A. Zagatto, M.A.Z. Arruda, A.O. Jacntho, I.L. Mattos, Anal. Chim. Acta 234 (1990) 153.
[24] J.C. Miller, J.N. Miller, Estadística para Química Analítica, 2a edición, Addison-Wesley Iberoamericana, 1993, pp. 87–103.