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Fluorimetric determination of niacin in foods by high-performance liquid chromatography with post-column derivatization

S. Lahély [a], M. Bergaentzlé [a], C. Hasselmann [a,b,*]

[a] *Département des Sciences de l'Aliment, Faculté de Pharmacie 74, route du Rhin, 67400 Illkirch, France*

[b] *Laboratoire Interrégional de la Direction Générale de la Concurrence, de la Consommation et de la Répression des Fraudes, chemin du Routoir, 67400 Illkirch, France*

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Abstract

A method to determine the content of niacin in various foods by liquid chromatography is proposed and includes hydrochloric acid hydrolysis of the sample, pre-column conversion of the different vitamers (NAD, NADP, nicotinamide and nicotinic acid) into nicotinic acid by alkaline hydrolysis, separation on a C18 stationary phase, post-column derivatization by UV irradiation in the presence of copper (II) ions and hydrogen peroxide, and fluorimetric detection. The proposed method leads to a good recovery rate (90–107%) and a satisfactory repeatability (coefficient of variation less than 4%). Owing to its very low quantification limit (0.2 μg g^{-1}) and the excellent resolution of the nicotinic acid peak, it could most probably be applied to the determination of niacin in any foodstuff.

1. Introduction

Dietary niacin (nicotinamide and nicotinic acid) is mainly present in the pyridine nucleotides nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP). In manufactured foods, these bound forms could be partially hydrolyzed into nicotinamide and nicotinic acid (Jacob and Swendseid, 1990). In plants, niacin may be bound to macromolecules and unavailable for mammals. In cereals, it is mainly bound to polysaccharides, but also to polypeptides and glycopeptides (sometimes referred to as niacinogen or niacytin) and only about 25% of these bound forms are susceptible to hydrolysis in the human organism by gastric juice (Van den Berg, 1997). However this low bioavailability in cereals could be increased by prior treatment with alkali (as for the preparation of tortillas in Central America where corn is cooked in lime).

Whatever the analytical technique used for niacin determination (microbiology, photometry, capillary electrophoresis, HPLC or GC), a prior hydrolysis of food is essential. If biologically active niacin is required, an acid hydrolysis is preferred, whereas if total niacin is required, an alkaline treatment is necessary to release non-bioavailable niacin (Finglas and Faulks, 1984).

At present, the method the most often used for niacin determination is the microbiological method using *Lactobacillus plantarum* (Anonymous, 1997a). This method is very sensitive, but time-consuming and lacks reproducibility. On the other hand, its specificity is uncertain (Snell and Wright, 1941). The colorimetric method based on the König reaction, also recommended by the AOAC (Anonymous, 1997b) despite the danger of direct handling of cyanogen bromide, presents a sensitivity lower than that of the microbiological method. Moreover, this method is not specific for niacin, because all pyridine derivatives so react. In fact, improvement of the specificity requires prior isolation of niacin.

Very recently, determination of nicotinic acid by capillary electrophoresis with UV detection was reported by Ward et al. (1997). However the poor sensitivity of the detector restricts the application of this method to the analysis of supplemented foodstuffs. Gas chromatography is scarcely used, owing to the low volatility of niacin and the necessity of preparing a volatile derivative. The method proposed by Tanaka et al. (1989) for nicotinamide determination is also only applicable to supplemented foods. Stennert and Maier (1996) proposed the use of a nitrogen-phosphorus detector for the nicotinic acid determination (after

* Corresponding author. Tel.: 0033 388 676 920; fax: 0033 388 676 944; e-mail: claude.hasselmann@pharma.u-strasbg.fr.

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derivatization as methyl ester) in coffee (which contains high amounts of this vitamin) but did not apply their method to other foodstuffs. On the contrary, numerous methods by high-performance liquid chromatography, using UV detection, have been proposed for the determination of niacin in foodstuffs (Ball, 1998), but the different pre-column clean-up procedures considered, sometimes complex to implement, like column switching (Van Niekerk et al., 1984), never allowed a good resolution of niacin, owing to the low specificity and the insufficient sensitivity of the detection mode. Electrochemical detection (used for nicotinic acid detection) did not give more satisfactory results (Kral, 1983). In fact, only fluorimetric detection, requiring a pre- or post-column derivatization of niacin (because this vitamin is not fluorescent), could allow a sensitive determination of niacin in food. Some derivatization reactions have been reported, but only tested on standard solutions of vitamins or serum samples, never on food samples. The reagents proposed by Tsuruta and Kohashi (1984) and Navas Diaz et al. (1993) react with the carboxylic function of nicotinic acid and thus would not be very specific for the analysis of a food matrix. They also offer other disadvantages: the reaction takes place in an organic medium (and the reagent is commercially unavailable) (Tsuruta and Kohashi, 1984) or is very time-consuming (2h) (Navas Diaz et al., 1993). On the other hand, the reaction reported by Mawatari et al. (1991) for the determination of both nicotinamide and nicotinic acid in human serum (UV irradiation with $\lambda > 300$ nm in the presence of hydrogen peroxide and copper (II) ions) which takes place in aqueous solution, seems very fast, thus suitable for post-column derivatization and, moreover, highly specific.

The aims of this study were to show that this derivatization reaction could be applied to food matrixes and to propose a specific and sensitive method by reverse phase HPLC/fluorimetry for the determination of niacin in any food.

2. Materials and methods

2.1. Reagents

Nicotinic acid standard stock solution. For a 1 g l^{-1} standard stock solution, 100 mg of nicotinic acid (99%, Merck, Nogent-sur-Marne, France) were dissolved in 100 ml distilled water.

Nicotinamide standard stock solution. For a 1 g l^{-1} standard stock solution, 100 mg of nicotinamide (Sigma Chemicals, St-Quentin Fallavier, France) were dissolved in 100 ml distilled water.

NAD standard stock solution. For a 0.5 g l^{-1} standard stock solution, 50 mg of NAD (99%, Sigma Chemicals) were dissolved in 100 ml distilled water.

NADP standard stock solution. For a 0.5 g l^{-1} standard stock solution, 50 mg of NADP (sodium salt, 99%, Sigma Chemicals) were dissolved in 100 ml distilled water.

Hydrochloric acid solution. For a 0.1 M solution, 4 ml of 37% hydrochloric acid (for analysis, Carlo Erba, Rueil-Malmaison, France) was diluted to 500 ml with distilled water.

Sodium hydroxide solution. For a 5 M solution, 20 g of sodium hydroxide (analytical grade, SDS, Peypin, France) were dissolved in 100 ml distilled water.

Copper(II) sulfate solution. For a 5.10^{-3} M solution, 0.12 g of copper(II) sulfate pentahydrate (99%, Merck) were dissolved in 100 ml distilled water.

Mobile phase. For a 0.07 M potassium dihydrogen phosphate, 0.075 M hydrogen peroxide and 5.10^{-6} M copper(II) sulfate solution, 4.77 g of potassium dihydrogen phosphate (for analysis, Merck) were dissolved in ca. 400 ml distilled water. 0.5 ml of copper(II) sulfate 5.10^{-3} M solution and 3.8 ml of 30% hydrogen peroxide (Aldrich, St-Quentin Fallavier, France) were added. The solution was made up to 500 ml with distilled water. After being shaken, the solution was filtered through a 0.45 μm cellulose acetate filter (Sartorius, Goettingen, Germany).

2.2. Sample preparation

Foods studied (beef liver, fruit juice, brewer's yeast, peanuts, tomato, biscuits, green peas, crystallized fruit, beer, pork and veal) were randomly selected at local sources. Fruit juice was supplemented with niacin. Brewer's yeast, biscuits and crystallized fruit contained guaranteed amounts of niacin.

A finely ground sample (1 – 5 g) was weighed into a conical flask and 0.1 M hydrochloric acid (30 ml) was added. The solution was heated in a water bath at 100°C for one hour. After being allowed to cool, the solution was made up to 50 ml with distilled water and then filtered through filter paper. A 25-ml aliquot was added to 5 ml of a 5 M sodium hydroxide solution. The solution was autoclaved at 120°C for 1h. After being allowed to cool, the pH of the solution was adjusted to pH 4.5 and the volume made up to 50 ml with distilled water. The solution was filtered first through filter paper and then through a cellulose nitrate filter (0.45 μm) (Sartorius). This filtrate was 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 (Varian) and a Model 470 scanning fluorescence detector (Waters, Milford, USA). Chromatographic

peaks were quantified using a Star Chromatography integrator (Varian).

A Lichrospher 100 RP 18 endcapped (5 mm i.d. × 250 mm; octadecylsilyl; 5 μm particle size; Merck) and a guard column RP 18 (4 mm i.d. × 4 mm; octadecylsilyl; 5 μm particle size; Merck) were used for all analyses.

The photochemical reaction was carried out in a PTFE tube (10 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 cm × 14 cm × 10 cm; Prolabo, Fontenay-sous-Bois, France).

2.3.2. Chromatographic conditions

These conditions were developed by Mawatari et al. (1991). Separation by reverse phase chromatography was accomplished isocratically with the mobile phase described above. The separation was performed at ambient temperature and at a flow rate of 1 ml min^{-1}. The fluorimetric detector operated at an excitation wavelength of 322 nm and at an emission wavelength of 380 nm. The injection volume was 20 μl.

The standard stock solution of nicotinic acid was diluted to 1/10 and 1/100 with distilled water, then again to obtain calibrated solutions containing 0.1 to 8 μg ml^{-1} of nicotinic acid.

For the recovery tests, known volumes of the standard solutions were added to the sample solution before the first hydrolysis.

Precision of the method was estimated by calculating standard deviations (Sr), relative standard deviations and repeatability (r = 2.8 Sr).

3. Results and discussion

Mawatari et al. (1991) did not propose any mechanism to explain the formation of a fluorescent compound from nicotinic acid and nicotinamide by UV irradiation in the presence of hydrogen peroxide and copper (II) ions. Most probably, the copper (II) ions catalyze the formation of OH radicals by hydrogen peroxide photolysis, which would then substitute a hydrogen atom of the pyridine ring in the ortho or para position; the enol thus formed would be in equilibrium with the corresponding ketone.

The chromatographic conditions proposed by these authors and applied to a standard solution of nicotinic acid, nicotinamide, NAD and NADP, showed that the peaks corresponding to NAD and NADP are extremely broad and their fluorescence intensities much lower than those of peaks corresponding to the free forms (Fig. 1). In other respects, the mobile phase used did not allow a good resolution of the NADP and nicotinamide peaks. The addition of a counter ion to the mobile phase, and thus of an organic solvent, in order to improve this resolution, led to a considerable reduction of the fluorescence intensity. Under these conditions, it seemed more discerning to propose a pre-column conversion of NAD, NADP and nicotinamide into nicotinic acid instead of trying to determine these different vitamers separately.

The conversion of nicotinamide into nicotinic acid by autoclaving (120°C) for one hour in the presence of 0.22 M calcium hydroxide (conditions sometimes used to hydrolyze bound forms of niacin (Tyler and Shrago, 1980; Van Niekerk et al., 1984)) occurred only with a yield lower than 80%. A complete conversion of standard solutions of NAD, NADP and nicotinamide into nicotinic acid under the same heating conditions was only achieved by using 0.1 M sodium hydroxide. In a food matrix, the sodium hydroxide concentration should be increased to 0.8 M to obtain such a result (verified for all foodstuffs studied).

Unfortunately, an alkaline treatment releases a large part of non-bioavailable forms of niacin in food (Van den Berg, 1997). Moreover, the use of sodium hydroxide induces a gelation of the samples. A prior acid hydrolysis (0.1M HCl in a water bath at 100°C for 1 h) was thus performed to release only bioavailable forms of niacin (Gregory, 1985). Such an acid hydrolysis (followed by an alkaline treatment) applied to yeast or beef liver samples (which contain high amounts of natural niacin) led effectively to niacin concentrations similar to those obtained under conditions simulating roughly those encountered in gastric digestion (0.1 M hydrochloric acid hydrolysis at 37°C for 3 h (followed by an alkaline treatment)) (respectively, 248 and 241 μg g^{-1} in yeast; 185 and 177 μg g^{-1} in beef liver).

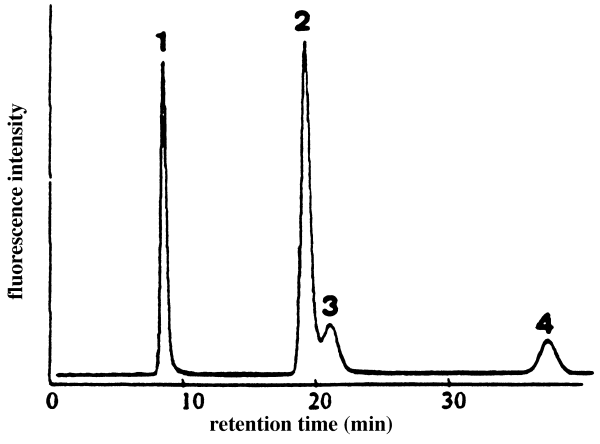

Fig. 1. Chromatographic analysis of a standard solution of nicotinic acid (1) (8.1 μmol ml^{-1}), nicotinamide (2) (8.2 μmol ml^{-1}), NADP (3) (12.3 μmol ml^{-1}) and NAD (4) (13.9 μmol ml^{-1}).

The proposed analytical protocol (hydrochloric acid hydrolysis, pre-column conversion of all the vitamers into nicotinic acid, reverse phase chromatography, post-column derivatization and fluorimetric detection) allowed an excellent resolution of the nicotinic acid peak (retention time 8.5 min) in all foods studied, proving the very good specificity of the post-column reaction (Fig. 2). A linear calibration curve ($y = 3.99x + 0.04$, regression coefficient $R^2 = 0.9994$) for peak area against concentration in the range of 0.01–8 μg ml^{-1} nicotinic acid was obtained.

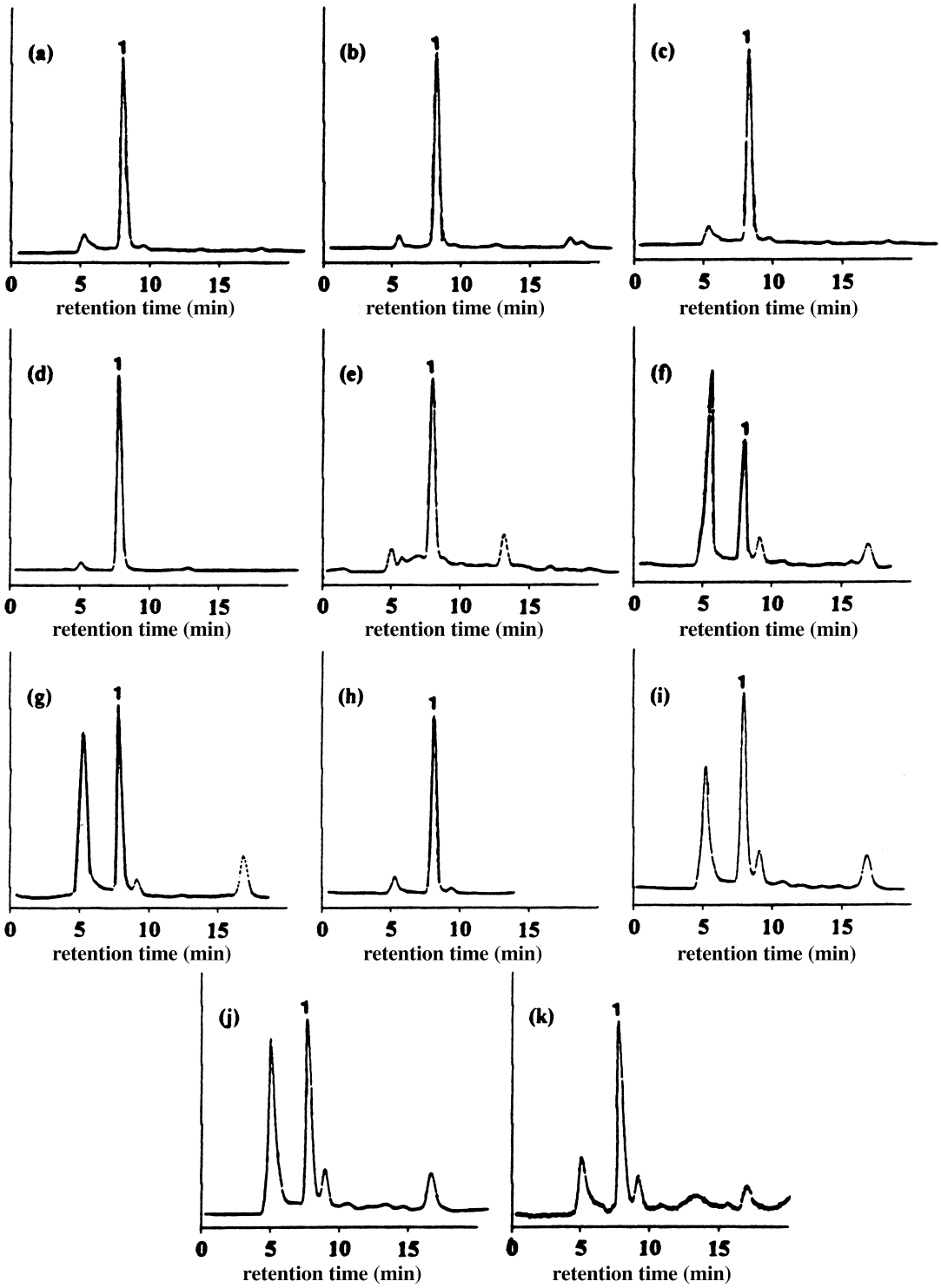

Fig. 2. Chromatographic separation of nicotinic acid (1) in yeast (a), peanut (b), biscuits (c), fruit juice (d), crystallized fruit (e), green peas (f), tomato (g) (see concentrations in Table 2), beef liver (183 μg g^{-1}) (h), veal (71 μg g^{-1}) (i), pork (55 μg g^{-1}) (j), beer (6 μg g^{-1}) (k).

Table 1
Recovery[a] (%) of nicotinamide and nicotinic acid added to various foodstuffs

Food	Nicotinic acid		Nicotinamide	
	Recovery rate	Standard deviation	Recovery rate	Standard deviation
Yeast	101	4	107	4
Fruit Juice	96	3	94	4
Green peas	102	6	105	4
Tomato	94	3	90	2

[a] Average of six replicates.

Table 2
Niacin concentrations[a] (μg g^{-1}) in various foodstuffs

Food	Niacin concentrations	Standard deviation	Coefficient of variation (%)	Repeatability
Yeast	218	6	2.6	16
Peanut	209	7	3.2	18
Biscuits	203	6	3.1	18
Fruit juice	142	3	2.3	9
Crystallized fruit	86	3	3.5	8
Green peas	16.8	0.6	3.5	2
Tomato	4.2	0.1	2.0	0.3

[a] Average of six replicates.

Recovery rates were always satisfactory (close to 100%) both for nicotinic acid and nicotinamide (Table 1). Repeatability was also satisfactory (coefficient of variation always lower than 4%) (Table 2).

Owing to the high sensitivity of the fluorimetric detection, the quantification limit obtained was estimated at approximately 0.2 μg g^{-1} (for a 5 g test sample). This protocol could most probably be applied to the determination of niacin in any foodstuff.

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