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Food Chemistry 78 (2002) 129–134

Food Chemistry

www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods Section

Enzymatic extraction procedure for the liquid chromatographic determination of niacin in foodstuffs

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Received 1 October 2001; received in revised form 28 February 2002; accepted 28 February 2002

Abstract

An enzymatic extraction procedure for the determination of the niacin present in foodstuffs by means of a NAD glycohydrolase (or NADase) has been proposed as a replacement for the hydrochloric acid hydrolysis usually used. The determination of nicotinic acid and nicotinamide by HPLC/fluorimetry [according to Lahély et al. (*Food Chemistry*, 1999, *65*, 129)] has made it possible to show that the acid treatment very definitely led to the release of forms of nicotinic acid not bioavailable in some foodstuffs (wheat flour, wheat germ, peanuts), unlike hydrolysis with NADase from *Neurospora crassa* [37 °C, 18 h, pH 4.5, 24 μg g^{-1} of sample (0.013 U g^{-1})] which led to both an accurate determination of the respective contents of nicotinic acid and nicotinamide and a reliable estimation of the bioavailable niacin in the different foodstuffs analysed. The combined addition of a protease and an amylase to the NADase, sometimes recommended when acid hydrolysis was used, proved to be superfluous.

Keywords: Niacin; Enzymatic extraction; NAD glycohydrolase; Foodstuffs

1. Introduction

A fluorimetric determination of niacin in foods by high performance liquid chromatography with post-column derivatization [UV irradiation in the presence of hydrogen peroxide and copper (II) ions] has recently been published by Lahély, Bergaentzlé, and Hasselmann (1999). Owing to its specificity the derivatization used, initially suggested by Mawatari, Inuma, and Watanabe (1991) for the determination of both nicotinamide and nicotinic acid in human serum, has made it possible to obtain an excellent chromatographic isolation of this vitamin without necessitating a preliminary purification of the samples analysed. It has also been possible to determine it with very good repeatability (RSDr $\leqslant 3.5\%$) and excellent sensitivity (quantification limit 0.2 μg g^{-1}), much better than that which it is possible to attain by direct UV absorption.

The original extraction protocol proposed for this procedure included not only hydrochloric acid hydrolysis in order to release the nicotinamide possibly present in the sample analysed in the form of nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP), but also an alkaline hydrolysis performed on the filtered acid extract in order to convert the nicotinamide present completely into nicotinic acid. Thus, ultimately, only this latter molecule needed to be isolated by liquid chromatography. In routine analyses performed on various samples of cereals such an alkaline treatment has, however, very often induced the formation of impurities, the presence of which then made the chromatographic isolation and quantification of the nicotinic acid impossible. It, therefore, seemed clearly preferable not to retain this alkaline hydrolysis step, which of course has the consequence of making necessary a chromatographic separation of nicotinic acid and nicotinamide and a separate determination of these two vitamers.

In fact, when the objective of the analysis is the determination of the supposedly bioavailable niacin (free nicotinic acid and nicotinamide, NAD, NADP), the most frequent extraction protocol used simply consists of hot hydrolysis of the sample by a dilute mineral acid (Ball, 1994, 1998). However, such a treatment is far removed from the physiological conditions of digestion

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0308-8146/02/$ - see front matter
PII: S0308-8146(02)00205-4

and would moreover be likely to release, at least partially, the nicotinic acid not bioavailable contained in wheat (Hepburn, 1971). It thus seemed judicious to propose the replacement of the usual acid treatment by specific extraction of niacin by means, for example, of an enzyme such as NAD glycohydrolase in order to hydrolyse only the bound forms of this vitamin clearly bioavailable (NAD and NADP).

2. Materials and methods

2.1. Reagents

The vitamin standards [nicotinic acid, nicotinamide, nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP)] were obtained from Sigma-Aldrich (Saint-Quentin Fallavier, France).

The following enzymes were used: NAD glycohydrolase or NADase [E.C. 3.2.2.5] from *Neurospora crassa* (Sigma-Aldrich, catalogue No.N-9879), papain [EC 3.4.22.2] (Prolabo, Fontenay-sous-Bois, France, catalogue No.26.146.180) and α-amylase [E.C. 3.2.1.1] from *Aspergillus oryzae* (Sigma-Aldrich, catalogue No. A-0273). The NADase solution was prepared as follows: 3 ml of enzyme were dissolved in 5 ml of 100 mM phosphate buffer (pH 6.8), then stored at −20 °C until use. The activities of the enzymes were measured at pH 4.5 (50 mM sodium acetate solution) and 37 °C according to the quality control protocols provided by Sigma-Aldrich (NADase activity: 1 unit hydrolyses 1 μmol of NAD to nicotinamide and ADP-ribose in 1 min; protease activity: 1 unit releases 1 μmol of tyrosine from casein in 1 min; amylase activity: 1 unit releases 1 mg of maltose from starch in 3 min).

All other chemicals used were of the highest purity available.

2.2. Sample preparation

The foods studied (frozen peas, frozen spinach, frozen French beans, fresh beef fillet, fresh pork escalope, sweet corn, yeast, wheat flour, wheat germs, rice and peanuts) were purchased at local sources. The various samples were finely ground before being weighed.

2.2.1. Extraction protocols

A finely ground sample (1 g for yeast, 5 g for other foodstuffs) was weighed in a conical flask.

2.2.1.1. Extraction with NADase (phosphate buffer pH 6.8). Fifty millilitres of 100 mM phosphate buffer, then 200 μl of NADase solution (i.e. 120 μg of NADase) were added to the sample. The mixture was incubated at 37 °C for 18 h. After incubation, the solution was made up to 100 ml with distilled water in a graduated flask.

In some experiments, the enzymatic treatment was omitted. The sample was simply solubilized in phosphate buffer (20 °C, 15 min) or incubated at 37 °C for 18 h (possibly after heating at 100 °C for 10 min).

2.2.1.2. Extraction with NADase (sodium acetate solution pH 4.5). Fifty millilitres of 50 mM sodium acetate solution were added to the sample [or to 200 μl of NAD (or NADP) standard solution (4.90 g l^{-1})]. The mixture was incubated at 37 °C for 18 h in the presence of NADase [200 μl (sample) or 100 μl (standard solution)]. After incubation, the solution was made up to 100 ml with distilled water in a graduated flask. In some experiments, the enzymatic treatment was omitted.

A preliminary incubation of the sample in dilute hydrochloric acid solution was sometimes performed. Fifty millilitres of 0.1 M hydrochloric acid were added to the sample in a 150 ml conical flask and the solution was adjusted to pH 2 with 2.5 M sodium acetate. It was then placed in an incubator at 37 °C for 3 h. After being allowed to cool, the solution was adjusted to pH 4.5 with 2.5 M sodium acetate.

2.2.1.3. Extraction with trienzymatic treatment (sodium acetate solution pH 4.5). Fifty millilitres of 50 mM sodium acetate solution, then 200 μl of NADase solution, 100 mg of papain, 500 μl of 1% glutathione and 100 mg of α-amylase were added to the sample. The mixture was incubated at 37 °C for 18 h. After incubation, the solution was made up to 100 ml with distilled water in a graduated flask.

2.2.1.4. Hydrochloric acid extraction. Fifty millilitres of 0.1 M hydrochloric acid were added to the sample [or to 200 μl of NAD (or NADP) standard solution (4.90 g l^{-1})] in a 150 ml conical flask. The solution was placed in a water bath at 100 °C for 1 h. After being allowed to cool, it was adjusted to pH 4.5 with 2.5 M sodium acetate and diluted to 100 ml in a graduated flask with distilled water.

The various solutions obtained after extraction were systematically filtered through filter paper, then through a 0.45 μm cellulose acetate filter. The filtrates were used for chromatographic investigation.

2.3. Chromatographic determination

2.3.1. Apparatus

The HPLC system consisted of a 3012 multisolvent delivery system (Varian, Les Ulis, France), a 9300 injection system (Varian) and a 9075 fluorescence detector (Varian). Chromatographic peaks were quantified using a Star chromatographic integrator (Varian).

An Uptisphere C_{18} HDO column (4.6 mm i.d.×150 mm; 5 μm particle size; Interchim, Montluçon, France)

and a guard column C_{18} HDO (4 mm i.d.×4 mm; 5 μm particle size; Interchim) were used for all analyses.

The photochemical reaction was carried out in a PTFE tube (5 m×0.5 mm i.d.) which was wound around a black light (300–400 nm; vapour lamp with a filter excluding the 254 nm line; power of 7 mW/cm^2; 65.8×14×10 cm; Prolabo).

2.3.2. Chromatographic conditions

The separation performed at ambient temperature and at a flow rate of 1 ml min^{-1} by reversed phase chromatography was accomplished isocratically. The mobile phase employed (0.07 M potassium dihydrogen phosphate, 0.075 M hydrogen peroxide and 5.10^{-6} M copper (II) sulfate solution) was that used by Lahély et al. (1999). The fluorimetric detector operated at an excitation wavelength of 322 nm and at an emission wavelength of 380 nm. The injection volume was 20 or 100 μl.

The data were quantified using external calibration. The standard solutions were diluted with distilled water to obtain calibrated solutions containing 0.01–5 μg ml^{-1} of nicotinic acid and nicotinamide.

2.4. Statistical method

A significance test for the comparison of the means of two samples (significance level $P=0.05$) (Miller & Miller, 1993) was used to compare the niacin concentration values obtained by performing two different extraction protocols.

3. Results and discussion

3.1. The chromatographic separation and detection of nicotinic acid and nicotinamide

Minor modifications were made to the chromatographic protocol proposed by Lahély et al. (1999) for the analysis of nicotinic acid alone. The excessive length (12 m) of the post-column reactor initially recommended by Mawatari et al. (1991), slightly shortened by Lahély et al. (1999) (10 m), was ultimately reduced to 5 m. In fact, a column of such length proved sufficient to permit optimal derivatization of nicotinic acid and nicotinamide. Furthermore, a high density reversed stationary phase, well adapted to the analysis of basic compounds (like nicotinamide), was selected. These modifications have made it possible to obtain chromatographic peaks of excellent quality for the two vitamers (Fig. 1). On the other hand, the mobile phase was not modified because the addition of an organic solvent, which would have permitted the relatively high retention time of nicotinamide (22 min) to be reduced, leads to a marked diminution of the fluorescence quantum yield of this vitamer (Mawatari et al., 1991).

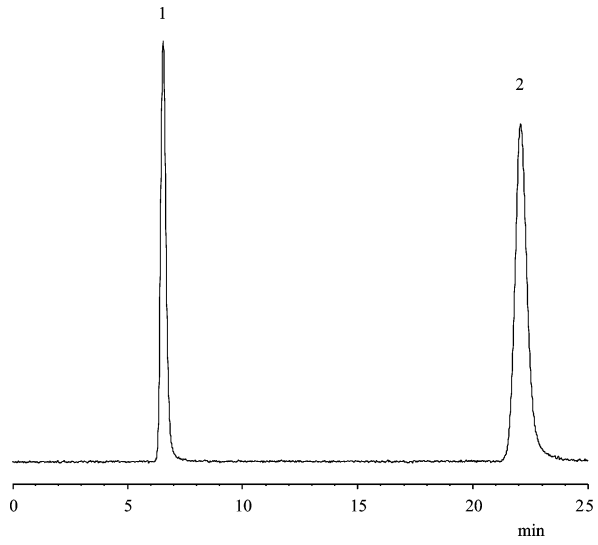

Fig. 1. Chromatographic analysis of a standard solution (2 μg ml^{-1}) of (1) nicotinic acid and (2) nicotinamide (injection volume 20 μl).

3.2. The enzymatic extraction conditions using the NAD glycohydrolase of Neurospora crassa

Of the two NAD glycohydrolases (or NADases) at present commercially available, only the NADase of *Neurospora crassa* is soluble in the aqueous phase. Consequently, it was selected for this study.

The initial assays performed on samples of yeast showed that the use of the enzymatic treatment for 18 h at 37 °C and at the pH optimum of the NADase (buffer pH 6.8) (treatment irrelevant in this case because the yeast apparently does not contain either NAD or NADP, see Table 2) led to erratic experimental contents of niacin, markedly lower than those obtained by a simple dissolution of the sample for 15 min in this

Table 1
Influence of the extraction protocol on the niacin concentration (μg g^{-1} of nicotinic acid equivalents) in a yeast sample[a]

Extraction protocol	Concentration
(1)[b]	181 (6)
(2)[c]	142 (32)
(3)[d]	138 (42)
(4)[e]	178 (12)
(5)[f]	185 (7)
(6)[g]	179 (5)

[a] Average of three determinations (standard deviations in brackets).
[b] (1) Absence of NADase (pH 6.8, 15 min, 20 °C).
[c] (2) NADase (120 μg) (pH 6.8, 18 h, 37 °C).
[d] (3) Absence of NADase (pH 6.8, 18 h, 37 °C).
[e] (4) Absence of NADase (pH 6.8, water bath at 100 °C, 10 min, then 18 h at 37 °C).
[f] (5) Absence of NADase (pH 4.5, 18 h, 37 °C).
[g] (6) NADase (120 μg) (pH 4.5, 18 h, 37 °C).

Table 2
Influence of the extraction protocol on the niacin concentration (expressed in $\mu g.g^{-1}$ of nicotinic acid equivalents) in various foodstuffs[a]

Food	Extraction protocol	Concentration					
		Nicotinic acid		Nicotinamide		Niacin	
Peas	(1)[b]	0.29	(0.01)	11.0	(0.1)	11.3	(0.1)
	(2)[c]	0.27	(0.03)	10.7	(0.3)	10.9	(0.3)
	(3)[d]	1.22	(0.08)	10.2	(0.4)	11.4	(0.4)
	(4)[e]	0.32	(0.05)	0.41	(0.01)	0.73	(0.05)
Spinach	(1)	0		0.72	(0.06)	0.72	(0.06)
	(2)	0		0.71	(0.05)	0.71	(0.05)
	(3)	0		0.69	(0.04)	0.69	(0.04)
	(4)	0		0		0	
French beans	(1)	0.19	(0.01)	2.8	(0.2)	3.0	(0.2)
	(2)	0.25	(0.03)	2.9	(0.2)	3.2	(0.2)
	(3)	0.37	(0.06)	2.6	(0.2)	3.0	(0.2)
	(4)	0		0		0	
Sweet corn	(1)	3.6	(0.2)	13.8	(1.0)	17.4	(1.0)
	(2)	–		–		–	
	(3)	4.3	(0.4)	12.7	(0.9)	17.0	(1.0)
	(4)	3.8	(0.3)	13.7	(0.3)	17.5	(0.4)
Rice	(1)	10.3	(0.2)	0		10.3	(0.2)
	(2)	9.9	(0.7)	0		9.9	(0.7)
	(3)	10.0	(0.5)	0		10.0	(0.5)
	(4)	9.8	(1.0)	0		9.8	(1.0)
Wheat flour	(1)	3.4	(0.1)	1.7	(0.1)	5.2	(0.1)
	(2)	3.2	(0.1)	1.7	(0.1)	5.0	(0.1)
	(3)	5.7	(0.4)	1.9	(0.1)	7.6	(0.4)
	(4)	3.5	(0.1)	0.44	(0.01)	4.0	(0.1)
Wheat germ	(1)	10.8	(0.2)	0		10.8	(0.2)
	(2)	11.0	(0.3)	0		11.0	(0.3)
	(3)	13.8	(0.5)	0		13.8	(0.5)
	(4)	11.1	(0.4)	0		11.1	(0.4)
Peanuts	(1)	26.5	(0.9)	3.7	(0.3)	30.2	(1.0)
	(2)	25.6	(0.3)	3.4	(0.3)	29.0	(0.4)
	(3)	93.4	(0.7)	1.9	(0.3)	95.8	(0.8)
	(4)	26.8	(0.4)	2.9	(0.2)	29.7	(0.5)
Yeast	(1)	17	(1)	182	(5)	199	(5)
	(2)	–		–		–	
	(3)	22.0	(0.3)	174	(5)	196	(5)
	(4)	17	(1)	177	(6)	194	(6)
Beef fillet	(1)	3.8	(0.2)	53	(1)	57	(1)
	(2)	3.5	(0.2)	50	(2)	54	(2)
	(3)	3.6	(0.7)	50	(2)	54	(2)
	(4)	3.51	(0.02)	52	(1)	56	(1)
Pork escalope	(1)	0		64	(2)	64	(2)
	(2)	–		–		–	
	(3)	0.2	(0.1)	57	(1)	58	(1)
	(4)	0		60	(2)	60	(2)

[a] Average of three determinations (standard deviations in brackets).
[b] (1) NADase (120 μg) (pH 4.5, 18 h, 37 °C).
[c] (2) NADase (120 μg), papain (100 mg), α-amylase (10 mg) (pH 4.5, 18 h, 37 °C).
[d] (3) HCl 0.1 M (water bath at 100 °C during 1 h).
[e] (4) Absence of NADase (pH 4.5, 18 h, 37 °C).

same buffer (Table 1). Such results, also obtained when the incubation was performed at 37 °C for 18 h in the absence of NADase, are very certainly explained by microbial destruction of niacin during this incubation. No diminution of the niacin content was in fact observed by initially subjecting the sample to thermal treatment (water bath at 100 °C for 10 min) (Table 1).

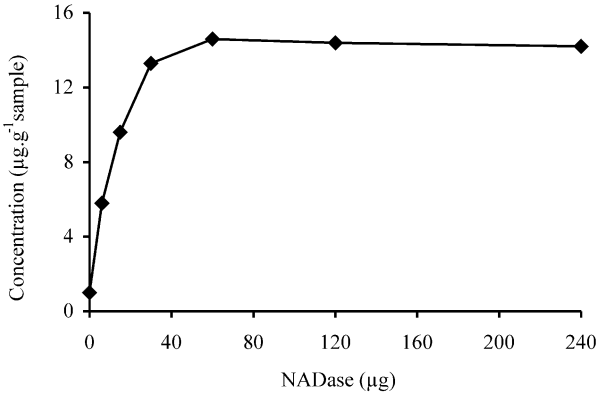

Fig. 2. Concentrations of niacin (in μg g^{-1} of nicotinic acid equivalents) in pea samples (sample weight 5 g) in relation to the quantity per sample of NADase (in μg) [incubation at 37 °C for 18 h in sodium acetate solution (pH 4.5)].

Since the NADase is active in the pH range 3–9 (Schomburg & Salzman, 1991), it seemed preferable to lower the pH of the extraction solution to 4.5 and thus avoid this microbial growth, rather than perform the extraction at the optimal pH of this enzyme and then be obliged to carry out a preliminary heat treatment of the sample. Under such extraction conditions (18 h, 37 °C, pH 4.5), the results obtained (concentration and accuracy) were, in fact, quite satisfactory [Table 1, lines (5) and (6)]. Under the same conditions, NAD and NADP (1.35 and 1.17 μmol respectively) were quantitatively transformed into nicotinamide during NADase digestion (60 μg) of standard solutions of these nucleotides. This enzymatic hydrolysis did not induce any subsequent transformation of nicotinamide into nicotinic acid.

The determination of the quantity of NADase necessary for the hydrolysis of the bioavailable, bound forms in foodstuffs was carried out using peas as test sample (5 g) on account of their NAD and NADP content (10.6 μg g^{-1}, expressed in nicotinic acid equivalents, i.e. 0.43 μmol per sample, expressed in NAD equivalents), markedly higher than those of the other foodstuffs studied containing these nucleotides (spinach, French beans, wheat flour, peanuts) [see Table 2, lines (1) and (4)]. The results obtained showed that the quantity of NADase necessary to attain a maximal niacin content was 60 μg (Fig. 2). Twice the quantity of NADase was finally used in the analytical protocol [24 μg g^{-1} (i.e. 0.013 U g^{-1}) of sample, the NADase used having an activity of 0.55 U mg^{-1}] and thus ought a priori always to be more than adequate to hydrolyse all of the NAD and NADP present in the analysed sample, whatever the nature of the foodstuff under study.

3.3. The possible combination of a protease and an amylase with the NADase

The main justification for the treatment in acid medium (0.1 M) on a water bath at 100 °C or in an autoclave at 120 °C, usually performed for the extraction of bioavailable niacin (Ball, 1998), is the conversion of NAD and NADP into nicotinamide. However, it also causes an at least partial hydrolysis of the proteins and starch possibly contained in the foodstuff analysed. Some authors, undoubtedly because they considered a more complete hydrolysis of these polymers necessary for a correct determination of niacin, have even coupled this acid hydrolysis with a diastase treatment (van Niekerk, Smit, Strydom, & Armbruster, 1984; Vidal-Valverde & Reche, 1991), sometimes combined with a papain treatment (Krishnan, Mahmud, & Matthees, 1999). Since the NADase recommended in this analytical protocol does not contain any protease or amylase impurity, it was legitimate to ask whether the combination of a protease (papain) and an amylase (α-amylase) with this enzyme could possibly prove to be essential for the determination of niacin in some foodstuffs. The addition of these enzymes was in fact not justified in any of the foodstuffs analysed [Table 2, lines (1) and (2)]. A simple treatment with the NADase was always sufficient, even when these foodstuffs contained large quantities of starch (rice, wheat flour) or proteins (wheat germ, peanuts, beef fillet).

3.4. The comparison of the niacin contents obtained as a function of the extraction protocol used. Advantages of the treatment with NADase

The niacin contents (nicotinic acid and nicotinamide) of various foodstuffs obtained by performing hydrolysis of the samples either with NADase or hot dilute hydrochloric acid are given in Table 2 [(lines (1) and (3)].

Acid hydrolysis led to niacin contents significantly higher than those obtained by performing enzymatic hydrolysis in the analysis of wheat flour, wheat germ and peanuts. Very likely, and as had already been suggested by Hepburn (1971), the increase in their niacin content (exclusively attributable to the increase in their nicotinic acid content) results from the release of bound vitamin forms, probably bioavailable only to a slight extent or not at all. In fact, it has never been possible to release these latter, even partially, by subjecting these foodstuff samples to a mild acid treatment (pH 2, 37 °C, 3 h), close to the physiological conditions of digestion, whether or not this is followed by the enzymatic treatment (Table 3). On the other hand, acid hydrolysis induced a slight degradation of nicotinamide in the pork escalope sample, thereby causing a moderate decrease of its niacin content.

In all of the other foodstuffs studied, the contents of niacin appeared not to depend on the extraction protocol

Table 3
Influence of the extraction protocol on the niacin concentration (expressed in μg g^{-1} of nicotinic acid equivalents) in various foodstuffs[a]

Food	Extraction protocol	Concentration					
		Nicotinic acid		Nicotinamide		Niacin	
Wheat flour	(1)[b]	3.0	(0.3)	0.47	(0.09)	3.5	(0.3)
	(2)[c]	3.10	(0.09)	1.9	(0.2)	5.0	(0.2)
	(3)[d]	5.5	(0.4)	1.9	(0.2)	7.5	(0.4)
	(4)[e]	2.94	(0.07)	1.82	(0.06)	4.79	(0.09)
	(5)[f]	3.01	(0.01)	1.7	(0.2)	4.7	(0.2)
Wheat germ	(1)	10.9	(0.6)	0		10.9	(0.6)
	(2)	10.9	(0.4)	0		10.9	(0.4)
	(3)	14.5	(0.6)	0		14.5	(0.6)
	(4)	11.1	(0.2)	0		11.1	(0.2)
	(5)	11.1	(0.7)	0		11.1	(0.7)
Peanuts	(1)	27	(1)	2.54	(0.04)	30	(1)
	(2)	27.5	(0.3)	2.85	(0.05)	30.4	(0.3)
	(3)	94	(3)	1.8	(0.2)	96	(3)
	(4)	28.0	(0.9)	2.01	(0.08)	30.1	(0.9)
	(5)	29.6	(0.6)	2.0	(0.1)	31.6	(0.6)

[a] Average of three determinations (standard deviations in brackets).
[b] (1) Absence of NADase (pH 4.5, 18 h, 37 °C).
[c] (2) NADase (120 μg) (pH 4.5, 18 h, 37 °C).
[d] (3) HCl 0.1 M (water bath at 100 °C during 1 h).
[e] (4) HCl pH 2 (3 h, 37 °C).
[f] (5) HCl pH 2 (3h, 37 °C) and NADase (120 μg, pH 4.5, 18 h, 37 °C).

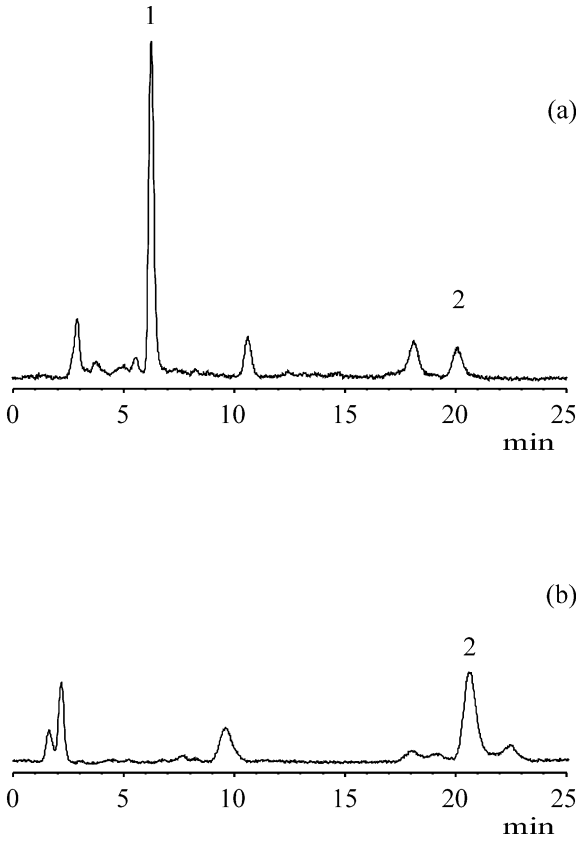

Fig. 3. Chromatographic isolation of (1) nicotinic acid and/or (2) nicotinamide in (a) peanuts (injection volume 20 μl) and (b) spinach (injection volume 100 μl) .

used. On analysis of peas, French beans and yeast, foodstuffs in which nicotinamide is by far the major vitamer (essentially or completely furnished by hydrolysis of NAD and NADP in the first two, pre-existing in the third), acid hydrolysis led, however, to nicotinic acid contents slightly higher than those obtained by performing enzymatic hydrolysis (Table 2). This excess of nicotinic acid does not result from an increase in the release of this vitamer subsequent to partial hydrolysis of the proteins and/or the starch of the food matrix, since the addition of a protease and an amylase to the NADase has never led to an increase of the nicotinic acid content in the foodstuffs analysed [see Table 2, lines (1) and (2)]. Most probably, it results from a partial conversion of the nicotinamide to nicotinic acid. By carrying out 0.1 M hydrochloric acid hydrolysis of standard solutions of NAD (1.35 μmol) and NADP (1.17 μmol) in a water bath at 100 °C for 1 h, it was in fact shown that about 10% of the nicotinamide formed during the total hydrolysis of these nucleotides were converted into nicotinic acid.

The extraction of niacin by means of NADase, a very specific enzyme the action of which seems to be strictly limited to the hydrolysis of NAD and NADP to nicotinamide, has always made it possible to obtain a very satisfactory subsequent chromatographic isolation of nicotinic acid and/or nicotinamide (Fig. 3), even when one of these compounds was present in very low concentration in the foodstuff analysed (Fig. 3b). Thus, it appears preferable to a hot hydrochloric acid hydrolysis for an accurate determination of the nicotinic acid and nicotinamide contents and for a good estimation of the bioavailable niacin in foodstuffs.

Acknowledgements

This work was supported by a grant (RA 97.13) from ACTIA (French Ministry of Agriculture and Fisheries).

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