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Determination of biotin in foods by high-performance liquid chromatography with post-column derivatization and fluorimetric detection

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Abstract

A method to determine the content of biotin in various foods by reverse phase liquid chromatography is proposed and includes extraction of the vitamers (*d*-biotin and *d*-biocytin) by enzymatic hydrolysis (papain) and takadiastase if necessary, post-column derivatization by avidin-FITC (fluorescein 5-isothiocyanate) and fluorimetric detection of the complex obtained. Enzymatic hydrolysis is preferred to sulfuric acid hydrolysis because it does not induce any degradation of the vitamin and allows a good estimation of the bioavailable forms. The proposed method has a good recovery rate (90–106%), a satisfactory repeatability (coefficient of variation less than 7%) and a very low detection limit (0.005 μg g^{-1}).

1. Introduction

Naturally active forms of biotin in foodstuffs are *d*-biotin and *d*-biocytin (Fig. 1). These two forms should therefore be determined in order to estimate the biotin nutritional potency of foods.

d-Biotin and *d*-biocytin occur in food in the free form but also covalently bound to proteins of the food matrix. In order to break these bonds, two kinds of treatment have been proposed: enzymatic hydrolysis with papain (Bitsch, Salz & Hötzel, 1986) or strong acid hydrolysis (1–3 M sulfuric acid by autoclaving at 121°C for 1–2 h) (Ball, 1998). This acid treatment, more frequently used than the enzymatic one, totally converts *d*-biocytin into *d*-biotin and permits therefore a total determination of biotin in foodstuffs containing *d*-biocytin, provided that the two vitamers have the same bioavailability. This hypothesis is very likely since biotinidase, present in pancreatic juice and intestinal mucosa of human organism, is capable of hydrolyzing *d*-biocytin to yield *d*-biotin (Ball, 1998), but still requires confirmation. On the contrary, the enzymatic hydrolysis with papain lets *d*-biocytin unchanged.

The methods of determination most commonly used in food analysis – by microbiology (using mainly *Lactobacillus plantarum*, Wright & Skeggs, 1944) or by photometry/fluorimetry (based upon the formation of a complex with avidin or streptavidin) (Rettenmaier, 1980; Bitsch, Salz & Hötzel, 1986; Reichert & Rubach, 1991) – are generally complex to implement and do not distinguish *d*-biotin from its inactive analogs. Moreover, during the microbiological determination, some food components (insaturated fatty acids, Axelrod, Mitz & Hofman, 1948), and other lipid compounds, Broquist & Snell, 1951) are able to favour the growth of the bacterial strain. In order to avoid an overestimate of biotin content and therefore to improve specificity of the determination, the prior isolation of *d*-biotin and *d*-biocytin from their food matrix seemed essential. As these vitamers (not very volatile) are water-soluble, high-performance liquid chromatography (HPLC) appeared particularly well suited for this isolation. However the usual content of biotin in most foods, from 5 to 50 ng g^{-1} according to Souci, Fachmann & Kraut, 1994, and the absence of strong chromophores, and therefore of fluorophores, in *d*-biotin's structure has precluded any direct sensitive detection of this vitamin by UV photometry or fluorimetry. The sensitivity of mass spectrometer is also inadequate (Careri, Cilloni, Lugari

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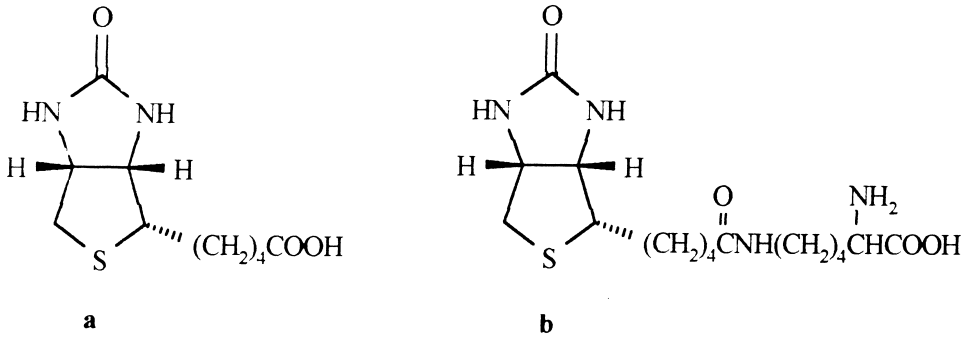

Fig. 1. Structures of *d*-biotin (a) and *d*-biocytin (b).

& Manini, 1996). A satisfactory detection limit could only be obtained after a pre- or post-column conversion of *d*-biotin and *d*-biocytin into fluorescent derivatives.

Derivatization of the carboxylic group of *d*-biotin by means of a fluorophore has been proposed for the determination of *d*-biotin in serum samples (Desbene, Coustal & Frappier, 1983; Röder, Engelbert & Troschütz, 1984; Hayakawa & Oizumi, 1987; Yoshida, Uetake, Nakai, Nimura & Kinoshita, 1988; Stein, Hahn, Lembcke & Rehner, 1992). The reactions proposed are always performed in an organic medium. They lead to the formation of numerous interfering products, are time-consuming (1–3 h) and lack specificity because of the abundance of carboxylic groups in food matrices. They cannot be recommended in food analysis. On the contrary, the fast complexation of *d*-biotin and *d*-biocytin with avidin or streptavidin appears to be very specific (Green, 1970). On that account, these proteins, covalently bound to a fluorescent marker (fluorescein 5-isothiocyanate or FITC) could be used as reagent for a post-column derivatization of *d*-biotin and *d*-biocytin, as suggested by Przyjazny, Hentz & Bachas, 1993 and Hentz & Bachas, 1995. These authors however only applied these reactions to the determination of *d*-biotin in two food supplements.

The objective of this study is to show that this derivatization reaction is of general application in food analysis and to propose a highly specific and sensitive chromatographic method for the determination of biotin in any food.

2. Materials and methods

2.1. Reagents

d-Biotin and d-biocytin standard stock solutions. For a 100 μg ml^{-1} standard stock solution, 10 mg of *d*-biotin (99%, Sigma Chemicals, St-Quentin Fallavier, France) or *d*-biocytin (Sigma Chemicals) were dissolved in 100 ml distilled water.

1 M *Sulfuric acid solution*. 1 ml of sulfuric acid (96%, for RPE, Carlo Erba, Rueil-Malmaison, France) was diluted to 20 ml with distilled water.

1.5 M *Sulfuric acid solution*. 1.5 ml of sulfuric acid (96%, for RPE) was diluted to 20 ml with distilled water.

Citrate buffer solution. 0.92 g of citric acid, monohydrate (99.7%, Sigma Chemicals) and 2.10 g of sodium monohydrogen phosphate, dihydrate (for analysis, Merck, Nogent-sur-Marne, France) were dissolved in 900 ml distilled water. This solution was adjusted to pH 5.7 with 1 M sulfuric acid, and then diluted to 1000 ml with distilled water (solution stable for 24 h only).

1% *Glutathione solution*. 0.1 g of reduced glutathione (98–100%, Sigma Chemicals) was dissolved in 10 ml distilled water (solution stable for 24 h only).

1% *EDTA solution*. 0.1 g EDTA, sodium salt, dihydrate (Sigma Chemicals) was dissolved in 10 ml distilled water (solution stable for 24 h only).

2% *Papain solution*. 1 g of papain powder (titer 80, Prolabo, Fontenay-sous-Bois, France) was dissolved in 50 ml of citrate buffer solution (solution stable for 5 days at 4°C).

0.1 M *Potassium monohydrogen phosphate solution*. 17.4 g of potassium monohydrogen phosphate, dihydrate (for analysis, Merck) were dissolved in 1000 ml distilled water.

0.1 M *Potassium dihydrogen phosphate solution*. 13.6 g of potassium dihydrogen phosphate (for analysis, Merck) were dissolved in 1000 ml distilled water.

0.1 M *Phosphate buffer solutions* (pH 6 *and* 7). 0.1 M potassium monohydrogen phosphate solution and 0.1 M potassium dihydrogen phosphate solution were mixed in such proportions that the final solutions had a pH of 6 and 7.

Reagent for post-column derivatization. 50 mg l^{-1} avidin-FITC (80% of protein, 2–4 mol FITC/mol avidin, Sigma Chemicals) stock solution (solution stable for 2 weeks at 4°C) was prepared in phosphate buffer solution (pH 7). 24 ml of this stock solution were diluted to 600 ml with the same phosphate buffer solution. This solution was filtered through a 0.45 μm acetate cellulose

filter (Sartorius, Goettingen, Germany) (solution stable for 8 h, screened from light).

2.2. Sample preparation

Foods studied (cereals, multivitamin tablet, fruit juice, infant milk, beef liver, tomato, peanuts, brewer's yeast, yogurt, carrot, pork, chicken and (raw) egg) were randomly selected at local sources. Cereals, multivitamin tablet, fruit juice and infant milk contained guaranteed amounts of biotin.

A finely ground sample (5–10 g) was weighed into a conical flask. Reduced glutathione (300 μl), EDTA (300 μl), citrate buffer (30 ml) and papain (3 ml) were added. If the sample analyzed contained high amounts of starch (cereals and brewer's yeast), 100 mg of takadiastase (from *Aspergillus Oryzae*, 60.5 U mg^{-1}, Buchs, Switzerland) were added. The solution was continuously shaken and incubated in an oven at 37°C overnight and, after being cooled, made up to 50 ml with distilled water. This solution was shaken and filtered first through filter paper and then through a cellulose nitrate filter (0.45 μm) before chromatographic injection.

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. $\times$ 250 mm; octadecylsilyl; 5 μm particle size; Merck) and a guard column RP 18 (4 mm i.d. $\times$ 4 mm; octadecylsilyl; 5 μm particle size; Merck) were used for all analyses.

The reagent for post-column derivatization, pumped with a Model 501 pump (Waters), was added to the column effluent through a T connector followed by a 10.0 m knitted open-tubular (KOT) reactor made of PTFE tubing (0.5 mm i.d., 14 mm helix diameter) prepared according to Selavka, Jino & Krull, 1987.

2.3.2. Chromatographic conditions

Separation by reverse phase chromatography was accomplished isocratically with a mobile phase consisting of 0.1 M phosphate buffer solution (pH 6) and methanol (for HPLC, Carlo Erba) (81:19 v/v). This solution was filtered through a 0.45 μm regenerated cellulose filter (Sartorius).

The separation was performed at ambient temperature and at a flow rate of 0.4 ml min^{-1}. The reagent for post-column derivatization was pumped with a flow rate of 1 ml min^{-1}.

The fluorimetric detector operated at an excitation wavelength of 490 nm and at an emission wavelength of 520 nm.

The injection volume varied from 20 to 200 μl. Data were quantified using external calibration. The stock standard solutions of *d*-biotin and *d*-biocytin were diluted to 1/10 and 1/100 with distilled water, then again to obtain calibrated solutions containing 0.001–0.5 μg ml^{-1} of *d*-biotin or *d*-biocytin.

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

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

3. Results and discussion

It was found that the mobile phase used in chromatographic procedure proposed by Przyjazny, Hentz & Bachas, 1993, for the determination of biotin should be slightly modified (increase of methanol ratio from 19% to 23%) in order to obtain a good separation of a standard solution of *d*-biotin and *d*-biocytin (Fig. 2a). The quantification limits obtained for the determination of these compounds, after post-column derivatization by avidin-FITC or streptavidin-FITC, were similar (150 pg) and close to the detection limits given by Przyjazny, Hentz & Bachas, 1993 (about 90 pg). Avidin-FITC was finally chosen as derivatization reagent because it is considerably cheaper than streptavidin-FITC.

The calibration curves obtained for *d*-biotin ($y = 19.00x^2 + 7.55x + 0.04$, with $R^2 = 0.9999$) and *d*-biocytin ($y = 300.0x^2 + 80.0x + 0.1$, with $R^2 = 1.0000$) were quadratic when the concentrations of these vitamers ranged from 0.001–0.5 μg ml^{-1}.

The application of this chromatographic method showed that the amounts of biotin obtained in several foods depend largely on the hydrolysis treatment used to break biotin-protein bonds (possibly present in foods). For example, no biotin could be detected in peanuts and raw egg using an enzymatic hydrolysis (papain, 37°C, 18 h), whereas using acid hydrolysis (1.5 M sulfuric acid by autoclaving at 120°C for 2 h), this vitamin was found in both foods (1.50 μg g^{-1} in peanuts and 0.40 μg g^{-1} in raw egg). This disagreement is probably due to the fact that biotin is very likely unavailable in these foods (Anderson, Baker & Mistry, 1978; Whitehead, Armstrong & Waddington, 1982; Frigg, 1984; Bonjour, 1984) and is released (partially or totally) by sulfuric acid hydrolysis but not by enzymatic hydrolysis.

A comparison of these two hydrolysis methods carried out on a beef liver sample showed on the contrary that enzymatic hydrolysis led to a biotin concentration markedly higher (1.47 μg g^{-1}) than that obtained after

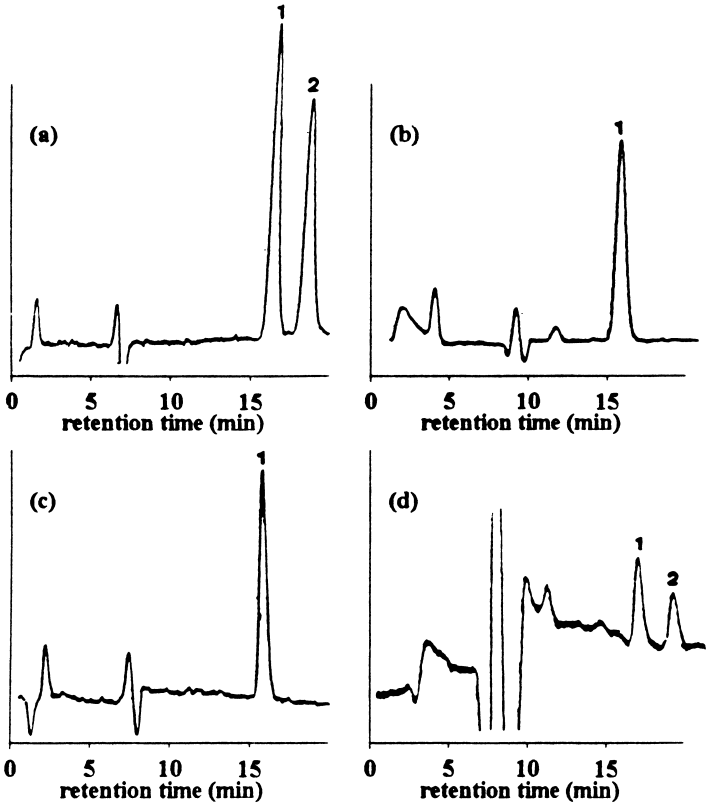

Fig. 2. Chromatographic separation of *d*-biotin (1) and *d*-biocytin (2) in a standard mixture (0.1 μg ml^{-1}) (a), cereals (b), beef liver (c) and tomato (c).

acid hydrolysis (treatment by autoclaving at 120°C for 2 h with 1 M H_2SO_4) (0.73 μg g^{-1}). A similar result was obtained with a fruit juice sample (0.15 μg g^{-1} of biotin after enzymatic hydrolysis, absence after acid hydrolysis). In the two food samples mentioned above (beef liver, fruit juice), the drop of biotin concentrations observed after acid hydrolysis could be explained (at least partially) by a degradation of *d*-biotin to varying extents during this treatment. In a standard solution, losses of *d*-biotin (15–20%) were actually ascertained after sulfuric acid hydrolysis (1.5 M H_2SO_4 by autoclaving at 120°C for 2 h). When *d*-biotin was added to a beef liver sample, slightly higher losses (15–30%) were noted.

Moreover, it also has been shown in beef liver samples that the *d*-biotin concentration obtained can vary to a great extent depending on the sulfuric acid hydrolysis conditions chosen. For example the concentration decreased by 40% when the sulfuric acid concentration was reduced from 1.5/1.0 M (by autoclaving at 120°C for 2 h). It also decreased by the same amount when the duration of autoclaving time was reduced by half (1 h instead of 2 h) in the presence of 1.5 M sulfuric acid. The determination of biotin in food carried out using sulfuric acid hydrolysis, appeared to be largely dependent on the duration of autoclaving and sulfuric acid concentration, and the values obtained are unreliable for beef liver. The maximal concentration was reached only after autoclaving for 3 h in presence of 3 M sulfuric acid. But, under such experimental conditions, a non-negligible quantity of vitamin initially present in the sample was destroyed. Under these conditions, the concentration obtained corresponded neither to bioavailable biotin, nor to total biotin. On the other hand, enzymatic

Table 1
Results of an interlaboratory study concerning the determination of biotin (two replicates) in a lyophilized pork liver sample (Van den Berg, 1995)

Laboratory	Hydrolysis conditions	Biotin concentration (μg g^{-1})
1	1 M H_2SO_4, 2 h, 120°C	1.1
		1.1
2	3 M H_2SO_4, 30 min, 121°C	1.3
		1.3
3	3 M H_2SO_4, 3 h, 130°C	0.3
		0.3
4	3 M H_2SO_4, 30 min, 120°C	1.1
		1.1
5	1.5 M H_2SO_4, 1 h, 121°C	1.7
		1.4
6	Papain, pH 5.5, 18 h, 37°C	2.3
		2.2
7	Papain, pH 5.0, 18 h, 37°C	2.3
		2.4

Table 2
Recovery rate[a] (%) of *d*-biotin and *d*-biocytin in various foodstuffs

Food	*d*-biotin		*d*-biocytin	
	Recovery rate	Standard deviation	Recovery rate	Standard deviation
Multivitamin tablet	96	4	–	–
Beef liver	98	3	–	–
Infant formula	99	6	104	5
Cereals	97	5	91	2
Fruit juice	92	2	97	2
Tomato	106	5	90	7

[a] Average of five replicates.

Table 3
Biotin concentrations[a] (μg g^{-1}) in various foodstuffs

Food	Biotin concentration	Standard deviation	Relative standard deviation (%)	Repeatability
Multivitamin tablet	107	6	5.4	16
Beef liver	1.26	0.07	5.7	0.2
Infant formula	0.46	0.03	2.7	0.03
Cereals[b]	0.411	0.005	1.3	0.02
Fruit juice	0.103	0.004	4.0	0.01
Tomato	0.014	0.001	6.4	0.003

[a] Average of ten replicates (only tomato's samples contained *d*-biocytin; in this case, biotin concentration corresponds to the sum of *d*-biotin and *d*-biocytin concentrations).
[b] Sample treated with takadiastase.

hydrolysis (with papain) does not induce any degradation of biotin in foods and led to good approximate determination of bioavailable biotin content.

All these results corroborate the observation already been made by participants to an interlaboratory study organized by the Community Bureau of Reference (Brussels), concerning the microbiological determination of biotin present in lyophilized pork liver (Van den Berg, 1995). In this study, the two laboratories which used an enzymatic hydrolysis actually obtained mean concentrations markedly higher (2.3 μg g^{-1}) than the five laboratories which used sulfuric hydrolysis (1.1 μg g^{-1}) (Table 1).

This enzymatic method, which implies the subsequent separation of both *d*-biotin and *d*-biocytin, was thus preferred to sulfuric acid hydrolysis. However, in foods containing high amounts of starch, such as cereals and yeast, addition of takadiastase was necessary.

The recovery rates of the analytical protocol tested (hydrolysis of the sample with papain, and possibly with takadiastase, isolation of *d*-biotin and *d*-biocytin by reverse phase chromatography, post-column derivatization with avidin-FITC and fluorimetric determination) were closely similar and varied between 92% and 106% for *d*-biotin and between 90% and 104% for *d*-biocytin (Table 2).

In any foodstuff studied, it was possible to obtain a good isolation of *d*-biocytin (if this compound was present) and *d*-biotin (see for example chromatograms (b), (c) and (d) in Fig. 2). When concentrations of *d*-biotin and *d*-biocytin were lower than 0.04 μg g^{-1} (quantification limit for a 20 μl injection volume), it was possible, owing to the excellent specificity of the detection mode, to increase the injection volume to 200 μl and therefore to determine biotin in foods at concentrations as low as 0.004 μg g^{-1}, without any problems of interference. The repeatability of the method also appeared to be very satisfactory (Table 3).

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