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Fluorimetric determination of pantothenic acid in foods by liquid chromatography with post-column derivatization

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Abstract

A method to determine the content of free pantothenic acid in various foods by reverse phase liquid chromatography–fluorimetry is reported. It includes a purification of the samples by successive passages through anion and cation exchange cartridges and a post-column derivatization of pantothenic acid as the fluorescent 1-alkylthio-2-alkylisoindole (reaction of β-alanin, formed by hot alkaline hydrolysis of pantothenic acid, with orthophthaldialdehyde in the presence of 3-mercaptopropionic acid). An enzymatic hydrolysis prior to the purification step (pepsin at 50 °C for 3 h, then pantetheinase and alkaline phosphatase at 20 °C for 18 h) made it possible to release the bound pantothenic acid and thus to obtain the total Vitamin B_5 content of these foodstuffs. The method proposed for the determination of free and bound pantothenic acid gives a good recovery rate (96–101%) and a satisfactory repeatability (R.S.D.$_r$ less than 8%). Owing to its low detection limit (0.65 $\mu g\, g^{-1}$) and the good resolution of the pantothenic acid peak, it could most probably be applied to the determination of this vitamin in any foodstuff.

Keywords: Food analysis; Derivatization, LC; Pantothenic acid

1. Introduction

Pantothenic acid exists in foodstuffs in its free form, as well as bound in coenzyme A (CoA) and acyl carrier protein (ACP). The determination of the total Vitamin B_5 content therefore requires a release of the vitamin from its bound forms. Neither acid nor alkaline hydrolysis can be used since pantothenic acid is degraded by such treatments. The only practicable alternative is enzymatic hydrolysis. The release of the pantothenic acid from CoA was successfully accomplished by the simultaneous action of alkaline phosphatase and pigeon liver pantetheinase [1]. Even though this enzymatic treatment does not release the vitamin from ACP [2,3], it is the most commonly used treatment for the determination of total Vitamin B_5 in foodstuffs, irrespective of the procedure subsequently used for the determination of pantothenic acid [4–7].

Determination of pantothenic acid in foods has most commonly been accomplished by microbiological assay [6,8], despite the fact that this assay is tedious, time-consuming, of low specificity and often exhibits relatively poor precision. Radioimmunoassay [4,9] and indirect enzyme immunoassay (ELISA) [6,10–12] have also been used. These methods, however, have disadvantages from a practical point of view (the use of radioisotopes and scintillation counting for radioimmunoassay and the acquisition of non-commercially available antisera for indirect enzyme immunoassay). Moreover, nothing can guarantee their specificity owing to the possibility of cross-reactions, most particularly in the food matrices.

Capillary electrophoretic and chromatographic methods, while much more specific than the preceding methods, have been subject to very little development owing to the particular physical properties of pantothenic acid: this molecule, of very low volatility and non-fluorescent, absorbs very weakly in the ultraviolet region, and only at wavelengths shorter than 210 nm.

A method for the determination of pantothenic acid in multivitamin supplements based on capillary electrophoresis

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(CE) and operated in micellar mode was developed by Schreiner et al. [13]. In other respects, a chiral resolution of native DL-pantothenic acid performed by CE using 2-hydroxypropyl-β-cyclodextrin as a chiral selector, was also found to be useful for quantitative chiral analysis of a soft drink containing pantothenic acid [14]. However, the poor sensitivity of the UV detector used in these two protocols restricts their application areas. On the other hand, Sádecká et al. [15] have recently proposed a capillary isotachophoretic method with detection by conductivity which would be sufficiently simple and sensitive for routine analysis of pantothenic acid at low concentrations.

In order to analyse pantothenic acid by gas chromatography, Tesmer et al. [16] and Davidek et al. [17] suggested a pre-column conversion of this vitamin into pantoyl lactone by means of hydrolysis in concentrated acid medium. According to Lahély [18], the recovery yield of this time-consuming method is not satisfactory and its repeatability proved to be mediocre. More recently, Rychlik [7,19] and Rychlik and Freisleben [20] have designed a stable isotope dilution assay using gas chromatography–mass spectrometry. This very sensitive method requiring, however, silylation of the pantothenic acid and the synthesis of a four-fold labeled isotopomer of the vitamin as internal standard, appears to be complex. The synthesis of this internal standard is also necessary in the elegant but nonetheless low precision method using a tandem liquid chromatography–mass spectrometry [21,22].

As for high performance liquid chromatography coupled to UV detection at $\lambda \leq 205$ nm, it could only be used for the determination of pantothenic acid in supplemented foodstuffs (milk and infant formulas) owing to the very low sensitivity of the mode of detection used [23,24].

The objective of this study was first to propose a post-column derivatization of pantothenic acid as a fluorescent compound (formation of β-alanine by hot alkaline hydrolysis of pantothenic acid and reaction with orthophthaldialdehyde), and then to design a complete, specific and sensitive method for the determination by means of HPLC-fluorimetry of free and bound pantothenic acid in a large variety of foodstuffs.

2. Experimental

2.1. Reagents

The vitamin standards (sodium D-pantothenate and coenzyme A (lithium salt, purity 94%)) were obtained from Sigma–Aldrich (Saint-Quentin Fallavier, France).

An acetone-dried pigeon liver powder (Sigma, catalogue No. L8376) containing pantetheinase (EC 3.5.1.) was purified by dialysis: the powder (1 g) was dissolved at 0 °C (ice bath) in 10 ml of 20 mM sodium hydrogen carbonate; the solution was centrifuged at 3500 rpm for 10 min; the supernatant was then introduced into a molecular porous membrane tubing (width 25 mm, diameter 16 mm; volume/length 2.0 ml/cm, Spectra/Por 4 membranes MWCO 12,000–14,000, Bioblock, Illkirch, France) placed in a 3000 ml volume of 20 mM sodium hydrogen carbonate for 15 h at 4 °C; the purified pantetheinase solution was distributed in 1 ml vials and stored frozen until it was used. Its activity, defined in an unofficial way as the number of μmol of pantethine hydrolysed in 1 h at 37 °C and pH 8 per ml of pantetheinase solution, was determined as follows: a volume of 250 μl of pantetheinase solution was added to 15 ml of 200 mM Tris buffer (pH 8) containing 100 μg of pantethine; the mixture was incubated at 37 °C for 4 h with continuous shaking; after incubation, the solution was made up to 50 ml with distilled water in a graduated flask, then filtered through a 0.45 μm cellulose acetate filter; residual pantethine and pantothenic acid were determined by HPLC/fluorimetry (see Section 2.3.2).

Other enzymes used were pronase (EC 3.4.24.31, Sigma, catalogue No. P5147), pepsin (EC 3.4.23.1, Sigma, catalogue No. P7125), papain (EC 3.4.22.2, Prolabo, Fontenay-sous-Bois, France, catalogue No. 26146.180), α-amylase (EC 3.2.1.1, Sigma, catalogue No. A6211) and alkaline phosphatase (EC 3.1.3.1, Sigma, catalogue No. P6772).

A comparative study of the alkaline phosphatase activity in relation to the incubation temperature was carried out as follows: a mixture of alkaline phosphatase (5 U) and coenzyme A (324 μg) in 15 ml of 200 mM Tris buffer (pH 8) was incubated at the chosen temperature for 6 h, then made up to 50 ml with distilled water; pantetheine and pantethine, molecules resulting from this enzymatic treatment, were determined by HPLC/fluorimetry (see Section 2.3.2).

All other chemicals used were of the highest purity available.

2.2. Sample preparation

The foods studied (yeast, pig liver, chicken meat, salmon, whole chicken egg, powdered milk, frozen spinach, frozen peas, frozen French beans, lentils and avocado) were purchased at local sources. Only powdered milk was supplemented with pantothenic acid. With the exception of dry products (powdered milk, yeast and lentils), the foodstuffs were stored frozen until they were analysed. The solid samples were finely ground. The sample amounts (1 g of yeast, or pig liver; 2 g of powdered milk; 5 g of avocado, lentils, chicken egg, chicken meat, or salmon; 7 g of other foodstuffs) took into consideration the estimated Vitamin B_5 content of the food studied [25].

2.2.1. Pantothenic acid extraction

The sample was weighed in a 100 ml conical flask. A 25 ml volume of 200 mM Tris buffer (pH 8) was added. The mixture was shaken for 5 min, made up to 50 ml with distilled water and centrifuged at 11,500 rpm for 10 min. The

supernatant was filtered through a 0.45 μm cellulose acetate filter.

2.2.2. Total Vitamin B_5 extraction

The sample was weighed in a 100 ml conical flask. A 15 ml volume of 50 mM acetate buffer (pH 4.5) and 1 ml of a pepsin solution (50 mg ml^{-1} (4500 U ml^{-1})) were added. The mixture was incubated at 50 °C for 3 h. The pH was then adjusted to pH 8 by addition of 5 M sodium hydroxide. A 10 ml volume of 200 mM Tris buffer (pH 8), 0.6 ml of an alkaline phosphatase solution (20 U ml^{-1}) and 2.5 ml of a pantetheinase solution (80 mU ml^{-1}) were added. The mixture was incubated at 20 °C for 18 h, then made up to 50 ml with distilled water and centrifuged at 11,500 rpm for 10 min. The supernatant was filtered through a 0.45 μm cellulose acetate filter. In some experiments, the protease treatment was omitted: a 25 ml volume of 200 mM Tris buffer containing the other enzymes (alkaline phosphatase (12 U) and pantetheinase (200 mU)) was added directly to the 100 ml conical flask containing the weighed sample.

2.2.3. Extract purification

A 5 ml volume of the extract (Sections 2.2.1 and 2.2.2) was loaded on to a strong anion exchange cartridge (SPE cartridge Chromafix 400-SB, Macherey-Nagel, Düren, Germany) previously washed successively with 5 ml of methanol and 5 ml of distilled water. The first 2 ml were rejected. The next three ml were collected and an aliquot (2 ml) was introduced in a 5 ml volumetric flask. A 0.25 ml volume of 250 mM hydrochloric acid was added. This solution was made up to 5 ml with a 300 mM phosphate buffer (pH 3) (whatever the foodstuff analysed, the pH was always included between 2.8 and 3.2). During the analysis of powdered milk, the samples were centrifuged at 11,500 rpm for 10 min.

The solution obtained (5 ml) was then loaded on to a strong cation exchange cartridge (SPE cartridge Chromafix 400-SA, Macherey-Nagel) previously washed successively with 5 ml of methanol and 5 ml of distilled water. The first 2 ml were rejected. The next three ml were collected and used for chromatographic investigation.

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, and a 363 fluorescence detector (Varian). Chromatographic peaks were quantified using a Star Chromatographic integrator (Varian). A Lichrospher 100 RP 18 endcapped (250 mm × 5 mm i.d.; octadecylsilyl; 5 μm particle size; Merck, Darmstadt, Germany) and a guard column RP 18 (4 mm × 4 mm i.d.; octadecylsilyl, 5 μm particle size; Merck) were used for all analyses.

The reagent for post-column derivatization (an aqueous solution containing 200 mM sodium hydroxide, 1.0 mM orthophthaldialdehyde, and 1.6 mM 3-mercaptopropionic acid), pumped with a Model 110 pump (Beckman-Coulter, Roissy, France), was added at a flow rate of 1 ml min^{-1} to the column effluent through a T connector followed by a PTFE reactor (0.5 mm i.d. × 40 m) simply wound around the metallic insulator of a Crococil column oven (Jasco, Nantes, France) at the set temperature of 99 °C. In fact, no reduction of peak broadening was noted after using a knitted coil.

2.3.2. Chromatographic conditions

The mobile phase used was a gradient of methanol and phosphate buffer (33 mM, pH 2.5). The proportion of methanol in the mobile phase was increased linearly from 0 to 10% during 25 min. Elution with the final composition of the mobile phase (90:10, v/v) was maintained for 8 min. The mobile phase was then immediately adjusted to its initial composition (phosphate buffer). The flow rate of the mobile phase was 1 ml min^{-1}.

For the separation of pantethine, pantetheine and pantothenic acid (see Section 2.1, determination of the enzyme activities), the mobile phase used was slightly different: the proportion of methanol in the mobile phase was increased linearly from 10 to 45% during 7 min; elution with the final composition (55:45, v/v) was maintained for 11 min. The mobile phase was then immediately adjusted to its initial composition (phosphate buffer (33 mM, pH 2.5)–methanol 90:10, v/v).

Fluorimetric detection was performed at an excitation wavelength of 345 nm and an emission wavelength of 455 nm.

The data were quantified using external calibration. The standard stock aqueous solution of pantothenic acid (1 g l^{-1}) was diluted with distilled water to obtain calibrated solutions containing 0.05–2.00 μg ml^{-1} of pantothenic acid. The standard deviations always referred to individual weighings.

For the recovery tests, a known quantity of a pantothenic acid standard (approximately half of the quantity of Vitamin B_5 present in the sample studied) was added before the extraction step.

2.4. Statistical method

A significance test for the comparison of the mean values of two samples (significance level $P = 0.05$) [26] was used to compare the pantothenic acid concentration values obtained by performing different extraction protocols.

3. Results and discussion

3.1. Post-column derivatization of pantothenic acid as fluorescent 1-alkylthio-2-alkylisoindole

In order to carry out the fluorimetric determination of pantothenic acid in preparations containing several

vitamins by flow injection analysis, Blanco et al. [27] chose to automate the conversion of this vitamin into fluorescent 1-alkylthio-2-alkylisoindole (resulting from the reaction of β-alanine, formed by alkaline hydrolysis of pantothenic acid, with orthophthaldialdehyde in the presence of 2-mercaptoethanol). According to these authors, the experimental conditions for obtaining a satisfactory hydrolysis yield of pantothenic acid to β-alanine (concentration of sodium hydroxide higher than 0.3 M and a temperature of 85 °C) would not be favourable for the subsequent revelation of the fluorescence properties of the isoindole formed. The pH of the hydrolysis solution would, in fact, be higher than its optimal fluorescence pH (included between 10.5 and 12.0). This compound would moreover be likely to be degraded at 85 °C. Blanco et al. [27] therefore used two reactors in series (and hence two pumps) in order to be able to carry out the second step of this reaction under more favourable conditions of pH and temperature.

Such a design was first suggested as a post-column derivatization device, each of the two reagents having the same flow rate as that of the mobile phase (1 ml min^{-1}). A high hydrolysis yield of pantothenic acid to β-alanine (83%) could, in fact, be obtained at lower concentrations of sodium hydroxide (200 mM in the reagent and hence 100 mM in the first reactor as a result of the dilution effect of the mobile phase), provided however the length of the reactor and the temperature of the oven in which the latter was placed were increased appreciably (see Table 1, columns 1 and 2). As regards the formation of the fluorescent derivative, it seemed judicious to increase the concentration of orthophthaldialdehyde and to replace the 2-mercaptoethanol by 3-mercaptopropionic acid (gain of sensitivity by a factor of 2). No adjustment of the pH was necessary at the entrance to the second reactor. The conditions proposed have ultimately made it possible to attain a limit of detection of 2.5 ng, very similar to that obtained by Blanco et al. [27] in flow injection analysis. The relationship of the fluorescence signal to the concentration was linear over the range 0.05–2 μg ml^{-1}, and the repeatability was quite satisfactory (R.S.D.$_r$ = 4.0% (n = 6)). In practice, the need to have two pumps available for the implementation of the post-column derivatization is difficult to accept for the performance of routine analyses and was, indeed, a very serious handicap.

Table 1
Experimental conditions for the conversion of pantothenic acid into 1-alkylthio-2-alkylisoindole in flow injection analysis with two separate reactors (1) [27] and in post-chromatographic column derivatization with two separate reactors (2) or only one reactor (3) (this work)

Parameter	(1)	(2)	(3)
Reactor 1			
Length (m)	10	40	40
Diameter (mm)	0.5	0.5	0.5
Temperature (°C)	85	99	99
Reactor 2			
Length (m)	3	3	–
Diameter (mm)	0.5	0.5	–
Temperature (°C)	20	20	–
Sodium hydroxide (mM)	600	200	200
3-Mercaptopropionic acid (mM)	–	0.6	1.6
Orthophthaldialdehyde (mM)	0.373	1.5	1.0
2-Mercaptoethanol (mM)	0.038	–	–
Boric acid (mM)	400	–	–

Consequently, carrying out the entire post-column conversion in only one reactor (device with one pump) was considered. This modification resulted in a slight diminution of the hydrolysis yield of pantothenic acid to β-alanine (76%), but also in a significant lowering of the fluorescence signal measured (about 50%), undoubtedly due to the partial destruction of the 3-mercaptopropionic acid under the conditions of pH and temperature chosen for carrying out the alkaline hydrolysis. In fact, this disadvantage could have been overcome to a large extent by increasing the concentration of this chemical compound in the derivatization reagent (see Table 1, columns 2 and 3). Since, moreover, the use of a device with one pump has made it possible to reduce to a very large extent the relatively wide baseline fluctuations produced during the chromatographic recording with a device with two pumps, the detection limit obtained under these conditions was finally identical to that obtained with the previous device. The linear range was not modified (0.05–2 μg ml^{-1}) and the repeatability of the measurement was improved (R.S.D.$_r$ = 0.5% (n = 6), R.S.D.$_r$ (day-to-day) = 2.9% (n = 6)). Consequently, this device was selected for the remainder of the work.

3.2. Determination of free pantothenic acid

Although the chromatographic isolation of pantothenic acid without prior purification was possible in an avocado sample by using a phosphate buffer (pH 2.5)–methanol gradient as mobile phase (Fig. 1a), such a result could not be obtained with other foodstuff samples owing to the presence of many interfering peaks (see Fig. 1b, for example). Their presence can probably be attributed to the modest selectivity of the post-column derivatization method chosen. Orthophthaldialdehyde is, in fact, capable of reacting with all the amino groups inevitably present in the different samples analysed. A prior purification of these latter, which included the removal of the chemical compounds possessing such a function thus proved essential for a general application of this analytical method.

The choice of a solid phase extraction on an anion exchange cartridge first suggested itself because the pantothenic acid in Tris buffer (pH 8) is in the anionic form whereas the amine functions are not charged. The vitamin unfortunately showed no affinity for the stationary phase under the experimental conditions selected.

It was then decided to acidify the solution to be purified in order to protonate the amine functions and render the pantothenic acid molecule neutral, then to pass this solution

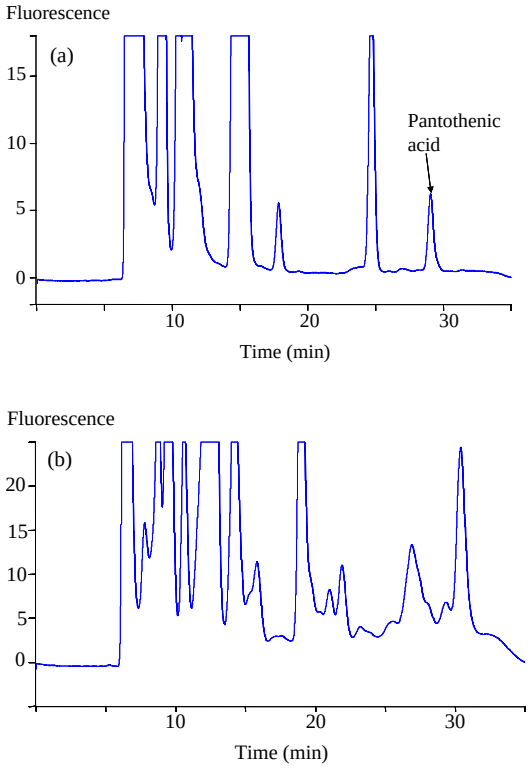

Fig. 1. Chromatograms obtained for the determination of free pantothenic acid in avocado (a) and powdered milk (b), without any purification of the sample.

through a cation exchange cartridge and thus retain the amine impurities. This mode of purification has actually made it possible to obtain a very satisfactory chromatographic isolation of pantothenic acid from all of the foodstuff samples analysed. Unfortunately, the recovery yield of the method, quite acceptable in the absence of purification (99.8% on analysis of the avocado sample), proved to be very low whatever the foodstuff analysed and particularly as the pH of the solution had been lowered prior to its passage through the cation exchange cartridge. Thus, whereas the recovery yield of the pantothenic acid from the avocado sample was always close to 100% when the pH of the solution to be purified had been maintained at 8, it was no more than 80, 72 and 50% when the pH of the solution had been lowered to 4, 3 and 2, respectively. Very probably, molecular interactions were induced between pantothenic acid and some foodstuff constituents during the acidification, interactions all the stronger as the medium was acidic, resulting in the loss of pantothenic acid during its passage through the cation exchange cartridge. However, it was possible to resolve this analytical problem by retaining (very probably) the foodstuff constituents interacting with pantothenic acid on an anion exchange cartridge prior to carrying out the acidification (to pH 3). The use of such a mode of purification has, in fact, made it possible to obtain recovery yields between 92 and 100% for all of the foodstuffs analysed.

Table 2
Liquid chromatographic determination of Vitamin B_5 contents in various foodstuffs[a] (expressed in $\mu g\,g^{-1}$ of pantothenic acid) in relation to the enzymatic treatment performed: (1) absence of enzymes (free pantothenic acid); (2) alkaline phosphatase–pantetheinase (20 °C, 18 h, pH 8) (pantothenic acid free and bound in CoA); (3) pepsin (50 °C, 3 h, pH 4.5), then alkaline phosphatase–pantetheinase (20 °C, 18 h, pH 8) (total Vitamin B_5)

Food	Concentration		
	(1)	(2)	(3)
Avocado	8.2 (0.2)[b]	8.4 (0.2)[b,c]	8.9 (0.3)[c]
Carrot	3.45 (0.05)[b]	3.6 (0.2)[b,c]	3.9 (0.3)[c]
French beans	1.8 (0.2)[b]	2.0 (0.2)[b]	2.5 (0.2)
Lentils	10.6 (0.4)	14.7 (0.4)	17.7 (0.3)
Peas	2.0 (0.2)	4.4 (0.2)	5.0 (0.2)
Spinach	0.80 (0.08)	1.10 (0.09)[c]	1.13 (0.03)[c]
Chicken meat	10.2 (0.2)	13.0 (0.1)	15.1 (0.8)
Pig liver	30.7 (0.1)	63 (2)	73 (2)
Salmon	11.2 (0.5)[b]	11.9 (0.4)[b]	15.3 (0.7)
Chicken egg	17.8 (0.2)	22.3 (0.5)[c]	23.0 (0.5)[c]
Powdered milk	46.3 (0.7)	54 (2)	57.1 (0.9)
Yeast	24.1 (0.7)	73 (3)	80 (3)

[a] Average of three determinations (standard deviation in parentheses).
[b,c] Not significantly different according to the statistical test for the comparison of the means of two samples (significance level $P = 0.05$).

The analytical protocol finally proposed (dissolution of pantothenic acid in Tris buffer (pH 8), purification by passage through an anion exchange cartridge, acidification to pH 3, purification by passage through a cation exchange cartridge, liquid chromatography with post-column derivatization) always made it possible to obtain a good isolation of the pantothenic acid present in the foodstuff samples tested and thus to determine correctly their free pantothenic acid contents (Table 2, column 1).

3.3. Determination of pantothenic acid, free and bound in CoA

The foodstuff samples were treated in a conventional manner by a mixture of alkaline phosphatase and pigeon liver pantetheinase in order to determine their free and coenzyme A-bound pantothenic acid contents.

The acetone-dried pigeon liver powder sold by Sigma unfortunately contains high quantities of pantothenic acid and must be purified before use. The method of purification by dialysis suggested during this study (see Section 2.1) proved to be simpler to use and more efficient than that initially recommended by Novelli and Schmetz [28] (treatment with an anion exchange resin) and still currently used [6,7]. It has, in fact, made it possible to obtain solutions of pantetheinase with residual contents of pantothenic acid of the order of $1\,\mu g\,ml^{-1}$, lower than those of the solutions purified on an anion exchange resin according to the protocol of Rychlik [7] ($2\,\mu g\,ml^{-1}$). The pantetheinase activities of the solutions analysed ($55–85\,mU\,ml^{-1}$) were moreover always higher than those of the solutions purified on an anion exchange resin ($22–60\,mU\,ml^{-1}$).

Using as substrate a standard solution of CoA, it was initially confirmed that the experimental conditions usually selected for the treatment of the foodstuff samples by alkaline phosphatase and pantetheinase (pH 8, 37 °C) were indeed optimal conditions for the hydrolysis of CoA into pantothenic acid.

The estimation of the quantity of enzymes necessary for this hydrolysis was carried out using pig liver as test sample (1 g) on account of its high CoA content (see Table 2). This sample was first heated on a water bath at 95 °C for 10 min in order to inhibit its endogenous enzymes. The results obtained showed that the quantities of enzymes necessary to attain a maximal pantothenic acid content were 4 U for alkaline phosphatase and 35 mU for pantetheinase. Approximately three times the quantities of enzymes (12 U of alkaline phosphatase and 100 mU of pantetheinase) thus ought a priori always to be more than adequate to hydrolyse all the CoA present in the analysed sample, whatever the nature of the foodstuff under study.

The incorporation of this enzymatic treatment in the analytical protocol previously worked out for the determination of free pantothenic acid gave very satisfactory results for the analyses of non-vegetable foodstuffs (pantothenic acid contents equal to or higher than the contents of free pantothenic acid, relative standard deviations included between 1.4 and 5.5% ($n = 3$) and recovery yields included between 93 and 99%). On the other hand, the results obtained for the analysis of samples of fruit and vegetables were quite erratic (pantothenic acid contents more often lower than those obtained previously for the determination of free pantothenic acid (−30% in the avocado and up to −70% in peas); very high relative standard deviations, up to 72% for the analysis of samples of lentils, and recovery yields systematically as much as 70% lower).

The analysis of pea samples had shown that a diminution of the incubation temperature during the enzymatic hydrolysis resulted in a very marked increase in the pantothenic acid content, with improvement in the repeatability of the measurements, and in a maximal content (4.4 μg ml^{-1}) for an incubation temperature of 20 °C (Fig. 2). Hence these results would indicate that the analytical difficulties encountered during an incubation at 37 °C very definitely resulted from a contamination of the solution by microorganisms or enzymes, since the deleterious effects observed were very rapidly attenuated when the incubation temperature was lowered and disappeared completely at 20 °C.

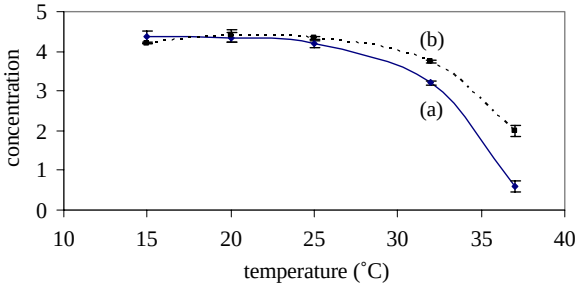

Fig. 2. Concentrations of pantothenic acid, free and bound in coenzyme A (in μg g^{-1}), in peas (sample weight 7 g) in relation to the temperature of the enzymatic hydrolysis. Incubation for 18 h in Tris buffer solution (pH 8), without (a) or with (b) prior autoclaving of the samples at 120 °C for 10 min.

The implication of the foodstuff substrate in this contamination during the analysis of all the samples of vegetable origin could be clearly established by carrying out on these foodstuffs the determination of free pantothenic acid, thus without any addition of enzymes, in various ways. In fact, very similar results (contents, precision, accuracy) to those given in Table 2 (column 1) were obtained when an incubation at 37 °C for 18 h was performed, provided it was preceded by autoclaving. On the other hand, without prior autoclaving this incubation led to aberrant results (fall in the free pantothenic acid contents, combined with a very wide scatter of the measurements). However, an autoclaving of the solution before the enzymatic treatment at 37 °C and pH 8 during the determination of pantothenic acid free and bound in CoA in pea samples only resulted in a moderate increase in the pantothenic acid content (from 0.6 to 2.0 μg g^{-1}) (Fig. 2). This result would indicate that the foodstuff substrate is only partially responsible for this contamination.

The increase, from 2.0 to 4.4 μg g^{-1}, of the pantothenic acid content, with improvement of the precision when the solution was autoclaved before incubation (in the presence of enzymes) and the incubation temperature was lowered from 37 to 20 °C (Fig. 2) indicates clearly that the analytical problems encountered during the analysis of pea samples are also linked to the addition of the enzymes (and does not depend on the method used for the purification of pantetheinase). A similar observation was made during the analysis of all of the other foodstuffs of vegetable origin.

A more detailed understanding of the analytical difficulties generated by the use of the enzymatic treatment during the analysis of vegetable substrates had not previously been further investigated, but these difficulties could finally be settled very easily by lowering the incubation temperature from 37 to 20 °C.

The activity of the alkaline phosphatase–pantetheinase mixture, measured by using as substrate a standard solution of CoA, was of course lower at 20 °C than at 37 °C, the fall observed, of the order of 50%, being entirely attributable to a loss of activity of the pantetheinase. However, it still proved more than sufficient to make possible the complete hydrolysis of CoA in a foodstuff like yeast, which contains a high concentration of this compound (see Table 2), since absolutely identical contents of pantothenic acid were obtained for this foodstuff, whether the enzymatic treatment was carried out at 37 or 20 °C.

Nonetheless, a new optimization of the necessary quantities of alkaline phosphatase and pantetheinase was carried out by using again pig liver as substrate. The results obtained (Fig. 3) show that for an incubation at 20 °C it is necessary to use approximately twice as much pantetheinase and as

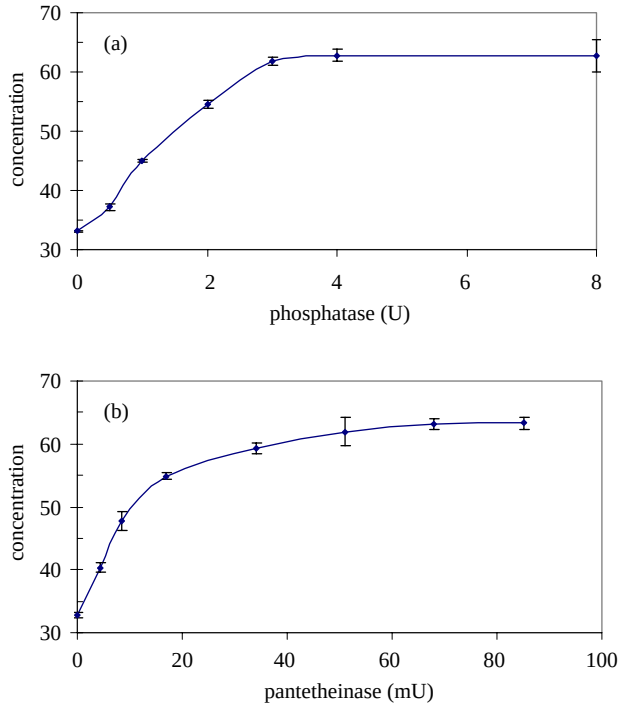

Fig. 3. Concentrations of pantothenic acid, free and bound in coenzyme A (in $\mu g\,g^{-1}$), in pig liver (sample weight 1 g) in relation to the quantities of alkaline phosphatase (in U) (a) or pantetheinase (in mU) (b) used per sample during the incubation at 20 °C for 18 h in Tris buffer solution (pH 8) in presence of an excess of the other enzyme (200 mU of pantetheinase (a) or 15 U of phosphatase (b)).

much alkaline phosphatase as for an incubation at 37 °C to attain a maximal pantothenic acid content, namely 65 mU of pantetheinase and 4 U of alkaline phosphatase, predictable values if reference is made to the results given in the preceding paragraph. However, as a precautionary measure, it was finally decided to recommend quantities of enzymes three times higher in the protocol retained (12 U of alkaline phosphatase and 200 mU of pantetheinase for an incubation at 20 °C and pH 8 for 18 h).

The analytical method suggested has thus made it possible to obtain very satisfactory results (Table 2, column (2)). In the non-vegetable samples, the contents of pantothenic acid obtained by carrying out an enzymatic treatment at 20 °C proved to be identical with those obtained when this treatment was performed at 37 °C. In the samples of vegetable origin, these contents were always higher than or equal to the contents of free pantothenic acid. Furthermore, the recovery yields of the method for all of the samples analysed (vegetable and non-vegetable) were always close to 100% and the relative standard deviations of repeatability ($R.S.D._r$) were included between 0.8% (chicken meat) and 10% (French beans). These results also show that CoA is the major pantothenic acid-containing compound in yeast, pig liver and peas, but that it was not found, on the other hand, in the avocado, carrots, French beans and salmon. In the four other non-supplemented foodstuffs studied (chicken egg, chicken meat, spinach, lentils), the percentage of pantothenic acid released from CoA is included between 20 and 30%. These results confirm that CoA is effectively the major pantothenic acid-containing compound in animal liver and yeast as has already been mentioned by Brown [29] and Gonthier et al. [6], but they seem partially to refute the general but unsupported allegation of Ball [30], according to which the majority of naturally occurring pantothenic acid would be in the form of CoA.

3.4. Determination of total Vitamin B_5

Very curiously, whereas the monographs of nutrition and analytical chemistry dealing with pantothenic acid always mention that in foodstuffs this vitamin is also bound in ACP, no analyst seems hitherto to have suggested methods of determination which make it possible to take into account the contribution of the ACP to the pantothenic acid content.

Considering the structure of this protein, a protease treatment prior to enzymatic hydrolysis by the pantetheinase-alkaline phosphatase mixture might be envisaged in order to hydrolyse the protein moiety in the ACP molecule and thus facilitate the subsequent action of the two other enzymes. Apparently, such a treatment had never been tried on a foodstuff sample. Only Majerus et al. [2], in the course of a study concerning the identification of the prosthetic group of this protein, showed that the hydrolysis of the ACP by pronase (Tris buffer pH 8, 45 °C, 24 h) led curiously to the formation of pantoyl lactone. This result might be explained by the presence of phosphatase and pantetheinase impurities in the pronase, then by the chemical conversion of the free pantothenic acid (non-volatile) to pantoyl lactone (volatile) during the passage of the sample into the injector of the gas chromatograph.

Pronase, the optimal activity of which is located at pH 8, was not selected for this study owing to the analytical problems encountered previously with foodstuff samples of vegetable origin used in an incubation at 37 °C at that pH (see Section 3.3). For this reason it seemed more judicious to use proteases active in acidic solution. A treatment by papain (acetate buffer pH 4.5, 37 °C, 18 h) was unsatisfactory owing to the appearance on the chromatogram of interfering peaks undoubtedly due to the presence of impurities in this enzyme. Pepsin did not raise such a problem. In this case, the hydrolysis was carried out for 3 h at pH 4.5 (acetate buffer) and at 50 °C, in order not to make the analytical protocol excessively long. The pH chosen (4.5) is not the optimal pH for pepsin activity (which is pH 2), but such a choice, justified by the risks of molecular interactions between pantothenic acid and foodstuff constituents in more acidic medium (see Section 3.2) led only to a very modest fall (slightly less than 20%) in the activity of this enzyme.

This prior treatment with pepsin led, in fact, to an increase in the content of pantothenic acid in a sample of chicken meat. The optimization of the quantity of pepsin to be used, carried out using this foodstuff substrate rich in proteins,

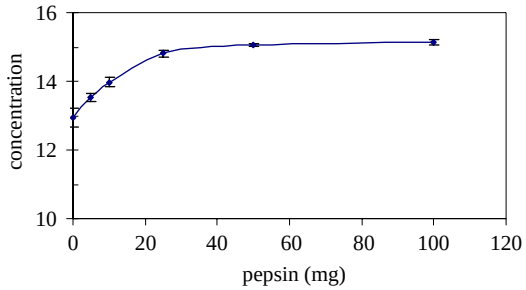

Fig. 4. Concentrations of total Vitamin B_5 (in $\mu g\,g^{-1}$) in chicken meat (sample weight 5 g) in relation to the quantities of pepsin (in mg) per sample (incubation at 50 °C for 3 h in acetate buffer pH 4.5).

showed that the addition of 50 mg of pepsin made it possible to obtain the maximal Vitamin B_5 content in this foodstuff (Fig. 4). In fact, the addition of this treatment with pepsin to the analytical protocol for the determination of Vitamin B_5 led, in the majority of the samples analysed (8 out of 12), to a not inconsiderable increase (of 5 to 22%) of their vitamin contents (Table 2, column 3). It is of course not possible to attribute this increase with certainty to the presence of ACP in these eight foodstuffs and to the efficiency of the enzymatic treatment envisaged to convert this ACP into pantothenic acid. The latter might also result from the rupture of possible interactions between proteins and pantothenic acid. It nevertheless remains a fact that the use of a protease treatment proved to be essential for the determination of the total Vitamin B_5 in most of the foodstuffs analysed (whereas an amylase treatment was always superfluous).

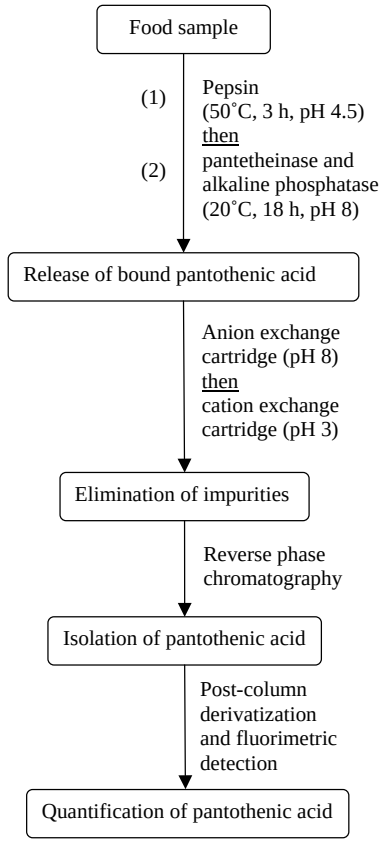

Fig. 5. Liquid chromatographic determination of total Vitamin B_5 content in foodstuffs (determination of free pantothenic acid with deletion of steps (1) and (2) and of free and bound in CoA pantothenic acid with deletion of step (1)).

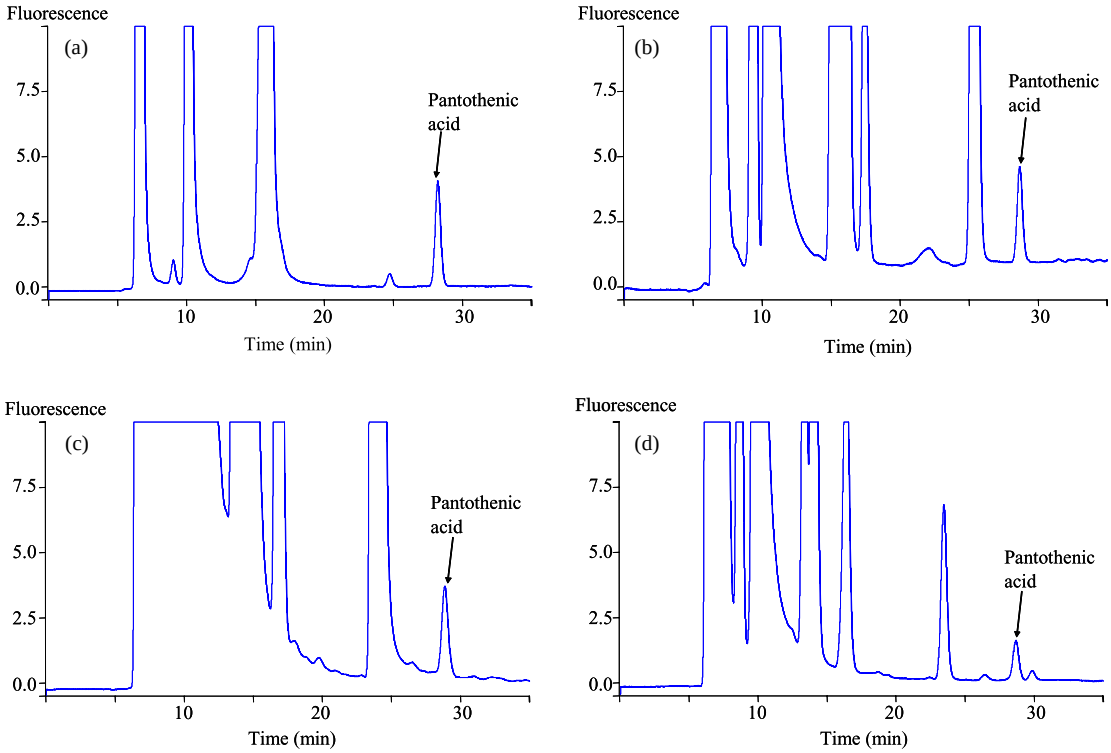

Fig. 6. Chromatograms obtained for the determination of total Vitamin B_5 in powdered milk (a); chicken egg (b); chicken meat (c); and peas (d).

The application of this complete protocol (Fig. 5) has always made it possible to obtain an excellent chromatographic isolation of pantothenic acid (Fig. 6). Whatever the foodstuff analysed, the recovery yield was included between 96 and 101% and its relative standard deviation for repeatability between 1.6 and 7.7%. For a sample weight of 5 g, its quantification limit was 0.65 $\mu g\,g^{-1}$. The Vitamin B_5 contents obtained are moreover of the same order of magnitude as those given in the literature [25].

4. Conclusion

The development of a post-column derivatization method for pantothenic acid as the fluorescent 1-alkylthio-2-alkylisoindole has made it possible to carry out a sufficiently sensitive determination of pantothenic acid, isolated beforehand by liquid chromatography, to permit its concentration to be determined in any foodstuff. A satisfactory chromatographic isolation, however, requires in spite of the use of a 200 mM phosphate buffer (pH 2.5)–methanol gradient as mobile phase, the recourse to a pre-column purification by successive passages of the solution to be analysed through anion and cation exchange cartridges. In the absence of an enzymatic treatment, the method proposed makes it possible to determine the free pantothenic acid. The estimation of the concentration of pantothenic acid bound in CoA is made possible by the enzymatic treatment with pantetheinase and alkaline phosphatase usually recommended. In the case of the analysis of vegetable substrates, it is essential that the incubation temperature for this enzymatic hydrolysis be brought from 37 to 20 °C in order to avoid the growth of microorganisms, prejudicial to the quality of the analytical results provided by the method. For the determination of the total Vitamin B_5, it has moreover proved essential to carry out a preliminary hydrolysis with pepsin (at pH 4.5) in order to release the pantothenic acid bound to proteins in a majority of the foodstuffs analysed.

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