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Determination of pantothenic acid in foods: influence of the extraction method

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The analysis of vitamin B_5 in foods requires the liberation of pantothenic acid present as bound forms such as CoA. Different methods are available to achieve this extraction. We have compared the influence of the extraction method on the quantification of pantothenic acid by both microbiological and immunological assays. Whatever the extraction method used, the two assays gave similar results and allow the quantification of pantothenic acid in foods. The equation of the regression curve between the immunological and microbiological assays was $y=0.309+0.936x$ and the correlation coefficient $r=0.969$. Only alkaline phosphatase associated with pantetheinase contained in a pigeon liver extract realized the total liberation of pantothenic acid from pure CoA. This level of liberation was, respectively, 39.5 and 22.3% with mylase and papain associated with taka-diastase. No hydrolysis was observed with buffer extraction without enzyme. Such a difference was not obtained from the extraction of foods. The quantity of pantothenic acid was only slightly modified by the extraction method used, either with supplemented or naturally vitamin rich foods. These results suggest the presence of endogenous enzymes that realize the whole or partly of the liberation of pantothenic acid if they are not destroyed by a heating treatment before the extraction procedure.

INTRODUCTION

Vitamin B_5 or pantothenic acid, a water-soluble vitamin of the B-group, occurs in all types of animal and plant tissues. Most of the vitamin is in bound form essentially as coenzyme A (CoA) or acyl carrier protein (ACP) that are the biologically active complexes. With the fashion of dietetic products, a lot of foodstuffs are supplemented in different vitamins which is achieved by addition of free pantothenate in the case of vitamin B_5. The control of the quantity of vitamins in general and of pantothenic acid, particularly, needs available, sensitive, specific and easy to perform methods.

The liberation of pantothenic acid from bound forms is always required for the total quantification of vitamin B_5 in foods by various techniques (microbiological, immunological or chemical). As pantothenic acid is sensitive to high and low pH, acidic and alkaline hydrolysis are not useful for this purpose. This liberation is performed by enzyme treatment. A number of enzymes have been proposed (taka-diastase, papain, mylase, clarase, alkaline phosphatase, pantetheinase,...) and are used alone or in association, for the determination of pantothenic acid in foods. In our laboratory, for routine analysis, mylase is used to liberate pantothenic acid. Others recommend a mixture of papain and taka-diastase (Tagliaferri, personal communication). These 2 methods were compared with the enzyme solution technique published by Novelli *et al.* (1949) modified by, among others Zook *et al.* (1956), Bell (1974), Walsh *et al.* (1979) and Tanner *et al.* (1993), using alkaline phosphatase and pantetheinase extracted from chicken and pigeon liver.

The purpose of our study was to determine the influence of the extraction technique on the measurement of pantothenic acid with the microbiological assay and the ELISA technique we have recently described (Gonthier *et al.*, 1998).

MATERIALS AND METHODS

Reagents

The chemical reagents, the calcium pantothenate standard (purity 99% min) and coenzyme A as sodium salt

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(purity 97%) were obtained from Sigma Chemical Company, France. For the enzymes, alkaline phosphatase (EC 3.1.3.1) (Type I from bovine intestine 4·3 units mg^{-1}, ref P 3877) and pigeon liver acetone powder (1 U mg^{-1}) containing a pantetheinase (EC 3.5.1.-) were from Sigma too. Taka-diastase (from *Aspergillus oryzae*) was from Serva, Germany (ref 35740 with α-amylase activity 36 U mg^{-1} and β-amylase activity 59 U mg^{-1}) and mylase ME (from *Aspergillus oryzae*) from United States Biochemical, USA (ref 19300). Papain (EC 3.4.22.2) (from *Carica papaya*) (ref 107144, 30000 USP-U mg^{-1}) and the growth medium, *Lactobacillus plantarum* and vitamin pantothenic acid broth for microbiology were purchased from Merck, Germany

Choice of foods

We have studied the influence of the extraction techniques on products naturally rich in vitamin B_5 and on supplemented products. Vitamin B_5 is generally added in the form of calcium pantothenate, less hygroscopic than the acidic form. A priori, no extraction is required for these foods, as vitamin B_5 is always free. At the present time, most of the vitamin B_5 measurements are being carried out on these products within the quality control in the food industry. Twenty-one foods were tested: 11 were naturally rich in vitamin B_5 (2 dried baker's yeasts [*Saccharomyces cerevisiae*], whole grain rice, calf liver, pig kidney, salmon, egg, Roquefort (blue) cheese, raw milk, lentils, avocado), 10 were calcium-pantothenate-supplemented. Each extraction was carried out twice, each result was obtained from the average of 2 determinations for the microbiological technique, and of 4 wells for the ELISA technique. Assay by both methods was performed from the same extraction solution.

Sampling

For the naturally-rich vitamin B_5 products, a 1 g sample was weighed and extracted. For the supplemented products, 15 g were first blended with 15 ml distilled water. A 2 g sample of mixture was then taken for assay. When the mixture obtained was not homogenous, 1 or 2 × 15 ml of water were added and the weight of the samples increased accordingly (3 or 4 g).

Extraction procedures

For the control extraction (buffer), 10 ml of 0·5 M acetate buffer (pH 4·5) were added to each sample. Tubes were incubated for 2 h at 50°C. For mylase extraction (mylase), 100 mg of mylase ME were added to 10 ml of acetate buffer. The mixture was kept for 2 h at 50°C. For papain-takadiastase extraction (PT), 100 mg of papain and 50 mg of taka-diastase were added to 10 ml acetate buffer. The mixture was incubated overnight at 42°C. For alkaline phosphatase-pantetheinase extraction (APP), 25 mg of alkaline phosphatase were dissolved in 10 ml 0.2 M Tris buffer (pH 8) containing 0·2 ml of a 10% pigeon liver extract solution with prior anion-exchange resin treatment (Tanner *et al.*, 1993). The enzyme solution was added to the sample, the mixture was then homogenized and incubated overnight at 37°C.

For all four methods, enzyme reaction was stopped by autoclaving for 15 min at 120°C, which led to coagulation of heat-labile proteins. The cooled extraction solution was adjusted to pH 7·4 with NaOH. The pH of APP was first lowered to 4·5, then after 30 min, adjusted to pH 7·4. Acidification allowed coagulation of acidic pH sensitive proteins, in particular caseins.

The extraction solution was adjusted to 100 ml with distilled water then filtrated. It was used for both techniques.

Immunological assay (Gonthier *et al.*, 1998)

The immunoplates (Nunc Polysorp) were coated overnight at 4°C using a 0·05 M PBS (pH 7·4) solution of a pantothenic acid-pig thyroglobulin conjugate (1 μg ml^{-1}). After washing 5 times in PBS containing 0.05% of tween 20 (PBS-Tw), 50 μl of extraction solution and 50 μl of anti-pantothenic acid antibody (1/1000) in PBS-Tw were added directly into the well. When the level of vitamin B_5 in the extraction solution was higher than 1000 ng ml^{-1}, the sample was diluted 1/10 in PBS-Tw before adding to the wells. After 1 h at room temperature, the plates were washed 5 times in PBS-Tw; 100 μl of a 1/2000 PBS-Tw solution of peroxidase-conjugated AffiniPure Goat anti rabbit IgG (H + L) antibody (Jackson ImmunoResearch Laboratories, USA) was added to each well.

After 1 h at room temperature, the plates were washed 4 times; 100 μl of an 0.02% 3-3′-5-5′ tetramethyl benzidine dihydrochloride (TMB) solution in citrate-phosphate buffer (pH 4) containing urea peroxide was added to each well. Colour reaction was stopped after 10 min by adding 100 μl of 0.5 M H_2SO_4. Results were read at 450 nm, with a 620 nm reference.

Microbiological assay (Skeggs and Wright, 1944)

2 ml of an extraction solution dilution containing 10–50 ng of pantothenic acid were added to 3 ml of growth medium. After shaking, the tubes were sterilized by autoclaving for 15 min at 115°C, aseptically inoculated by one drop of a *Lactobacillus plantarum* suspension and incubated for 20 h at 37°C. Organism growth was established by measuring the optical density of growth medium at 620 nm.

Standard range

For both techniques, a standard range of unextracted calcium pantothenate was used. For ELISA, calcium

pantothenate was diluted from 0 to 10 000 ng ml^{-1} in PBS-Tw. For microbiological measurement, dilution was carried out in distilled water from 0 to 25 ng ml^{-1}.

Control of extraction and measurement reproducibility

To evaluate the overall extraction-measurement reproducibility, the same sample of dried baker's yeast was analysed 5 times on 5 different days, using each of the 4 extraction techniques and the 2 assay methods.

Control of the different extraction procedures

To measure the levels of pantothenic acid in the various enzymes, blank values were tested by each extraction method. The sample was replaced by 1 ml distilled water. Extractions were carried out as previously described, except that the final volume was adjusted to 50 ml instead of 100 ml.

To determine the percentage of recovery, 1 ml of a 10 μg ml^{-1} calcium pantothenate solution in H_2O was extracted using all 4 methods.

To determine the percentage of CoA hydrolysis, 1 ml of a 35 μg ml^{-1} free acid CoA solution in H_2O was treated using the various extraction methods.

RESULTS

Comparison of the two assay methods

Analysis of the reproducibility of the 2 assay methods (Table 1) showed intra- and inter-assay coefficients of variation that were lower for the microbiological method than for the ELISA technique. This can be related to a lower slope of the standard curve of the second method (Fig. 1).

The foods tested contained significantly different quantities of pantothenate (0.35 to 25.31 mg for 100 g). To facilitate the interpretation of results, the foods were artificially divided into 2 groups according to their vitamin contents (compare Fig. 3(a) and (b)).

The results of the 2 methods were highly correlated. The equation for the regression curve between the immunological and microbiological assays was: $y = 0.309 + 0.936x$. The correlation coefficient was $r = 0.969$ (Fig. 2).

Control and influence of extraction procedure

The microbiological assay was used to study the influence of extraction procedure because of the lower coefficients of variation observed.

The pantothenate content of the buffer and APP was nil or very low. The slightly positive values obtained for the 2 extraction solutions can be explained by the dubious measurements close to 0. Higher levels of exogenous vitamins were recovered in mylase and PT (Table 2).

Whatever the method used, no free vitamin was destroyed during the extraction phase. For all four methods, the calcium pantothenate recovery levels ranged from 99.67 to 103.90% (Table 2).

The CoA sample (35 μg of free acid) was equivalent to 10 μg calcium pantothenate. The percentage of CoA hydrolysis was calculated from the actual amount of pantothenate liberation. It varied strongly according to the extraction procedure: *ca* 0% with buffer, *ca* 20% with PT, *ca* 40% with mylase and close to 100% with APP (Table 2).

The influence of the extraction procedure on the quantification of pantothenate in different foods was shown in Fig. 3(a) and (b). Apart from the 2 baker's yeasts, calf liver and 2 supplemented products (mixed

Table 1. Repeatability and reproducibility of the assay of pantothenate for a baker's yeast according to the extraction and quantification method

	Buffer		Mylase		Papain-Takadiastase		Alk. Phosphatase-Pantetheinase	
	microbio	immuno	microbio	immuno	microbio	immuno	microbio	immuno
m	4.67	4.58	5.34	4.6	4.47	4.63	11.07	11.91
CV_r	2.92	9.29	10.23	20.82	3.15	10.58	0.2	14.21
m	4.66	4.08	5.37	4.27	4.87	4.73	11.6	11.11
CV_r	0.22	11.89	0.95	20.90	4.71	18.34	1.57	9.36
m	3.85	3.68	5.18	3.89	3.98	4.98	10.9	12.41
CV_r	4.22	21.53	6.15	4.72	4.96	13.21	0.88	10.13
m	3.58	2.96	4.28	3.31	3.75	3.29	9.45	8.33
CV_r	0.79	19.51	7.78	13.80	3.52	9.84	8.19	19.53
m	3.98	5.24	4.8	5.68	3.98	6.05	9.11	13.1
CV_r	0.87	14.52	3.12	20.02	8.96	13.15	8.69	19.51
M	4.15	4.11	4.99	4.35	4.21	4.74	10.43	11.37
CV_R	10.64	21.10	8.22	20.34	9.62	20.82	9.30	16.26

m: mean of pantothenate level (mg 100g^{-1}) obtained with 2 determinations for the microbiological assay (microbio) and with 4 wells for the ELISA (immuno).
M: mean of 5 determinations on 5 different days.
CV_r: Intra-assay coefficient of variation (%).
CV_R: Inter-assay coefficient of variation (%).

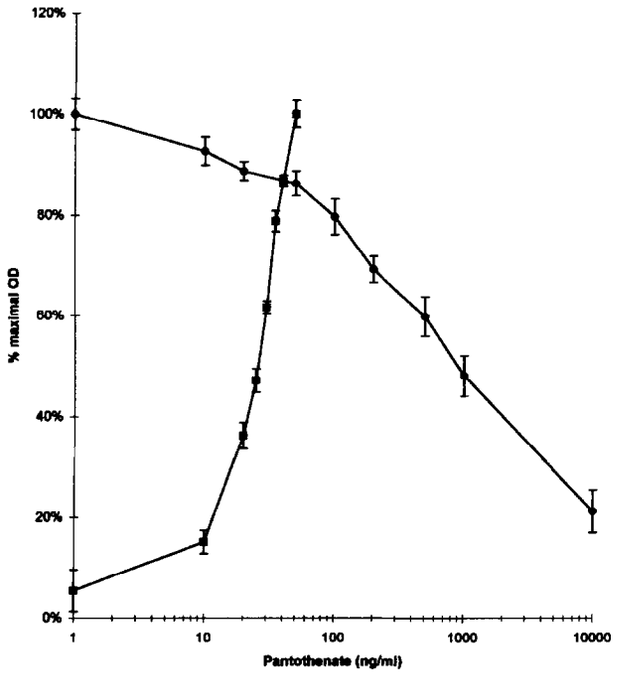

Fig. 1. Standard curves for microbiological (-■-) and immunological (-●-) assays.

and soup), the difference in the levels of vitamin B_5 per food according to the extraction method used was smaller than 10%. For the yeasts, APP extraction gave results 2 fold higher than the others. For the liver, buffer extraction gave significantly lower values. APP extraction had the same effect on 2 supplemented foods (mixed and soup).

DISCUSSION

In this study, we have compared our recently developed ELISA with the microbiological method routinely used in our laboratory. Until now, the microbiological assay was the most commonly used method for quantification of pantothenic acid in biological samples. Few attempts were made to develop immunological methods, RIA (Wyse *et al.*, 1979) or direct (Smith *et al.*, 1981) or indirect (Finglas *et al.*, 1988; Song *et al.*, 1990) ELISA.

Although the two dosage methods used different principles, they produced results that were comparable whatever the vitamin amount (Fig. 2). The same conclusion had been drawn from a previous comparative study of the radio-immunoassay and microbiological methods for the determination of pantothenic acid in 75 foods (Walsh *et al.*, 1979).

The inter- and intra-assay coefficients of variation for the microbiological method were lower than those obtained with the ELISA technique. The slope of the standard curve of the immunological method was low, which has to be related to the relatively low affinity of the antibody to free pantothenic acid (Gonthier *et al.*, 1998). This gentle slope explained the higher coefficients

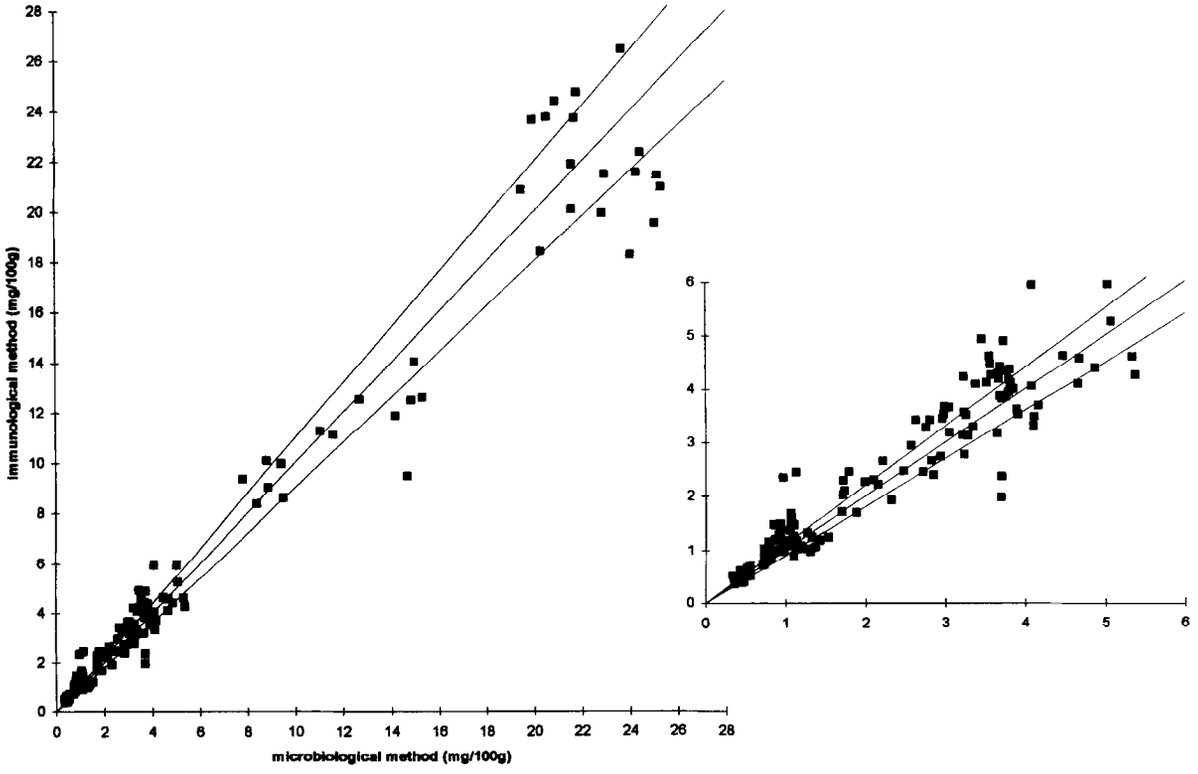

Fig. 2. Comparison of pantothenate values for 21 foods extracted by 4 procedures, each assayed by immunological and microbiological methods. The lines correspond to $y = x$ and $y = x \pm 10\%$.

Table 2. Control of the extraction parameters by the microbiological method. (mean ± standard deviation)

	Pantothenate in the enzyme solution ($ng\,ml^{-1}$ in 50 ml)	Percentage of the recovery of pantothenate	Percentage of hydrolysis of CoA
buffer	0.49 ± 0.13	99.67 ± 2.05	1.47 ± 1.47
mylase	9.45 ± 0.32	102.64 ± 4.17	39.50 ± 2.24
papain-takadiastase	12.43 ± 0.13	103.90 ± 5.25	22.28 ± 2.48
alk. phosphatase-pantetheinase	1.07 ± 0.07	99.67 ± 0.76	95.18 ± 2.50

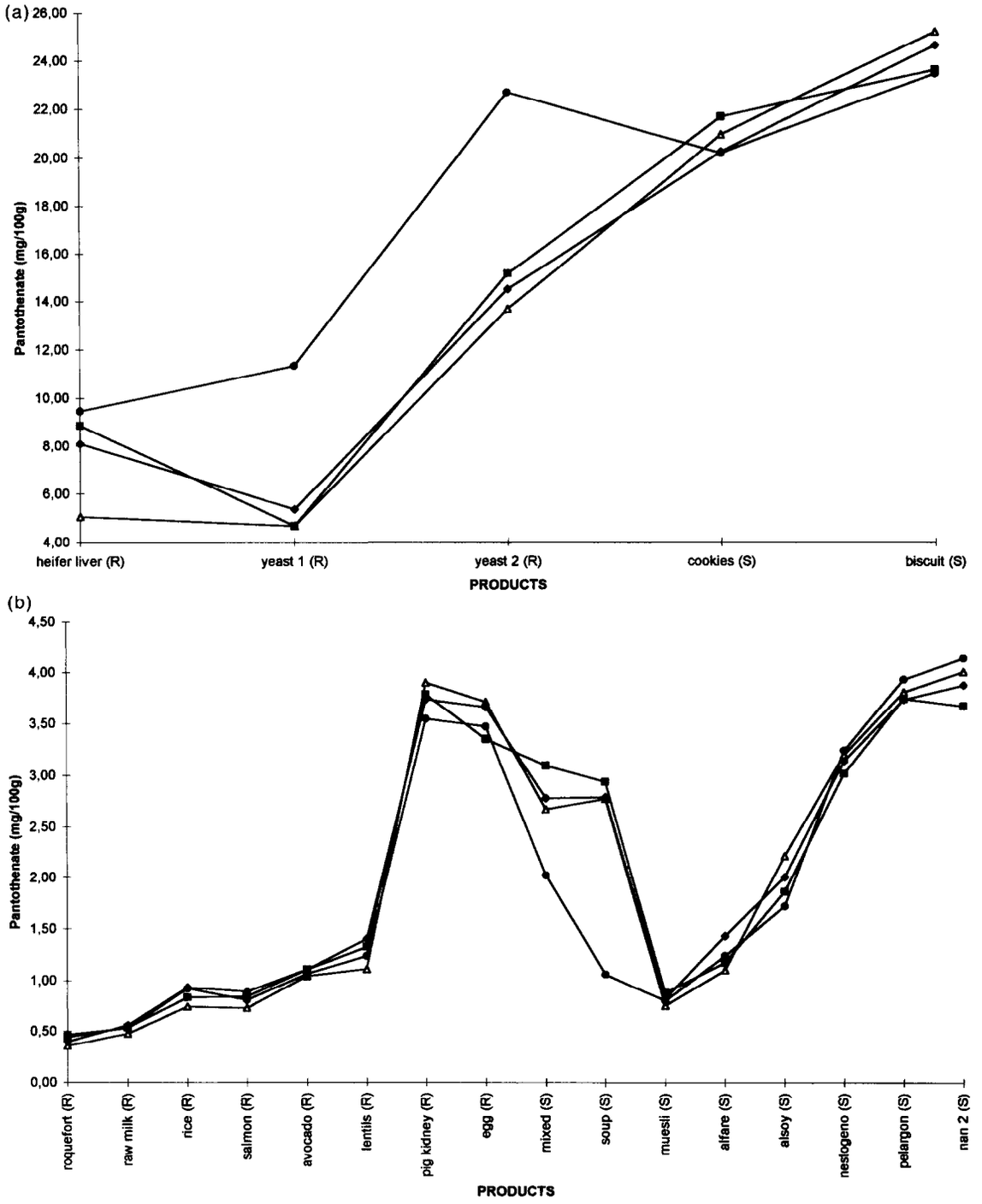

Fig. 3. (a) and (b) Comparison of the quantity of pantothenate of foods obtained with microbiological assay according to extraction procedure: (-Δ-) buffer extraction, (-◆-) mylase extraction, (-■-) papain-takadiastase extraction and (-●-) phosphatase-alkaline-pantetheinase extraction. (R): naturally rich in vitamin B_5 products. (S): supplemented products.

of variation: a little variation of the optical density corresponded to a bigger variation of the quantity of vitamin. The inter-assay variation coefficients of the microbiological method (approx. 10%) were comparable to those reported from a collaborative study on the dosage of pantothenic acid (Tanner *et al.*, 1993). The variation coefficients of the immunological method (approx. 20%) were close to those observed by Finglas *et al.* (1988). A reproducibility of 6% was obtained by Song *et al.* (1990) using an ELISA method with standard solution of pantothenic acid, but they did not take into account the extraction step and matrix influences.

As the immunological method is faster and easier, it can be used instead of the microbiological method for the routine control of supplemented food preparation processes that do not necessarily require an elevated sensitivity.

In foods, the liberation of pantothenic acid from bound forms is required. The specificity of the enzymes used to liberate vitamin B_5 varies. Mylase and clarase have a non-specific amylase activity. Besides this activity, taka-diastase also has proteolytic and lipidolytic activities. Papain has endoproteolytic and amidase activities; it also catalyses hydrolysis of the amino acid esters, preferably basic ones. Alkaline phosphatase (calf intestinal) demonstrates an ortho- and pyrophosphate activity that is essential for CoA hydrolysis (Wittwer *et al.*, 1989). The specificity of the pantetheinase substrate depends on its origin: from mammalian kidney, it hydrolyzes pantetheine but not CoA (Dupré and Cavallini, 1979; Wittwer *et al.*, 1986); that present in avian liver extracts is less specific and allows hydrolysis of the 2 substrates. However, the results may be altered by the presence of phosphatases in the extracts which may lead to the hydrolysis of CoA to pantetheine that would later be converted specifically into pantothenic acid (Wittwer *et al.*, 1989).

APP contained no vitamin B_5. The levels of pantothenic acid content were higher for mylase and PT but were not significant when dilutions were greater than 1/100. Such dilutions are used for foods that contain more than 0·2–0·3 mg of calcium pantothenate for 100 g, which was the case for all the foods in our study. This exogenous addition was observed on the percentage recovery of calcium pantothenate that was slightly higher for these 2 extractions (mylase, PT) than for the other two (buffer, APP). Only the APP extraction achieved the total liberation of the pantothenic acid from CoA. In contrast, there was no hydrolysis by control extraction with buffer.

Apart from 2 products (mixed and soup), there was no significant difference in pantothenate content of the supplemented foods whatever the liberation technique used. Most of the vitamin was free and did not require enzyme hydrolysis before measurement. Hydrolysis concerned only possible bound forms of the vitamin present within the food matrix. Our results demonstrate that the quantity of endogenous vitamin was negligible when compared to the amount of vitamin added. The endogenous vitamin may be destroyed by the treatment applied to the foods (heating, dehydration, lyophilization). The main practical problem encountered in quantifying these foods lies in obtaining representative samples of the foods. For these types of foodstuffs, dried vitamins are normally added then stirred into the rest of the pre-dehydrated food. This gives a non-homogenous distribution of the vitamins within the foods, which means that a 1 g sample was not sufficient. By increasing sample size and pre-diluting in water (15 g of sample + 15 ml water), the coefficient of variation within the same assay batch was divided by 2 (data not shown).

For 2 products (mixed and soup) which contained mainly dehydrated vegetable flakes, there was a marked decrease in pantothenate with APP release. This was also found with both microbiological and immunological methods. No satisfactory explanation can be given for this loss of vitamin B_5.

Apart from the yeasts (2 types of dried baker's yeast-*Saccharomyces cerevisiae*) and the liver, the differences in pantothenate content of the naturally-rich products according to the extraction technique used were not as significantly important as expected. The variations were minimal when compared to those obtained by Neilands and Strong (1948) and those observed with CoA hydrolysis. Walsh *et al.* (1979) suggest a possible liberation of pantothenate during bacterial growth linked either to bacterial enzyme liberation or to a non-enzymatic mechanism. If liberation occurs in this way, it also concerns other bound forms of the vitamin, but not CoA, as hydrolysis by the buffer would not have yielded negative results. It is generally accepted that CoA is quantitatively the most important bound form of the biological products. Annous and Song (1995) have shown that blood did not contain CoA but 4′-phosphopantothenic acid and pantetheine that could be used directly by the bacteria (Bird, 1963). However, it seems unlikely that there was no CoA in the foods analysed. In rats fed a regular diet, there were, in different organs, (heart, kidney or muscle), as many moles of free pantothenic acid as moles of CoA. In the liver, the quantity of CoA (in nmol) was 20 fold higher than of pantothenic acid (Reibel *et al.*, 1982). The levels of free pantothenic acid in natural products is generally low, from 10% to 30% maximum. In contrast, 90% of the pantothenic acid in human milk is in free form (Song *et al.*, 1984; Guilarte, 1989).

Liberation of the vitamin during quantification cannot be envisaged with the ELISA technique (much shorter incubation time, absence of bacterial enzymes). However, cross-reactions between anti-pantothenic-acid antibodies and very similar forms (such as 4′-phosphopantothenic acid and pantetheine) may occur, which would increase the levels of pantothenate actually liberated. Such compounds have not been tested with our

antibody but we have demonstrated that it did not respond to CoA and panthenol (Gonthier *et al.*, 1998). Other antibodies obtained using similar immunogenic conjugates by two different teams (Wyse *et al.*, 1979; Morris *et al.*, 1988) also failed to recognize pantetheine. The cross-reactivity was ca 100 fold less with 4′-phosphopantothenic acid than with pantothenic acid (Srinivasan *et al.*, 1981). The cross-reactions with 4′-phosphopantothenic acid and pantetheine would be facilitated because of the substitution that occurs on the terminal hydroxyl group of the pantothenic acid for both the immunogenic conjugate and 4′-phosphopantothenic acid.

The various techniques for the liberation of pantothenic may be unreliable. Although the assay results we obtained for the various foods are comparable to those published in the composition tables, the microbiological method with a prior extraction phase would need to be compared with a biological measurement using growth of chicks as a measurement parameter. For the same sample of brewer's yeast, Latymer and Coates (1982) observed a level of 5·94 mg $100g^{-1}$ using a biological assay, and 4·22 mg $100\,g^{-1}$ using a microbiological assay after pre-treatment by alkaline phosphatase and pantetheinase. Although liberation is not complete, it can however lead to quantification of about 70% of free vitamin.

Another suggestion to explain this low difference of pantothenic acid contents in function of the extraction method was the presence of endogenous enzymes that allow total or partial liberation of vitamin B_5. CoA is unable to penetrate biological membranes (Domschke *et al.*, 1971). In every cell there is also a permanent cycle of synthesis and degradation between pantothenic acid and CoA using different routes. CoA is converted to pantetheine by non-specific enzymes including an acid phosphatase present in the lysosomes (Bremer *et al.*, 1972) and a pyrophosphatase also active on nucleotides (Skrede, 1973). Pantetheine is hydrolyzed in pantothenic acid by a more or less specific amidase, pantetheinase. These pantetheinases were described in blood (Wittwer *et al.*, 1986). Others were found in pigeon and chicken liver (Neilands and Strong, 1948), pig kidney (Wittwer *et al.*, 1983) and horse kidney (Dupré and Cavallini, 1979). A hydrolytic activity of pantetheine was observed in various rat tissues, with varying results according to the tissues evaluated: it was high in the kidney, heart and small intestine, lower in the liver and muscle (Wittwer *et al.*, 1986). The optimum pH of those enzymes vary according to their origin and hydrolyzed substrate. Pig pantetheinase activity was greater at pH 9–9·5 (Wittwer *et al.*, 1986), whereas horse pantetheinase hydrolyzed pantetheine at pH 4–4·5 (Dupré and Cavallini, 1979) and pantetheine-3-pyruvate at pH 7·5–8·5 (Dupré *et al.*, 1984). The conventional extraction procedures with alkaline phosphatase and pantetheinase recommend heating (for 10–15 min at 121–123°C) the samples before extraction. Pre-treatment by heating is not systematically described with mylase, papain and taka-diastase. To obviate further variation factors between the various extraction methods and to limit heating which may partly destroy vitamin B_5, we decided not to perform preliminary autoclaving. Thus the enzymes naturally present in the foods were not systematically destroyed.

This endogenous enzyme activity was tested with 2 samples. CoA was added in excess to yeast and pig kidney samples and extracted with buffer. No hydrolysis was observed with yeast sample. With the kidney sample, ca 70% of the added CoA was hydrolysed. Therefore, in 2 h at 50°C in a buffer at pH 4·5, the endogenous enzymes led to liberation of a quantity of pantothenate equivalent to APP extraction. Pig kidney produced only a slight difference in pantothenate content according to the extraction technique used. We theorize that this may be related to the high pantetheinase activity measured in the kidney. In contrast, the bigger difference between buffer and APP values for the liver may be related to the low activity of the pantetheinase observed by Wittwer *et al.* (1986) in this tissue.

To explain why yeast differs from other foods, we can note that yeast is able to synthesize pantothenic acid, whereas animal cells cannot, which suggests that it does not contain all the enzymes required to liberate pantothenic acid from the combined forms of the vitamin. The yeasts we tested were dried yeasts. The enzymes may have been destroyed during dehydration, yet we obtained the same results when wet yeast was used. Higher plants are also able to synthesize pantothenic acid (Julliard, 1994), However, variability in such large numbers was not demonstrated with the plants we analysed (whole grain rice, lentils, avocado).

We have noted that 90% of the pantothenic acid in human milk is in free form. This rate was determined by the quantity of pantothenic acid measured after buffer and APP extraction by an RIA method (Song *et al.*, 1984) and a Radiometric Microbiological Assay (Guilarte, 1989). This rate may be falsified by the action of endogenous enzymes. Between 30 and 35% of the vitamin in cows' milk is lost by ultra-high temperature sterilization and storage (Gorner *et al.*, 1980). A part or the totality of the loss may be a result of the inactivation of the endogenous enzymes during sterilization treatment and thus to an artefact of analysis and not to a real destruction of the pantothenic acid.

In conclusion, the two methods for quantification of pantothenic acid we used gave comparable results. ELISA was faster and easier; it can be used instead of the microbiological assay when high sensitivity is not required. For the calcium pantothenate-supplemented foods, enzymatic extraction seems unnecessary. In some cases, it may lead to unexplained vitamin loss. For the naturally-rich vitamin B_5 foods, higher levels were obtained with APP extraction. In some foods, it seems evident that the total or partial liberation of pantothenic

acid is caused by the presence of endogenous enzymes. Their activity differs according to the foods making it more difficult to use than in blood. In our opinion, it is advisable not to destroy these enzymes by an initial heat treatment prior to achieving the extraction.

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REFERENCES

Annous, K. F. and Song, W. O. (1995) Pantothenic acid uptake and metabolism by red blood cells of rats. *Journal of Nutrition* **125**, 2586–2593.

Bell, J. G. (1974) Microbiological assay of vitamins of the B group in foodstuffs. *Laboratory Practice* **23**, 235–241.

Bird, O. D. (1963) Pantothenic acid and related compounds. In *Analytical Microbiology*, pp. 497–517. Academic Press, New York

Bremer, J., Wojtczak, A. and Skrede, S. (1972) Leakage and destruction of CoA in isolated mitochondria. *European Journal of Biochemistry* **25**, 190–197.

Dupré, S. and Cavallini, D. (1979) Purification and properties of a pantetheine-hydrolysing enzyme from horse kidney. In *Methods in Enzymology*, eds D. McCormick and L. Wright, Vol. 62, pp. 262–267. Academic Press, New York.

Dupré, S., Chiaraluce, R., Nardini, M., Cannella, C., Ricci, G. and Cavallini, D. (1984) Continuous spectrophotometric assay of pantetheinase activity. *Analytical Biochemistry* **142**, 175–181.

Domschke, W., Liersch, M. and Decker, K. (1971) Lack of permeation of coenzyme A from blood into liver cells. *Hoppe-Seyler's Zeitschrift für Physiologische Chemie* **352**, 85–88.

Finglas, P. M., Faulks, R. M., Morris, H. C., Scott, K. J. and Morgan, M. R. A. (1988) The development of an enzyme-linked immunosorbent assay (ELISA) for the analysis of pantothenic acid and analogues, Part. II: determination of pantothenic acid in foods by ELISA. *Journal of Micronutritient Analysis* **4**, 47–59.

Gonthier, A., Boullanger, P., Fayol, V. and Hartmann, D. J. (1998) Development of an ELISA for pantothenic acid (vitamin B_5) for application in nutrition and biological fields. *Journal of Immunoassay* (in press).

Gorner, F. and Uherova, R. (1980) Vitamin changes in ultra high temperature sterilized milk during storage. *Nahrung* **24**, 373–379.

Guilarte, T. R. (1989) A radiometric microbiological assay for pantothenic acid in biological fluids. *Analytical Biochemistry* **178**, 63–66.

Julliard, J. H. (1994) Purification and characterization of oxopantoyl lactone reductase from higher plants: role in pantothenate synthesis. *Botanica Acta* **107**, 191–200.

Latymer, E. A. and Coates, M. E. (1982) The availability to the chick of pantothenic acid in foods. *British Journal of Nutrition* **47**, 131–137.

Morris, H. C., Finglas, P. M., Faulks, R. M. and Morgan, M. R. A. (1988) The development of an enzyme-linked immunosorbent assay (ELISA) for the analysis of pantothenic acid and analogues, Part. I: production of antibodies and establishment of ELISA systems. *Journal of Micronutrition Analytical* **4**, 33–46.

Neilands, J. B. and Strong, F. M. (1948) The enzymatic liberation of pantothenic acid. *Archives of Biochemistry* **19**, 287–291.

Novelli, G. D., Kaplan, N. O. and Lipmann, F. (1949) The liberation of pantothenic acid from coenzyme A. *Journal of Biological Chemistry* **177**, 97–107.

Reibel, D. K., Wyse, B. W., Berkich, D. A. and Neely, J. R. (1982) Coenzyme A metabolism in pantothenic acid-deficient rats. *Journal of Nutrition* **112**, 1144–1150.

Skeggs, H. R. and Wright, L. D. (1944) The use of *Lactobacillus arabinosus* in the microbiological determination of pantothenic acid. *Journal of Biological Chemistry* **156**, 21–26.

Skrede, S. (1973) The degradation of CoA: subcellular localization and kinetic properties of CoA and dephospho-CoA pyrophosphatase. *European Journal of Biochemistry* **38**, 401–407.

Smith, A. H., Wyse, B. W. and Hansen, R. G. (1981) The development of an ELISA for pantothenate. *Federal Proceedings* **40**(3886), 915.

Song, W. O., Chan, G. M., Wyse, B. W. and Hansen, R. G. (1984) Effect of pantothenic acid status on the content of the vitamin in human milk. *American Journal of Clinical Nutrition* **40**, 317–324.

Song, W. O., Smith, A., Wittwer, C. T., Wyse, B. W. and Hansen, R. G. (1990) Determination of plasma pantothenic acid by indirect enzyme linked immunosorbent assay. *Nutrition Research* **10**, 439–448.

Srinivasan, V., Christensen, N., Wyse, B. W. and Hansen, R. G. (1981) Pantotrenic acid nutritional status in the elderly-institutionalized and noninstitutionalized. *American Journal of Chemical Nutrition* **34**, 1736–1742.

Tanner, J. T., Barnett, S. A. and Mountford, M. K. (1993) Analysis of milk-based infant formula. Phase V. Vitamins A and E, folic acid and pantothenic acid: food and drug administration-infant formula council: collaborative study. *Journal of the Association of Official Analytical Chemists* **76**, 399–413.

Walsh, J. H., Wyse, B. W. and Hansen, R. G. (1979) A comparison of microbiological and radioimmunoassay methods for the determination of pantothenic acid in foods. *Journal of Food Biochemistry* **3**, 175–189.

Wittwer, C. T., Wyse, B. W. and Hansen, R. G. (1982) Assay of the enzymatic hydrolysis of pantetheine. *Analytical Biochemistry* **122**, 213–222.

Wittwer, C. T., Burkhard, D., Ririe, K., Rasmussen, R., Brown, J., Wyse, B. W. and Hansen, R. G. (1983) Purification and properties of a pantetheine-hydrolysing enzyme from pig kidney. *Journal of Biological Chemistry* **258**, 9733–9738.

Wittwer, C. T., Wyse, B. W. and Hansen, R. G. (1986) Enzymatic hydrolysis of pantetheine. In *Methods in Enzymology*, eds D. McCormick and L. Wright. Vol. 122, pp. 36–43. Academic Press, New York.

Wittwer, C. T., Schweitzer, C., Pearson, J., Song, W. O., Windham, C. T., Wyse, B. W. and Hansen, R. G. (1989) Enzymes for liberation of pantothenic acid in blood: use of plasma pantetheinase. *American Journal of Clinical Nutrition* **50**, 1072–1078.

Wyse, B. W., Wittwer, C. T. and Hansen, R. G. (1979) Radioimmunoassay for pantothenic acid in blood and other tissues. *Clinical Chemistry* **25**, 108–111.

Zook, E. G., MacArthur, M. J. and Toepfer, E. W. (1956) *Pantothenic Acid in Foods*, USDA Handbook 97, Washington, D.C.