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Journal of Chromatography A, 1081 (2005) 182–189
JOURNAL OF CHROMATOGRAPHY A
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α-Ribazole, a fluorescent marker for the liquid chromatographic determination of vitamin B_{12} in foodstuffs

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Received 18 April 2005; received in revised form 9 May 2005; accepted 24 May 2005

Abstract

A method to determine the contents of free vitamin B_{12} in various foods by reversed phase liquid chromatography-fluorimetry is reported. It includes a purification of the samples by passage through an immunoaffinity column and a pre-column conversion of vitamin B_{12} into the fluorescent α-ribazole (successive treatments of the extract with 2.5 M sodium hydroxide (at 100 °C for 15 min) and alkaline phosphatase (7.5 U) at 37 °C and pH 8 for 16 h). An enzymatic hydrolysis prior to the purification step (pepsin at 37 °C and pH 4 for 3 h) made it possible to release the vitamin B_{12} bound to proteins and thus to obtain the total vitamin B_{12} contents of these foodstuffs. The method proposed for the determination of free and bound vitamin B_{12} gives a good recovery rate (95–100%) and a satisfactory repeatability (R.S.D.r between 1.0 and 5.4%). Owing to its low quantification limit (3 ng g^{-1}) and the good resolution of the α-ribazole peak, it could most probably be applied to the determination of this vitamin in any foodstuff.

Keywords: Food analysis; Vitamin B_{12}; α-Ribazole; Liquid chromatography

1. Introduction

The term vitamin B_{12} is used as the generic description for all cobalamins that exhibit anti-pernicious anaemic activity. The predominant forms of cobalamin present in foodstuffs include hydroxocobalamin, adenosylcobalamin, methylcobalamin, and occasionally, the non-naturally occurring cyanocobalamin used for food supplementation (Fig. 1).

Cobalamins exist in foodstuffs in their free forms as well as bound to proteins. Their release from proteins is most generally obtained by autoclaving the food sample (at 121 °C), first dissolved in a buffered solution (pH 4.5) in the presence of cyanide [1,2] or metabisulfite [3,4] ions. A protease treatment (papain) has also been recommended by Shenoy and Ramasarma [5].

The determination of vitamin B_{12} in foods is most commonly accomplished by microbiological assay using *Lactobacillus leishmanii* as test organism [3], despite the fact that this sensitive assay is tedious, time-consuming, of poor precision and often exhibits relatively low specificity [6]. Radioisotopic dilution has also been used [1,4,7]. This method, based on the very high affinity of vitamin B_{12} for a protein (intrinsic factor), is simple and rapid but nonetheless expensive (its requirements are radio-labelled cyanocobalamin and intrinsic factor of very high purity). A method based on the interaction of the non-intrinsic factor (R protein) with the analyte (protein binding assay) and associated with detection via surface plasmon resonance spectroscopy, recently proposed by Indyk et al. [2], is sufficiently sensitive to permit the determination of vitamin B_{12} in milk and meat products. However, it also is expensive and, according to the authors, lacks specificity (all of the cobalamins, active forms and inactive analogs, present in the foodstuffs are determined).

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doi:10.1016/j.chroma.2005.05.066

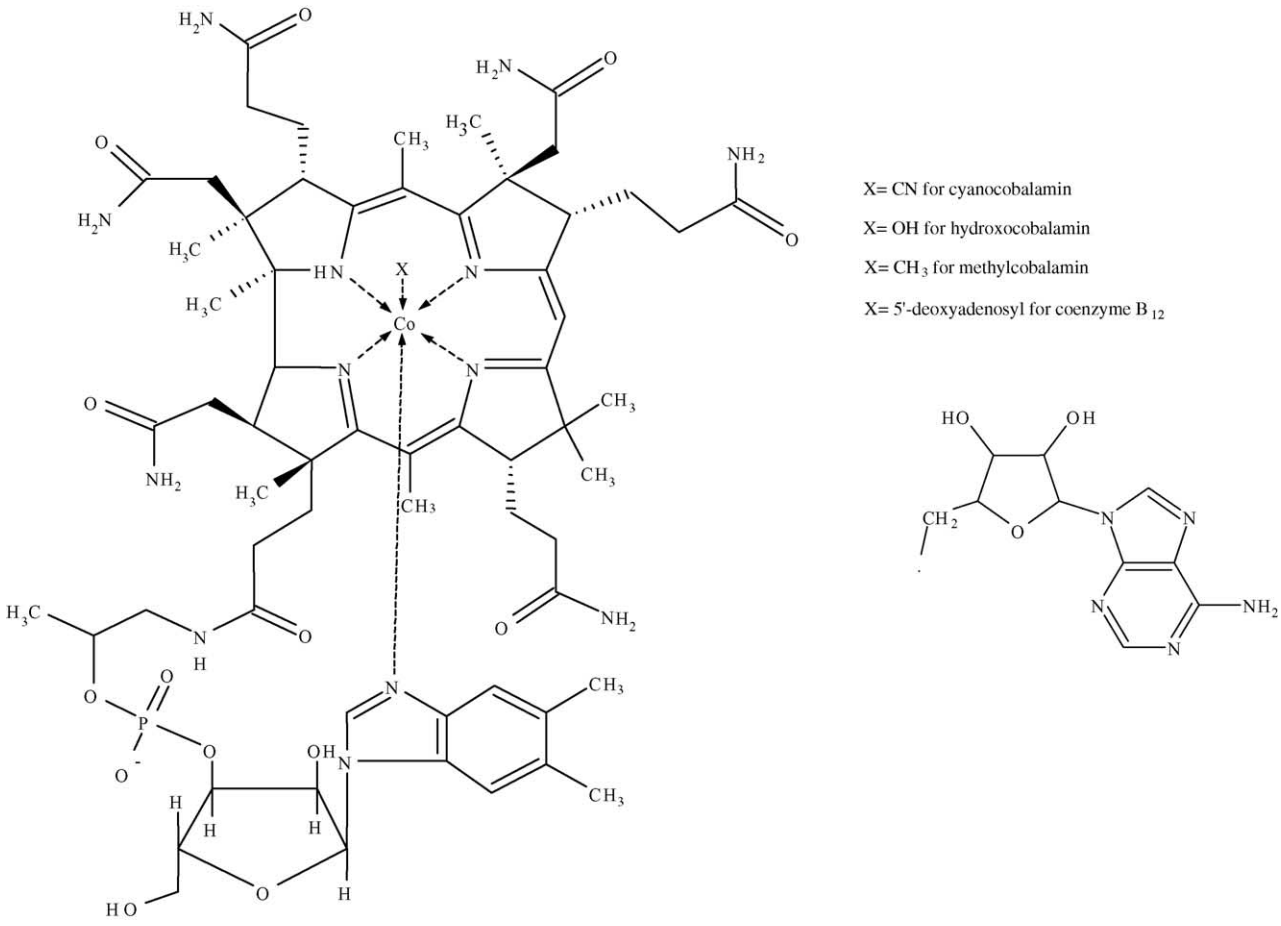

Fig. 1. Vitamin B_{12} chemical structures.

Capillary electrophoretic and chromatographic methods, while more specific than the preceding methods, have been subject to very little development owing to the low sensitivity of the various modes of detection proposed (UV or visible photometry [8–10], atomic absorption [11], ICP–AES [12] or ICP–MS [13,14]), and they are far too insensitive for the determination of vitamin B_{12} in non-supplemented foodstuffs. As this molecule is not naturally fluorescent, contrary to the assertion of Li and Chen [15] and Li et al. [16], the choice of a fluorimetric mode of detection, certainly better adapted than those previously mentioned to the determination of very low contents of vitamin B_{12} in foodstuffs ($1-600\,ng\,g^{-1}$) [17], requires, however, the prior conversion of this vitamin into a fluorescent compound.

It was thus decided to attempt to release one of the characteristic fragments of vitamin B_{12} (5,6-dimethylbenzimidazole, 1-α-D-ribofuranosyl-5,6-dimethylbenzimidazole (α-ribazole) or α-ribazole phosphate) by means of chemical and/or enzymatic hydrolysis, which has the effect of rendering it fluorescent [18], and of then using it as marker for the determination of this vitamin. Since, however, these fragments are implicated in the metabolism of vitamin B_{12} [19] and thus undoubtedly pre-exist in some food samples, it will obviously be essential to perform a very specific purification of the extracts before carrying out the hydrolytic fragmentation. If these two major analytical difficulties can be overcome, the subsequent objective of this study will be to design a complete, specific and sensitive method for the determination by HPLC-fluorimetry of free and total vitamin B_{12} in a large variety of foodstuffs (meat, fish, egg, milk).

2. Experimental

2.1. Reagents

The vitamin standards (cyanocobalamin, hydroxocobalamin, adenosylcobalamin and methylcobalamin) were obtained from Sigma–Aldrich (Saint-Quentin Fallavier, France).

The following enzymes were used: pepsin (EC 3.4.23.1, Sigma, catalogue N° P 7125) and alkaline phosphatase (EC 3.1.3.1, Sigma, catalogue N° P 6772, lyophilized powder from bovine intestinal mucosa). All other chemicals used were of the highest purity available.

2.2. Sample preparation

The foods studied (pig liver, whole chicken egg, beef fillet, fresh salmon, fresh mackerel, powdered milk) were purchased at local sources. Only powdered milk was supplemented with vitamin B_{12}. With the exception of powdered milk and whole chicken egg, the foodstuffs were stored frozen until they were analysed. The solid samples were finely ground and the eggs were beaten up just before their analysis. The sample amounts (2–8 g) took into consideration the estimated vitamin B_{12} content of the food studied [17].

2.2.1. Free vitamin B_{12} extraction

The sample was weighed in a 100 ml conical flask. A 25 ml volume of distilled water was added. The mixture was shaken for 5 min, made up to 50 ml with distilled water and centrifuged at 8000 × *g* for 10 min. The supernatant was filtered through a 0.45 μm cellulose acetate filter.

2.2.2. Total vitamin B_{12} extraction

The sample was weighed in a 100 ml conical flask. A 25 ml volume of 50 mM acetate buffer (pH 4) and 2 ml of a pepsin solution (50 mg ml^{-1} (4500 U ml^{-1})) were added. The mixture was incubated at 37 °C for 3 h. The pH was then adjusted to 7 by addition of 5 M sodium hydroxide. The mixture was made up to 50 ml with distilled water and centrifuged at 8000 × *g* for 10 min. The supernatant was filtered through a 0.45 μm cellulose acetate filter.

2.2.3. Purification of the extract on an immunoaffinity column

A definite volume of the extract (Section 2.2.1 or Section 2.2.2) (from 0.5 to 3 ml, depending on its vitamin B_{12} content) was loaded on to an immunoaffinity column (6 mm × 8 mm i.d; prototype, R-Biopharm, Saint-Didier au Mont d′Or, France) previously washed successively with 5 ml of distilled water and 5 ml of 100 mM phosphate buffer (pH 7). The column was washed successively with 10 ml of 100 mM phosphate buffer (pH 7) and 5 ml of distilled water and dried by passing through 10 ml of air with a syringe. The vitamin B_{12} was then eluted with 3 ml of methanol. The eluate was concentrated to dryness under a nitrogen stream.

2.2.4. Conversion of vitamin B_{12} into α-ribazole

A 500 μl volume of distilled water and 500 μl of 5 M sodium hydroxide were added to the dried extract obtained in Section 2.2.3. The solution was placed in a water bath at 100 °C for 15 min. After being allowed to cool, 500 μl of 5 M hydrochloric acid and 1.5 ml of 300 M Tris buffer (pH 8) containing 7.5 U of alkaline phosphatase were added. The mixture (3 ml) was incubated at 37 °C for 16 h. The supernatant was filtered through a 0.45 μm cellulose acetate filter.

2.3. Chromatographic determination

2.3.1. Apparatus

The HPLC system consisted of a 9012 multisolvent delivery system (Varian, Les Ulis, France), a 9300 injection system (Varian), a 363 fluorescence detector (Varian) and a 330 UV–vis diode arrays detector (Varian). Chromatographic peaks were quantified using a star chromatographic integrator (Varian). A Lichrospher 100 RP 18 endcapped (250 mm × 4 mm i.d.; octadecylsilyl, 5 μm particle size; Merck) were used for all analyses.

2.3.2. Chromatographic conditions

The mobile phase used was a gradient of methanol and distilled water. The proportion of methanol in the mobile phase was increased linearly from 40 to 80% during 20 min. Elution with the final composition of the mobile phase (20:80, v/v) was maintained for 10 min. The mobile phase was then immediately adjusted to its initial composition (water–methanol 60:40, v/v). The flow rate was 1 ml min^{-1} and the injection 100 μl.

Fluorimetric detection was performed at an excitation wavelength of 250 nm and an emission wavelength of 312 nm.

The data were quantified using external calibration. Standard aqueous solutions of cyanocobalamin, prepared by dilution with distilled water of a standard stock aqueous solution (1 g l^{-1}) of this vitamer, were hydrolyzed (sodium hydroxide, then alkaline phosphatase, see Section 2.2.4) to obtain calibrated solutions of α-ribazole (concentrations comprised between 0.15 and 15.0 ng ml^{-1}, expressed in cyanocobalamin equivalents). The standard deviation always referred to individual weighings.

For the recovery tests, a known quantity of a cyanocobalamin standard (approximately half of the quantity of vitamin B_{12} present in the sample studied) was added before the extraction step.

3. Results and discussion

3.1. Choice of α-ribazole as fluorescent marker of vitamin B_{12}

Studies of by no means recent date, often conducted in the context of structural studies of vitamin B_{12}, have shown that recourse to very drastic conditions of acidic hydrolysis of this vitamin was necessary to obtain α-ribazole and 5,6-dimethylbenzimidazole, whereas α-ribazole phosphate could be produced in the form of two isomers under much less rigorous conditions [20–23].

Preliminary results indeed showed that even after 6 h of hydrolysis at 100 °C a concentration of 2.5 M hydrochloric acid was insufficient to give rise to the formation of α-ribazole and/or 5,6-dimethylbenzimidazole from cyanocobalamin. On the other hand, appreciable quantities of two fluorescent chemical compounds with very similar retention times (7.9

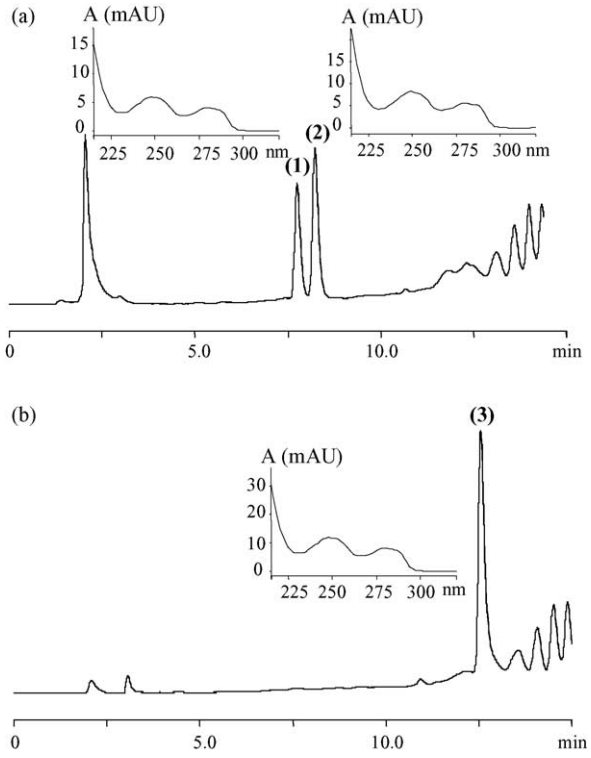

Fig. 2. Chromatographic analysis of a standard solution (100 ng ml^{-1}) of cyanocobalamin after hydrolysis with NaOH 2.5 M at 100 °C for 15 min without (a) or with (b) subsequent phosphatase alkaline hydrolysis [eluent, gradient of phosphate buffer (33 mM, pH 7) and acetonitrile (after an isocratic step of 2 min, the proportion of acetonitrile in the mobile phase was increased linearly from 10 to 70% during 28 min); flow rate, 1.0 ml min^{-1}; fluorimetric detection at 312 nm, with excitation at 250 nm]. Peaks (1) and (2) α-ribazole phosphate isomers, (3) α-ribazole.

and 8.5 min, see Fig. 2) and the same UV absorption and fluorescence emission spectra, which were moreover identical with those of α-ribazole [18,24], could be obtained under such conditions within 5 min. Without doubt, they are the two isomers of α-ribazole phosphate.

According to Armitage et al. [20], α-ribazole phosphate can also be obtained by alkaline hydrolysis ($Ba(OH)_2$, NaOH) of vitamin B_{12}. Not only has it been possible to confirm this result, it has also been shown that under identical conditions of hydrolysis (100 °C, 5 min, HCl or NaOH 2.5 M), the yield of α-ribazole phosphate formed by alkaline hydrolysis was about three fold higher than that obtained on acidic hydrolysis. In all subsequent assays of the conversion of vitamin B_{12} into α-ribazole phosphate, alkaline hydrolysis was thus preferred to acidic hydrolysis.

The optimisation of this conversion, attained without exceeding (for practical reasons) a hydrolysis temperature of 100 °C, showed that a maximum hydrolysis yield was obtained after treatment with 2.5 M sodium hydroxide for 15 min. No increase or decrease of this yield was observed by raising the sodium hydroxide concentration to 4 M or by prolonging the time of hydrolysis to 2 h. The chromatogram of a standard solution of cyanocobalamin hydrolysed under these optimal conditions (NaOH 2.5 M, 100 °C, 15 min) shows moreover a quite satisfactory separation of the two isomers of α-ribazole phosphate (Fig. 2a).

However, the choice of α-ribazole phosphate as fluorescent marker of vitamin B_{12} does not seem to be very judicious, mainly because of the inevitable and troublesome formation of two isomers, each of which will require separate determination. The sensitivity of the method will, moreover, be diminished and the risks of chromatographic interferences increased.

Although the direct formation by acidic or basic hydrolysis of the two other potential fluorescent markers of vitamin B_{12}, α-ribazole and 5,6-dimethylbenzimidazole, appears to be inconceivable in the light of the preliminary results obtained, an enzymatic dephosphorylation of the isomers of α-ribazole phosphate to α-ribazole is, on the other hand, readily conceivable. The addition of a large excess of alkaline phosphatase (7.5 U) to the solution obtained after alkaline hydrolysis of cyanocobalamin and adjustment of the pH to 8 in fact led, after incubation at 37 °C for 16 h, to the complete disappearance from the chromatogram of the two peaks attributed to the isomers of α-ribazole phosphate and to the appearance of fluorescent α-ribazole (Fig. 2b), characterized by its UV absorption and fluorescence emission spectra. This compound thus seems to be relatively easy to obtain in two steps (alkaline and enzymatic hydrolyses) and was thus finally preferred to the isomers of α-ribazole phosphate as fluorescent marker of vitamin B_{12} during this study.

The initial need to separate the two isomers of α-ribazole phosphate by means of reversed phase liquid chromatography had led to the choice of a 33 mM phosphate buffer (pH 7)-acetonitrile gradient as mobile phase (see Fig. 2). The use of such a gradient, which had sometimes led to the malfunctioning of the chromatographic unit (excess pressure caused by the precipitation of salts) was no longer justified for the simple isolation of α-ribazole and was replaced by a water–methanol gradient (see Section 2.3.2). The choice of this gradient made it possible to obtain an extremely sharp chromatographic peak for α-ribazole (Fig. 3), which had not been possible by using a water–acetonitrile gradient (obtention of a broad tailing peak).

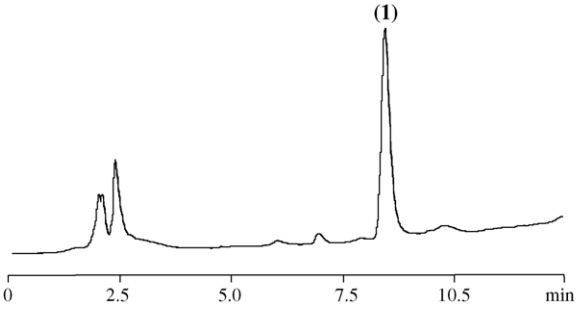

Fig. 3. Chromatographic analysis of a standard solution (10 ng ml^{-1}) of cyanocobalamin after alkaline treatment (NaOH 2.5 M at 100 °C for 15 min) and subsequent alkaline phosphatase hydrolysis; fluorimetric detection at 312 nm, with excitation at 250 nm. Peak (1) α-ribazole.

The fluorimetric detection of α-ribazole has ultimately made it possible to attain a limit of quantification of 0.033 pmol (45 pg, expressed in cyanocobalamin equivalents). The relationship of the fluorescence signal to the concentration of cyanocobalamin was linear over the range 0.5–15 ng ml^{-1}. The repeatability of the protocol proposed (alkaline hydrolysis, alkaline phosphatase treatment, reversed phase chromatography with gradient elution and fluorometric detection) was quite satisfactory (R.S.D.r = 2.3%) (n = 6), R.S.D.r (day to day) = 2.0% (n = 6)). Considering that the pre-column hydrolyses employed introduce a dilution of the extract to be analysed by a factor of 6, the minimal quantifiable content in a foodstuff (expressed in cyanocobalamin[1] equivalents) would then be 17 ng g^{-1}. The method would thus be adequate to enable the vitamin B_{12} content to be determined in the foodstuffs, the consumption of which makes it possible to meet most of the daily needs of the human organism for this vitamin [17].

Completely identical results with respect to the yield of α-ribazole formed, the linearity of the fluorescence signal to the concentration of vitamin B_{12} standard and the repeatability of the suggested protocol were obtained by replacing the standard solution of cyanocobalamin by a standard solution of methylcobalamin, adenosylcobalamin or hydroxocobalamin.

3.2. Purification of the samples by solid phase extraction on an immunoaffinity column

The preliminary application of the analytical protocol developed to a foodstuff sample (in this case, pig liver) showed unambiguously, however, that it was necessary to purify this sample before carrying out the alkaline hydrolysis.

In a first step, this purification was undertaken by means of a solid phase extraction on a Chromafix C_{18ec} cartridge (400 mg, Macherey-Nagel, Hoerdt, France), a procedure already used by Iwase and Ono [10] and Lambert et al. [25]. The protocol suggested, which incidentally entails a concentration of the extract to be analyzed by a factor of 2 (deposition of 1 ml of extract to be purified on the cartridge, washing with 5 ml of distilled water, then with 5 ml of methanol, evaporation to dryness of the methanolic extract and taking up of this extract in 500 μl of distilled water), made it in fact possible to obtain a satisfactory chromatographic isolation of α-ribazole. According to the results obtained, the concentration of vitamin B_{12} in the sample of pig liver would be 124 ng g^{-1}. Analyses of this same sample of pig liver carried out by first omitting the alkaline hydrolysis in the analytical protocol, then both the alkaline hydrolysis and the treatment with alkaline phosphatase have however shown that the latter contained naturally α-ribazole phosphate (81 ng g^{-1}) and α-ribazole (17 ng g^{-1}) and that its actual content of free vitamin B_{12} was in fact only 26 ng g^{-1}.

[1] In the remainder of the article, the mass concentrations (ng g^{-1}) indicated will always be expressed in cyanocobalamin equivalents.

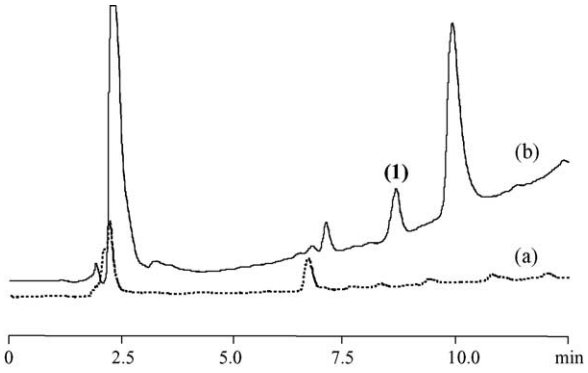

Fig. 4. Chromatograms obtained for the determination of vitamin B_{12} in pig liver after immunoaffinity purification followed by an alkaline phosphatase hydrolysis with (a) or without (b) omission of the prior alkaline treatment (NaOH 2.5 M at 100 °C for 15 min); fluorimetric detection at 312 nm, with excitation at 250 nm. Peak (1) α-ribazole.

In order to avoid an overestimation of the vitamin B_{12} content it thus appeared absolutely essential to carry out a much more specific purification of the extract obtained before the alkaline hydrolysis, in any case sufficiently specific to separate vitamin B_{12} from α-ribazole phosphate and α-ribazole which might naturally be present in the foodstuff sample analyzed.

Such a purification could be obtained by replacing the C_{18} cartridge previously used by an immunoaffinity column (see Section 2.2.3). In the absence of alkaline hydrolysis, no trace of α-ribazole was in fact found in the extract of a sample of pig liver analyzed by chromatography when the protocol included passage through an immunoaffinity column (Fig. 4a). By applying the complete protocol (with alkaline hydrolysis), it is therefore possible to obtain directly the effective content of free vitamin B_{12} of this foodstuff, namely 25 ng g^{-1} (Fig. 4b), thus confirming the result given above. The recovery of the suggested protocol (purification on an immunoaffinity column, alkaline hydrolysis, treatment with alkaline phosphatase and reversed phase liquid chromatography-fluorimetry) as measured on this sample of pig liver was very satisfactory (98%). This purification has, moreover, made it possible to concentrate the foodstuff extract by a factor ranging up to 6 for the samples with very low vitamin B_{12} contents and hence, ultimately, to lower the minimal quantifiable concentration of vitamin B_{12} in these foodstuffs to about 3 ng g^{-1}.

3.3. Release of protein-bound vitamin B_{12}

The autoclaving treatment generally used to cleave vitamin B_{12}-proteins bonds [1–4] is in fact very far removed from the physiological conditions of digestion. Furthermore, the need to handle potassium cyanide appeared quite undesirable. Protease hydrolysis, not with papain, because the impurities contained in this enzyme are sources of chromatographic interferences [26], but with pepsin (37 °C, buffered pH 4 solution) was thus preferred to autoclaving.

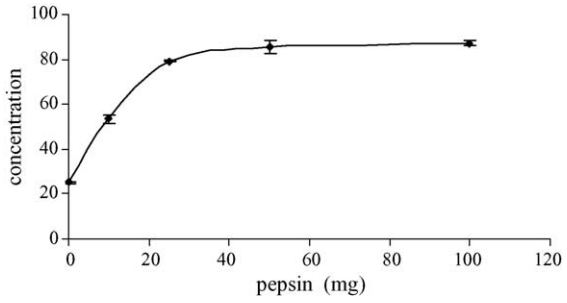

Fig. 5. Concentrations of total vitamin B_{12} (in ng g^{-1} of cyanocobalamin equivalents) in pig liver (sample weight 5 g) in relation to the quantity of pepsin (in mg) used per sample during the incubation at 37 °C for 3 h in acetate buffer solution (pH 4).

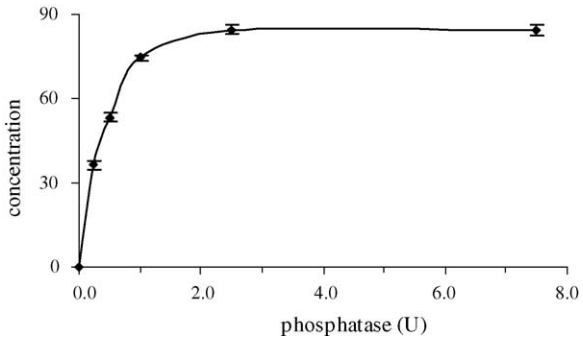

Fig. 6. Concentrations of total vitamin B_{12} (in ng g^{-1} of cyanocobalamin equivalents) in pig liver (sample weight 5 g) in relation to the quantity of alkaline phosphatase (in U) used per sample during the incubation at 37 °C for 16 h in Tris buffer solution (pH 8).

The optimization of this treatment was carried out on a sample of pig liver (5 g). The concentration of vitamin B_{12} found in this foodstuff matrix was increased regularly with the quantity of pepsin added, until a maximal concentration of (86 ± 5) ng g^{-1} ($n = 3$) was attained for an addition of 50 mg (4500 U) of enzyme (Fig. 5). This maximal concentration proved to be identical with that obtained by autoclaving this matrix in the presence of potassium cyanide according to the protocol of Muhammad et al. [1] (85 ± 6 ng g^{-1}, $n = 3$). In order to guarantee complete release of the vitamin B_{12} bound to proteins irrespective of the foodstuff analyzed, it was finally recommended to use twice the quantity of pepsin (100 mg, i.e. 9000 U) per sample.

3.4. Optimization of the dephosphorylation of α-ribazole phosphate to α-ribazole by means of alkaline phosphatase

In the experiments previously mentioned, a large excess of alkaline phosphatase (7.5 U) was always used to convert α-ribazole phosphate to α-ribazole, since no optimization of the quantity of enzyme really necessary could in fact be made in the absence of a protocol making possible the determination of the vitamin B_{12} bound to proteins (this excess was simply attested by the fact that a supplementary addition of this enzyme did not lead to a further increase in the concentration of the vitamin B_{12} measured).

The optimization of the quantity of alkaline phosphatase to be used was attained using pig liver as test foodstuff (sample weight 5 g) on account of its high vitamin B_{12} content. This latter quite obviously increased rapidly with the quantity of enzyme added, then reached a plateau (86 ng g^{-1}) after 2.5 U of alkaline phosphatase had been added (Fig. 6).

By comparing this result with that obtained with a standard cyanocobalamin solution of identical concentration, it appears that scarcely 5% of the alkaline phosphatase added (i.e. 0.10 U) was used in the pig liver sample to dephosphorylate α-ribazole phosphate, the remainder of the enzyme having served to dephosphorylate other constituents of the foodstuff matrix. As a precaution, it was then decided to triple the quantity of alkaline phosphatase previously found to be adequate to reach the concentration plateau and thus, finally, to employ 7.5 U in the complete protocol retained (Fig. 7).

3.5. Determination of free and total vitamin B_{12} in foodstuffs

The application of this protocol, the quantification limit of which was 3 ng g^{-1}, has always made it possible to obtain an excellent chromatographic isolation of α-ribazole (Fig. 8) and

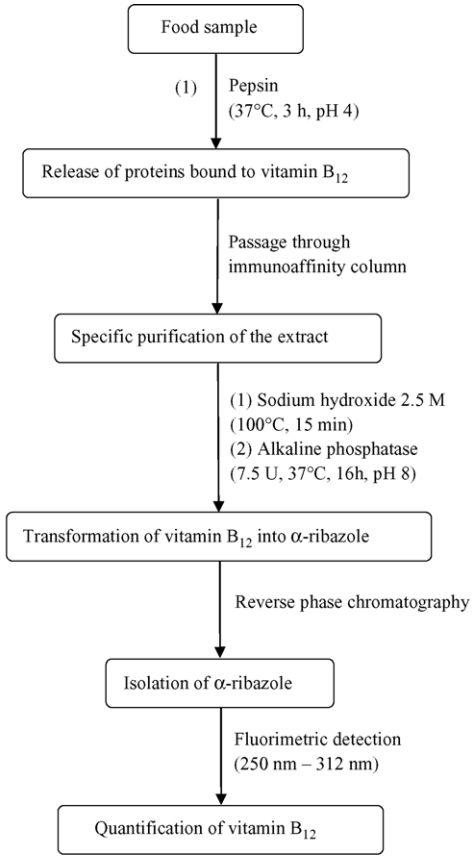

Fig. 7. Liquid chromatographic determination of total vitamin B_{12} content in foodstuffs (determination of free vitamin B_{12} with deletion of step (1)).

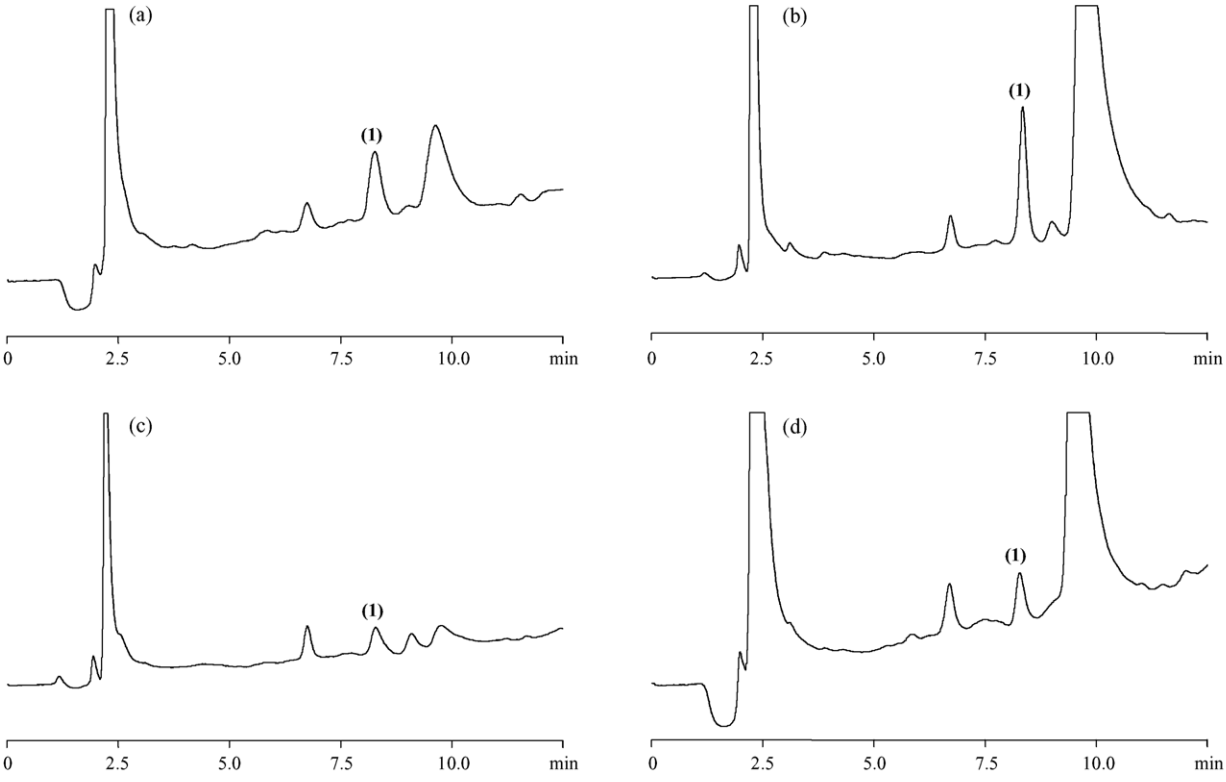

Fig. 8. Chromatograms obtained for the determination of total vitamin B_{12} in beef fillet (40 ng g^{-1}) (a), fresh mackerel (140 ng g^{-1}) (b), powdered milk (15.1 ng g^{-1}) (c) and whole chicken egg (19.7 ng g^{-1}) (d), fluorimetric detection at 312 nm, with excitation at 250 nm. Peak (1) α-ribazole.

Table 1
Liquid chromatographic determination of vitamin B_{12} contents in various foodstuffs[a] (expressed in ng g^{-1} of cyanocobalamin equivalents)

Food	Free vitamin B_{12}		Total vitamin B_{12}	
	Concentration	Recovery[a,b] (%)	Concentration	Recovery[a,b] (%)
Beef fillet	12.4 (0.5)	98 (1)	40 (1)	97 (2)
Pig liver	25.0 (0.3)	99 (6)	86 (3)	97 (4)
Fresh mackerel	21.8 (0.7)	100 (4)	140 (5)	95 (9)
Fresh salmon	11.2 (0.3)	98 (1)	26.5 (0.5)	100 (3)
Powdered milk	14.2 (0.5)	99 (6)	15.1 (0.8)	99 (9)
Whole chicken egg	17.6 (0.1)	97 (1)	19.7 (0.3)	96 (4)

[a] Average of three determinations (standard deviation in parentheses).
[b] Addition (to the sample weighing) of approximately half of the quantity of vitamin B_{12} present in the sample studied.

hence to measure the free (if the hydrolysis step with pepsin is deleted) and total contents of vitamin B_{12} correctly in the various foodstuffs studied (meat, fish, eggs, powdered milk) (Table 1). Whatever the foodstuff analysed, the recovery rate was always included between 97 and 100% (free vitamin B_{12}) or 95 and 100% (total vitamin B_{12}) and the relative standard deviation was included between 1.0 and 4.2% (free vitamin B_{12}) or 1.7 and 5.4% (total vitamin B_{12}).

Since powdered milk is a supplemented foodstuff (addition of cyanocobalamin), it is not surprising that the vitamin B_{12} found in this foodstuff is exclusively in the free form. In all of the other foodstuffs studied (non-supplemented), a considerable proportion of the vitamin B_{12} is bound to proteins, particularly in the meat and fish samples. The total vitamin B_{12} contents obtained are, moreover, of the same order of magnitude as those given in the literature [17], with the exception of the pig liver sample. The mean content obtained in this case (86 ng g^{-1}) is, in fact, markedly lower than the contents reported by Souci et.al. [17] (between 230 and 550 ng g^{-1}).

4. Conclusion

The pre-column formation of fluorescent α-ribazole from vitamin B_{12} (non-fluorescent) by means of the coupling of an enzymatic hydrolysis (alkaline phosphatase) to an alkaline hydrolysis has made possible the determination of this vitamin by HPLC-fluorimetry with a sensitivity such that the resulting analytical method can be applied to all foodstuff samples. Since, however, α-ribazole may be naturally present

in certain foodstuffs, in order to guarantee the specificity of the method it is absolutely essential to first separate the vitamin B_{12} from α-ribazole and α-ribazole phosphate pre-existing in the foodstuff sample by solid phase extraction on an immunoaffinity column. The column prototype used in the course of this work, which will very soon be commercially available, has proved to be particularly efficacious.

The fact that at present no manufacturer of chemical products sells α-ribazole in itself does not pose a problem. The external calibration can in fact be carried out without difficulty using standard solutions of cyanocobalamin subjected to the pre-column conversion. Nonetheless, efforts to make this chemical compound commercially available ought to be encouraged.

Acknowledgements

This work was supported by grants from ACTIA (RA 01-08) (French Ministry of Agriculture and Fisheries) and Nestlé S.A. (Switzerland). We thank R-Biopharm, France, for supplying the immunoaffinity columns.

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