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Review

Analytical techniques for determining biotin

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Abstract

Biotin is a vitamin of the B-complex, which plays an important biochemical role in every living cell. In the recent years, the interest in this vitamin has been rekindled, mainly due to its association with serious human disorders, such as the inherited syndrome multiple carboxylase deficiency, which can be successfully treated with biotin administration. Diagnosis of biotin deficiency as well as monitoring of biotin levels in biological fluids of patients receiving biotin treatment is crucial. Equally important is the determination of biotin levels in pharmaceutical preparations as well as in food and food supplement products, which constitute the main source of biotin in humans. Several analytical methods for measuring biotin in various samples, e.g. human fluids, pharmaceutical formulations, food material etc., have been reported in the literature. In this review, the most representative of these methods are presented, and their characteristics are evaluated. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Reviews; Food analysis; Pharmaceutical analysis; Biotin; Vitamins

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1. Introduction

Biotin (hexahydro-2-oxo-1 H-thieno[3,4-d]imidazole-4-pentanoic acid) is a water-soluble vitamin belonging to the B-complex, which is found in small quantities in all living cells. It exists in eight isomer forms, but only D-(+)-biotin is biologically active.

Biotin is synthesized in various bacteria and higher plants [1,2]. However, several microorganisms as well as higher animals are not capable of synthesizing biotin and their needs in this vitamin are met by dietary intake [3]. In addition, biotin can be biosynthesized in the intestinal tract of higher animals by symbiotic microorganisms [3,4]. In the intestine, biotin is absorbed through a saturable transportation system, while at great biotin concentrations, passive diffusion predominates. Exit of biotin from the enterocyte is carrier-mediated. As some studies suggest, the enzyme biotinidase, which is involved in the in vivo recycling of the vitamin, serves as a carrier protein for the transport of biotin into the cell as well as a biotin – binding protein in serum [5].

In nature, biotin is found either free or covalently bound to proteins or peptides. The catabolism of free biotin in microorganism cells has been studied extensively. On the other side, little is known concerning catabolism of free biotin in mammalian cells. It is generally believed that biotin is catabolized by β-oxidation at its side chain. This leads to the formation of bisnorbiotin, tetranorbiotin and related metabolites. The sulfur in the heterocyclic ring can become oxidized to biotin-L-sulfoxide, biotin-D-sulfoxide and biotin sulfone. In mammals, degradation of the heterocyclic ring, leading mainly to dethiobiotin, is quantitatively minor. The structural formulae of biotin and main biotin metabolites are shown in Fig. 1. These metabolites, together with non-identified biotin derivatives and biotin, are urine excreted. Regarding the metabolism of biotin covalently bound to peptides and proteins, it is believed that they are initially hydrolyzed to small biotinyl peptides, which consequently release biotin through biotinidase action [5].

Intracellularly, the most important biochemical function of biotin is that of the coenzyme of certain enzymes, known as carboxylases, transcarboxylases

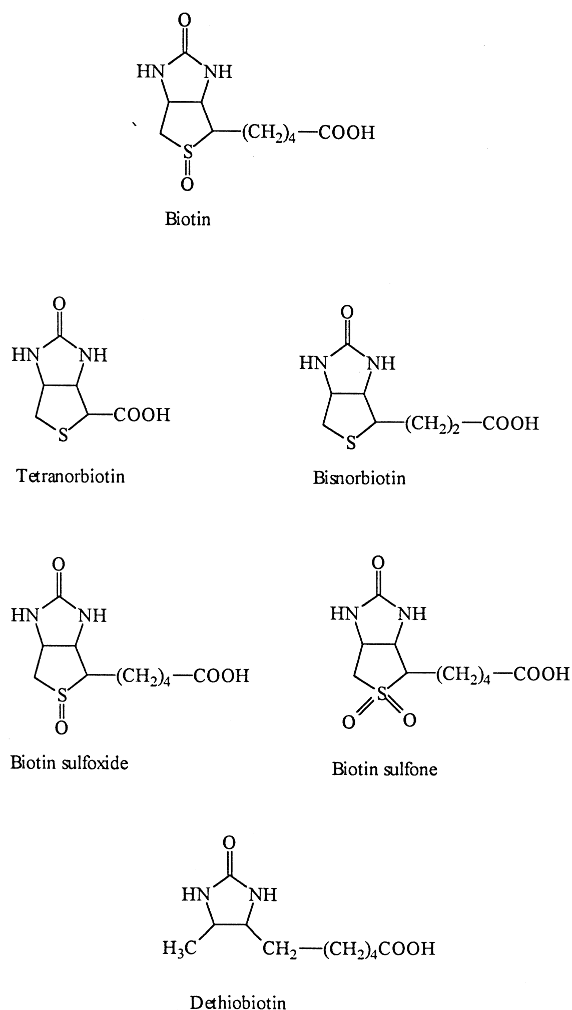

Fig. 1. Structural formulae of biotin and its main metabolites.

and decarboxylases, which catalyze the incorporation, transfer or elimination, respectively, of a carboxyl group to (or, from) a biomolecule. These biotinyl enzymes catalyze significant metabolic reactions and are involved in important biochemical processes, such as gluconeogenesis, lipogenesis, aminoacid metabolism and energy transduction [5]. In animal cells, the following biotinyl carboxylases have been found: acetyl-coenzyme A(CoA) carboxylase (E.C. 6.4.1.2), pyruvate carboxylase (E.C. 6.4.1.1), propionyl-CoA carboxylase (E.C. 6.4.1.3),

and β-methylcrotonyl-CoA carboxylase (E.C. 6.4.1.4). In biotinyl enzymes, biotin is covalently attached via its carboxyl group to a lysyl ε-amino group of the corresponding apoenzyme, the reaction being catalyzed by certain enzymes reported as biotin holocarboxylase synthetases [6]. Biotin is liberated from biotinyl enzymes through the catalytic action of the enzyme biotinidase [7]. Biotin deficiency can lead to decreased catalytic activity of biotinyl enzymes, which may be manifested by clinical symptoms, mainly neurological and dermatological ones [3]. Particularly severe are the neurological symptoms, which, along with other findings, may indicate that biotin is especially important for the correct functioning of nervous tissue [8,9]. Biotin has been demonstrated to increase guanylate cyclase activity in primary cultures of rat hepatocytes [10,11], leading to an increase in hepatic glucokinase activity and mRNA levels [12,13]; it has also been shown to affect glucokinase activity and mRNA levels in cultured pancreatic beta cells, which may indicate the vitamin's direct influence on glucose utilisation [14]. Moreover, biotin has been reported to enter the human cell nucleus, where it may be involved in histone biotinylation [15], although this function has not yet been completely elucidated. Recent findings indicate that biotin levels and expression of related genes are decreased in cancer tissues [16].

The richest dietary sources of biotin include liver, kidneys, heart, pancreas, poultry, egg yolk and milk. Smaller amounts are found in plants, mainly in seeds [17]. The dietary biotin intake in humans living in western countries has been estimated to be 35 to 70 $\mu g\ day^{-1}$, which seems to be absorbed almost completely [5]. A dietary intake of 35 μg of biotin per day is considered adequate for healthy adults. However, certain population groups, such as pregnant or lactating women, patients under anticonvulsant therapy, etc., may need increased biotin intake [5]. Vegetarian diet does not seem to affect biotin status [18].

Low biotin intake has been reported to result in serious biochemical disorders in animal organisms, such as reduced carboxylase activity, inhibition of protein and RNA synthesis, reduced antibody production, etc. There are indications of biotin involvement in severe animal syndromes, such as the avian Fatty Liver and Kidney Syndrome (FLKS) and the trout 'blue slime' disease [3,17,19,20].

Prolonged biotin deprivation in humans leads to pathological symptoms, which are eliminated when biotin is administered [3,21].

Biotin deficiency has been related to serious human disease states, such as the inherited metabolic syndrome Multiple Carboxylase Deficiency (MCD). This syndrome, which is accompanied by serious clinical symptoms, can be caused by defective activity of the enzymes involved in the in vivo biotin recycling [22,23]. Other human malfunctions, e.g. Sudden Infant Death Syndrome (SIDS), seborrheic dermatitis of infancy, Leiner disease and Rett syndrome have been associated with biotin deficiency status [3,24].

Administration of biotin in pharmacological doses leads to elimination or at least alleviation of clinical symptoms in neonates with MCD [23]. Moreover, in cases of patients with non-insulin dependent diabetes mellitus, administration of biotin has been reported to lead to significant decrease in blood glucose levels [25]. Biotin treatment has been reported to improve metabolic malfunctions occuring in patients with sternocostoclavicular hyperostosis [26]. Biotin has also been reported to alleviate clinical symptoms of patients under long-term hemodialysis treatment [27]. As reported, biotin has been used successfully for the treatment of acne, seborrheic eczema and alopecia [3]. So far there has been no indication of adverse effects due to high doses of biotin administration in humans.

Decreased biotin status, as compared to that of normal population, has been reported to occur in several population groups, such as pregnant women [28,29]. An active transport mechanism of maternal biotin seems to exist in favor of the fetus [30]. Neonate serum biotin levels were found to depend on the kind of milk they were fed with [31]. The content of human milk in biotin and biotin metabolites has been extensively studied [32–34]. Decreased circulating biotin levels have been also reported in patients on total parenteral nutrition [35–37], patients receiving certain anticonvulsants [38], gastrectomized patients, alcoholics and children with severe burns [3]. The exact clinical consequences or practical

utility of this finding are not, however, completely clarified.

It is evident that diagnosis of biotin deficiency as well as monitoring of biotin levels of patients receiving biotin treatment is very crucial. Equally important is the determination of biotin levels in food and food supplement products. For this reason, analytical methods have been developed, in order to determine biotin in biological fluids, as well as various kinds of food products and pharmaceutical preparations containing biotin.

2. Analytical techniques for biotin

The analytical techniques reported so far in the literature for determining biotin can be divided into the following main categories: microbiological methods, biological methods (bioassays), physicochemical methods and binding assays.

2.1. Microbiological methods

These highly sensitive methods (10^{-9} g l^{-1}) are among the first assays developed for determining biotin. They are based on the principle that growth of many microorganisms depends upon the presence of biotin in the culture media, since this vitamin is an essential nutrient for the microorganism under cultivation. Thus, the microbiological determination of biotin is achieved by inocculating a certain microorganism culture with standard solutions or unknown samples of the vitamin, and then 'quantifying' the culture development, e.g. by turbidimetry or titration [3,39]. Many microorganisms have been applied to the development of microbiological assays for biotin, e.g. *Lactobacillus plantarum, Lactobacillus casei, Saccharomyces cerevisiae, Ochromonas danica, Micrococcus sodonensis, Neurospora crassa, Rhizobium trifolii, Escherichia coli C162,* etc. [40,41]. Microbiological methods have also been developed based on the combined use of microorganisms and radiolabelled compounds. For example, the incorporation of $^{14}CO_2$ in microorganism cells is determined in the presence of varying quantities of biotin [42]. In addition, a method has been reported which determines the amount of $^{14}CO_2$ (metabolic product of L-[1-^{14}C]-methionine) generated by *Kloeckera brevis* cells, in the presence of varying quantities of biotin [43]. Microbiological methods have been applied to determining biotin in various materials, including food products, seawater and biological fluids, such as serum, urine and tissue extracts [40,44–46].

2.2. Bioassays

These assays, which are usually less sensitive than the microbiological ones, were mainly applied to the determination of biotin in food material, more specifically the bioavailable fraction. In biotin bioassays, rats or chickens, whose development has been inhibited by artificial biotin deprivation, are fed with pure biotin, as reference, or with samples of unknown vitamin content [3,39]. The determination of the concentration of the unknown sample is based on the animal development curve, which expresses weight gain in relation to the logarithm of the quantity of biotin administered.

In biotin bioassays one could also include methods that determine biotin indirectly through its biological function, i.e. by assessing the activity of a special biotin – dependent enzyme, e.g. pyruvate carboxylase or biotinyl CoA – synthetase, in the presence of increasing concentrations of biotin [3,47–49].

2.3. Physicochemical methods

Physicochemical methods for determining biotin, due to their rather limited sensitivity, are mainly applied to the measurement of the vitamin in samples with high biotin concentrations, such as pharmaceutical multi-vitamin preparations. These include methods of spectrophotometry, colorimetry, polarography, thin layer chromatography, gas chromatography, liquid chromatography, capillary zone electrophoresis (Table 1) and high-performance liquid chromatography (Table 2).

Among the first physicochemical methods developed to determine biotin was the spectrophotometric one developed by Green in the mid 60's [50]. This method is based on the red shift (from 280 nm to 233 nm) observed on the absorption spectrum of the tryptophan residues of avidin, a glycoprotein that specifically and stoichiometrically binds biotin with a K_{aff} of 10^{-15} mol l^{-1} [51], upon binding of biotin. A

Table 1
Physicochemical methods for biotin determination: Non-HPLC methods

Type of method	Method description	Original application(s)	Ref.
Spectrophotometric	Measurement of absorbance at 233 nm of tryptophane residues of avidin in avidin–biotin complex		[50]
Spectrophotometric (Colorimetric)	Measurement of decrease in absorbance at 500 nm after dissociation of avidin–HABA complex		[50]
Spectrophotometric (Colorimetric)	Measurement of absorbance at 533 nm of colored product of reaction of biotin with *p*-dimethylaminocinnamaldehyde		[52]
Spectrophotometric (Colorimetric)	Measurement of absorbance at 520 nm of iodine produced after oxidation of the S atom of biotin with potassium iodate		[53]
Spectrophotometric	Measurement of absorbance at 350–352 nm of tri-iodide produced after reaction of biotin with periodate		[54]
Polarographic	DMF–water and 0.05 *M* KNO_3 mixtures of biotin were used for polarographic determination		[55]
Paper chromatography	Biotin, was identified using various solvent and visualizing spraying systems		[56]
Gas chromatography	Biotin, was transformed to a silyl ester derivative and underwent gas chromatography	Agricultural premixes and pharmaceutical preparations	[57,58]
Thin-layer chromatography	Biotin was visualized by spraying with *p*-dimethylaminocinnamaldehyde	Multi-vitamin preparations	[59]
Liquid chromatography	A Vydak HS C_{18} column was used for the separation of biotin which was detected at low wavelength	Multi-vitamin preparations containing at least 300 μg of biotin each	[60]
Liquid chromatography–Mass spectrometry	Biotin and dethiobiotin were derivitized as methyl esters, separated on a HP RP 8 column and detected by MS. Detection limit was lower than 10 ng.	Extracts of *Bacillus sphaericus*	[61]
Capillary zone electrophoresis	Solid biotin preparations were dissolved in double-distilled water and filtered before analysis.	Pharmaceutical formulations	[62]

colorimetric-spectrophotometric assay for determining biotin was developed by Green, also [50]. This assay is based on the removal of the dye *p*-hydroxyazobenzene-2'-carboxylic acid (HABA) from the avidin-HABA complex and the subsequent decrease in the corresponding absorbance values at 500 nm, in the presence of increasing amounts of biotin. Among the first colorimetric assays developed for biotin was also that of McCormick and Roth [52]. This is based on the reaction of biotin with *p*-dimethylaminocinnamaldehyde and the subsequent photometric determination of the colored product at 533 nm. Another colorimetric assay for biotin was that involving the oxidation of the sulfur atom in the biotin molecule to the corresponding sulphone with potassium iodate and the concurrent reduction of iodate to iodine, which is then extracted in cyclohexane and its absorbance measured at 520 nm [53]. A later spectrophotometric assay for biotin measurement, characterized by enhanced sensitivity down to the microgram level, is based on the reaction of biotin with periodate, consequent reaction of the iodate formed with iodide, and photometric determination of the tri-iodide finally produced [54].

Polarography in DMF–water and 0.05 *M* KNO_3 mixtures was also used to measure biotin down to the microgram level [55].

Paper chromatographic data using various solvent systems were also reported for biotin [56].

Determination of biotin by gas chromatography, after formation of the biotin silyl-esters, was reported in the literature in the early 1970's [57,58]. Gas chromatography was applied to determining biotin in agricultural premixes and pharmaceutical injectable preparations [57].

A thin-layer chromatography method was also described for determining biotin [59]. This method uses spraying with *p*-dimethylaminocinnamaldehyde for visualizing biotin and was applied to the determination of biotin in multi-vitamin preparations.

A liquid chromatography method, based on the use of a Vydak HS C_{18} column, was reported [60]. In

Table 2
Physicochemical methods for biotin determination (continued): HPLC methods

Detection system	Type of column	Derivatization	Detection limit	Original application(s)	Ref.
UV or Fluorescence	μBondapak C_{18}	BAP or Mmc esters	BAP: 10^{-8} g Mmc: 10^{-9} g	Pharmaceutical products	[63]
Fluorescence	Nucleosil C_{18}	9-Anthryl-diazomethane	1 ng	–	[64]
UV	μBondapak C_{18} Aquapore AX-300/ Partisil SAX	–	10^{-6} g	–	[65,66]
Electrochemical detection	LiChrosorb RP 18 Hiber	–	5 – 10 ng	Multivitamin preparations	[67]
UV	Zorbax ODS	–	–	Multivitamin preparations	[68]
Fluoresence	LiChrosorb Si 60 TSK-gel 80 TM	1-Pyrenyl-diazomethane	Normal-phase: 100 fmol injection^{-1} Reversed-phase: 10 fmol injection^{-1}	Pharmaceutical preparations, Serum spiked with biotin	[69]
UV	Supelcosil LC-8-DB	–	–	Almond cultivars	[70]
UV	TSK gel ODS – 80 TM	Thiamine esters	10 ng	Pharmaceutical preparations	[71]
Fluorescence	Shandon Hypersil Hypersil ODS	Panacyl ester	Normal-phase: 10 pmol Reversed-phase: 100 pmol	Rat tissues	[72]
Fluorescence	Microsorb C_{18}	Post column: Avidin – FITC	$1.8 \cdot 10^{-8}$ M	Multivitamin preparations Horse-feed supplement Cell culture extract Infant formula	[73,74]
Fluorescence	Microsorb C_{18}	Post column: Avidin – ANS	$5.0 \cdot 10^{-7}$ M	Commercial vitamin tablets Horse-feed supplement	[74]

this method, biotin was detected spectrophotometrically at low UV wavelength. The method was applied to the measurement of biotin in multivitamin tablets containing at least 300 μg of biotin each. A liquid chromatography – mass spectrometry method for determining biotin was also described, measuring as low as 10 ng of the vitamin [61]. The method was applied to measuring biotin in extracts of *Bacillus sphaericus* cultures.

A capillary zone electrophoresis method has been described in the literature [62]. This method was applied to the analysis of biotin in pharmaceutical formulations.

Various high-performance liquid chromatography (HPLC) methods for determining biotin have been described so far, some of which are presented in Table 2. Various columns and elution protocols have been used in these methods. Biotin detection was achieved without any previous derivatization (UV) or after proper derivatization (UV, fluorescence), either pre- or post- column, in an attempt to decrease assay detection limit. HPLC methods could clearly discriminate between biotin and its main analogues, e.g. dethiobiotin, biotin sulfoxides and biotin sulfone. The above methods have been applied mainly to the determination of biotin in pharmaceutical multivitamin preparations. However, some of them have been used for analyzing more complicated samples, e.g. food material. In some reports sample pretreatment was necessary, depending on the sample analyzed and the assay protocol developed.

Among the HPLC methods developed for biotin in early 1980's was that of Desbene et al. [63], which used a reversed-phase μBondapak C_{18} column. Biotin was detected after pre-column derivatization with either UV absorbing (ω-4-dibromoacetophenone) or fluorescent (4-bromomethylmethoxy-coumarin) reagents. Detection limit was 10^{-8} g ml^{-1} and 10^{-9} g ml^{-1}, respectively. The method developed could discriminate between biotin

and its main analogues, e.g. dethiobiotin, biotin sulfoxides and biotin sulfone. In another method [64], biotin was reacted with 9-anthryldiazomethane and the fluorescent derivative was determined by HPLC using a reverse phase Nucleosil C_{18} column. The lowest biotin amount to be detected was estimated to 1 ng. The method could be applied to the analysis of pharmaceutical products. Underivatized biotin, biotin synthetic analogs and intermediates of the vitamin's commercial synthesis were analysed by reversed-phase and anion-exchange HPLC in a method later reported [65,66]. Separation of complex biotin mixtures was best accomplished by RP-HPLC (μBondapak C_{18} column). Anion-exchange HPLC (Aquapore AX-300 and Partisil SAX columns) was successful in separating simple mixtures in a relatively short time and also in separating sulfoxide isomers. The detection limit of the RP-HPLC separation was 10^{-6} g ml^{-1}. Underivatized biotin was analyzed by reversed-phase HPLC (LiChrosorb RP 18 Hiber column), using electrochemical, instead of UV, detection [67]. The lowest biotin amount to be detected was estimated to be 5–10 ng. The method was applied to the determination of biotin in multivitamin pharmaceutical preparations. Underivatized biotin was also analyzed with reversed-phase HPLC (Zorbax ODS column), using low UV (203 nm) detection [68]. The method was applied to the determination of biotin in multivitamin-multimineral tablets. The samples were pre-cleaned on a Sep-Pak Florisil column. In another method [69], biotin was reacted with 1-pyrenyldiazomethane (no catalyst was necessary) and the fluorescent derivative was analyzed with normal (LiChrosorb Si 60) and reverse phase (TSK-gel 80 TM) HPLC. The detection limit was 100 fmol injection^{-1} or 10 fmol injection^{-1}, respectively. The method was applied to the determination of biotin in multivitamin tablets as well as in serum spiked with biotin. An HPLC method, using a Supelcosil LC-8-DB column and UV detection, was applied to the determination of underivatized biotin in Italian almond cultivars [70]. Prior to HPLC analysis, samples were de-fatted with *n*-hexane extraction and pretreated on a cation-exchange extraction column. In another HPLC method, using TSK gel ODS – 80 TM column, biotin was detected after post-column derivatization and fluorescence measurement [71]. Derivatization consisted of chlorination of the biotin amide bond and consequent reaction with thiamine to a fluorescent thiochrome. The detection limit was 10 ng injection^{-1}. The method could be applied directly to pharmaceutical preparations without any sample pretreatment. In an HPLC method reported in the early 1990's, in which either normal or reverse phase column was used, biotin was detected after pre-column derivatization with panacyl bromide [(*p*-(9-anthroyloxy)phenacyl bromide], in the presence of crown ether catalyst, as a fluorescent label [72]. Lowest amounts of biotin detected by normal (Shandon Hypersil column) and reverse phase (Hypersil ODS) HPLC was about 10 pmol and 100 pmol, respectively. The method was applied to the determination of biotin in biological samples, i.e. rat intestinal tissues. Prior to HPLC, samples were extracted with chloroacetic acid and purified by a combination of solid-phase extraction on C_{18} cartridges, ion-exchange chromatography and thin-layer chromatography. More complicated systems for analyzing biotin, which combine HPLC separation and post column detection based on avidin binding, have also been described [73,74]. Briefly, in a reversed-phase HPLC method of this type, the biotin containing HPLC effluent was reacted with avidin labelled with fluorescein isothiocyanate (FITC), which leads to an enhancement of the fluorescence intensity at 520 nm. The assay detection limit was $1.8\cdot10^{-8}$ *M*. The system was applied to the determination of biotin in liquid vitamin preparations and horse-feed supplement. In another approach, the biotin containing HPLC effluent competes with the fluorescent probe 2-anilinonaphthalene-6-sulfonic acid (2,6-ANS) for the avidin binding sites. On release from avidin, the fluorescence emission of 2,6-ANS is shifted toward the red region, yielding a decrease in fluorescence intensity at 438 nm, which is proportional to the analyte concentration. The detection limit was $5.0\cdot10^{-7}$ *M*. The above system was applied to the determination of biotin in liquid vitamin preparations, vitamin tablets, horse-feed supplement and a liquid infant formula.

2.4. Binding assays

During the last 25 years, many attempts have been made to apply binding assays to the determination of biotin. Biotin binding assays are based on the same principle as immunoassays, which are widely used in clinical laboratory practice, differing from them only

in the fact that they utilize a specific binding protein instead of an antibody. Binding assays, like immunoassays, have many advantages such as high sensitivity, and rapid and convenient performance.

In all biotin binding assays reported so far, the specific binding protein used is the glycoprotein avidin or the structurally similar protein streptavidin [51]. As already mentioned, both avidin and streptavidin exhibit an extremely high K_{aff} along with high specificity in their binding to the biotin molecule. Due to their exceptional characteristics, the avidin – biotin (or, streptavidin – biotin) system has been used in a lot of applications in the fields of biochemistry, biotechnology, immunochemistry, immunology, molecular biology, etc [75,76].

In addition to avidin or streptavidin binding protein, many other reagents, e.g. various labels and separation means, have been used in the biotin binding assays developed so far. Thus, radioactive isotopes, enzymes, chemiluminescent or fluorescent substances or latex agglutination particles have been used as assay labels. Many of the assays developed are heterogeneous solid-phase systems. However, several homogeneous systems, mainly based on enzyme, fluorescence and bioluminescence labels, have also been described. Some of the most representative biotin binding assays are presented in Tables 3 and 4.

Many biotin metabolites bind to avidin and streptavidin, though typically with lesser affinity. Thus, although binding assays can determine biotin very specifically, e.g. among many other vitamins, they cannot 'totally' discriminate between biotin and biotin metabolites; such discrimination may, however, be desirable, especially if biological fluids are analyzed, e.g. during pharmacokinetic studies, etc. Thus, analytical systems which combine a binding assay with a HPLC methodology were described by Mock [77]. These analyses were applied to the measurement of biotin in human urine, serum and milk [34,78,79]

2.5. Other suggested analytical methods for biotin

More sophisticated assay systems, which use the binding protein avidin or streptavidin, have been developed during the last 15 years. For instance, a bioaffinity sensor method was described. The method uses a suitable metastable molecular complex consisting of HABA bound to a membrane and catalase labelled avidin. The metastable complex dissociates upon exposure to biotin in solution, which can be thus quantitated (10^{-9} – 10^{-7} g ml^{-1}) [111]. Another biotin assay system was developed based on the competition between biotin labelled with the electrically active compound daunomycin and biotin in standard solutions for avidin binding sites [112]. The assay developed is homogeneous, since daunomycin labelled biotin renders electrically inactive upon binding to avidin, as can be seen by the corresponding electrode response. Daunomycin was substituted by another, less toxic, electroactive compound, namely Nile Blue, in another electroactive binding assay system developed [113]. Biotin was also measured through direct electrical detection in another binding assay system [114]. According to its principle, the uptake of conjugated to HRP biotin (B-HRP) by an avidin containing, electron conducting redox hydrogel film on the surface of an electrode rotating in a H_2O_2 solution and poised at +0.1 V (Ag/Cl), produces a substantial electrocatalytic current. The current, resulting from H_2O_2 electroreduction to water, signals promptly the occurrence of the reaction between avidin and B-HRP. Dissolved biotin can be measured through its blocking of the binding of B-HRP to the avidin redox hydrogel-coated electrode. Similar systems have also been reported [115]. Recently, an 'electrically wired' amperometric immunosensor assay was developed for the determination of biotin [116]. The method was based on the use of a redox polymer {$[Os(bpy)_2(PVP)_{10}Cl]Cl$, where bpy=bipyridyl, PVP=poly-4-vinylpyridine} co-immobilized with an anti-biotin antibody on an electrode surface. The polymer transfers electrons between the electrode surface and biotin bound to the anti-biotin antibody on the sensing surface. The current produced is expressed vs. biotin concentration (detection limit: $6 \cdot 10^{-7}$ *M*).

3. Discussion

Microbiological assays were the first assay methods developed for determining biotin. These assays are very sensitive. However, they are not always

Table 3
Radioisotopic binding assays for determining biotin

Detection signal	Tracer	Assay type	Reference
β-Radiation	[^{14}C]Biotin	Heterogeneous liquid-phase (bentonite suspension)	[80]
β-Radiation	[^{14}C]Biotin	Heterogeneous liquid-phase ($ZnSO_4$ solution)	[81]
β-Radiation	[^{14}C]Biotin	Heterogeneous solid-phase (avidin-cellulose disks)	[82]
β-Radiation	[^{3}H]Biotin	Heterogenous liquid-phase (dextran-coated charcoal)	[83]
β-Radiation	[^{3}H]Biotin	Heterogeneous solid-phase (nitrocellulose filters)	[84]
β-Radiation	[^{3}H]Biotin	Heterogeneous liquid-phase (bentonite suspension)	[85]
β-Radiation	[^{3}H]Biotin	Heterogeneous solid-phase (anti-goat IgG linked to agarose)	[86]
γ-Radiation	[^{125}I]Biotinyl-derivative	Heterogenous liquid-phase (dextran-coated charcoal)	[87]
γ-Radiation	[^{125}I]Biotinyl-derivative	Heterogeneous solid-phase (avidin-cellulose)	[88]
γ-Radiation	[^{125}I]Biotinyl-derivative	Heterogeneous liquid-phase [(i)avidin/anti-avidin antibody, (ii)PEG precipitation]	i.[28] ii. [89,90]
γ-Radiation	[^{125}I]Biotinyl-derivative	Heterogeneous solid-phase (streptavidin-Sepharose 4B)	[91]
γ-Radiation	[^{125}I]Avidin	Heterogenous solid phase (biotinylated albumin-coated microplates)	[92]

highly specific, since other nutrients, e.g. fatty acids, may promote certain microorganisms' growth, thus interfering with assay results [40]. In addition, microbiological assays are rather tedious and time-consuming, which renders them not ideal for routine analysis of great numbers of samples. Nevertheless, microbiological assays were applied to the determination of biotin in various samples, such as food material, human biological fluids, tissue extracts, etc. [40,46].

A few bioassay systems have been proposed in the literature for determining biotin. These assays were mainly applied to the estimation of bioavailable biotin present in food samples [3].

Physicochemical assays for determining biotin were first developed in the mid 60's. Their sensitivity varies from rather limited to rather high, more specifically from the μg level of the first developed spectrophotometric assays to the pg level of the recently developed HPLC methods using fluorometric detection. These assays are considerably simpler and faster than the microbiological ones, but not as simple and fast as the binding assays, which are therefore considered advantageous for the routine

Table 4
Non radioisotopic binding assays for determining biotin

Detection signal	Tracer	Assay type	Ref.
Enzyme activity (potentiometric measurement)	Biotin-lysozyme	Homogeneous	[93]
Enzyme activity (spectrophotometric measurement)	Biotin-G6 PDH	Homogeneous	[94]
Enzyme activity (spectrophotometric measurement)	Biotin-AP	Heterogeneous solid-phase (biotinylated BSA coated microplates)	[95]
Enzyme activity (spectrophotometric measurement)	Biotin-pyrophosphatase	Heterogeneous solid-phase (biotinylated IgG coated microplates)	[96]
Enzyme activity (spectrophotometric measurement)	Biotin-HRP	Heterogeneous solid-phase (streptavidin coated microplates)	[97]
Enzyme activity (spectrophotometric measurement)	Biotin and HRP immobilized on unilamelar vesicles	Heterogeneous solid-phase (anti-biotin antibody coated plates)	[98]
Enzyme activity (spectrophotometric measurement)	Streptavidin-HRP	Heterogeneous solid-phase (biotinylated IgG coated microplates)	[99]
Enzyme activity (spectrophotometric measurement)	Streptavidin-HRP	Heterogeneous solid-phase (biotinylated IgG coated microplates)	[100]
Enzyme activity (spectrophotometric measurement)	Streptavidin-HRP	Heterogeneous solid-phase (biotinylated BSA coated microplates)	[101]
Latex agglutination (turbidity measurement)	Biotin-coated latex particles	Homogeneous	[102]
Fluorescence	Biotin-fluorescein	Homogeneous	[103]
Fluorescence	Avidin-ANS	Homogeneous	[104]
Fluorescence	Avidin-fluorescein	Homogeneous	[105]
Fluorescence[a]	Streptavidin-fluorescein	Homogeneous	[106]
Chemiluminescence	Biotin-isoluminol	Homogeneous	[107]
Chemiluminescence (energy transfer)	Biotin-ABEI Avidin-fluorescein	Homogeneous	[108]
Biochemiluminescence	Biotin-aequorin	Homogeneous	
Biochemiluminescence	Biotin-aequorin	Heterogeneous solid-phase (avidin coated solid particles)	[109] [110]

[a] This fluorescence binding assay has been applied to a flow injection analysis system.

analysis of great numbers of samples. However, the main advantage of physicochemical assays over the other biotin analytical methods is their better discriminating capacity between biotin and biotin metabolites, e.g. biotin sulfoxides, etc. Several physicochemical methods have been applied to the measurement of biotin in various samples, including food material, biological fluids and, especially, pharmaceutical preparations, e.g. multivitamin tablets.

Biotin binding assays are very sensitive. In addition, since they are usually characterized by a simple assay protocol and can be used for the analysis of a great number of samples in a short period of time, they are considered ideal for every-day routine laboratory use. The first biotin binding assays developed were radioactive ones. These assays were applied to determining biotin in various samples, such as food material, tissue extracts, and, especially,

human fluids, e.g. human serum, plasma, CSF and urine, obtained from various population groups. Non-radioactive biotin binding assays were later developed. These assays presented the advantage of not using radioactive tracers, while they retained their high sensitivity characteristics. Few of these assays were, however, applied to the measurement of biotin in unknown samples, especially complex biological ones. Concerning specificity of biotin binding assays, this is very high as far as discrimination capacity between biotin and other biological substances, different from biotin metabolites, is concerned –even if these substances show structural similarities with the biotin molecule, e.g. urea and uric acid, etc. However, biotin binding assays cannot discriminate equally well between biotin and biotin metabolites, as already mentioned. To overcome this problem, some researchers developed and used systems combining HPLC and binding assay methodologies.

Some more sophisticated analytical systems for determining biotin have been reported in the literature. These systems usually use avidin or streptavidin among their main reagents along with specially designed complex detection system. Functioning of these systems has not, however, been evaluated in unknown samples.

Special sample treatment is sometimes necessary prior to the assay performance [117], depending on the nature of the sample, or the assay principle and protocol, or sometimes on both. For instance, pretreatment is necessary for preparing various kinds of samples to be analyzed by the *Lactobacillus plantarum* microbiological assay. Thus, pharmaceuticals and premixes are homogenized and solubilized in diluted alkali, while food materials and other natural products are heat extracted prior to the assay [117]. Pretreatment is also necessary before analyzing natural samples, mainly food material, by various physicochemical methods as already described. Serum sample pretreatment has also been included in the protocol of radioisotopic binding assays which use a radiolabelled biotinyl amide as the assay tracer, so as to inactivate any serum biotinidase activity that might lead to tracer cleavage and affect assay results [87,28].

The measurement of biotin levels in various kinds of samples, among which food material, pharmaceutical preparations, human biological fluids, culture supernates, etc., might provide important information, of either theoretical or practical interest, in a lot of different studies, e.g. nutritional, biochemical, pharmacokinetic or pharmacological ones. Many analytical methods have been proposed in the literature for measuring biotin in standard solutions and unknown samples. One should carefully investigate certain parameters, e.g. expected biotin levels in the sample to be analyzed, presence and kind of biotin-resembling substances in it, special sample pretreatment if necessary (e.g. extraction, ultrasonication, pre-cleaning through a Sep-Pak column, preparation of derivatives to facilitate detection etc), desirable number of samples analyzed per time period, etc., before selecting the most appropriate assay methodology.

4. Nomenclature

ABEI	aminobutylethylisoluminol
2,6-ANS	2-anilinonapthalene-6-sulfonic acid
AP	alkaline phosphatase
B	Biotin
BAP	Bromoacetophenone
BSA	Bovine serum albumin
CSF	Cerebrospinal Fluid
DMF	dimethyloformamide
FITC	fluorescein isothiocyanate
FLKS	Fatty Liver and Kidney Syndrome
G6 PDH	6-phosphoric glucose dehydrogenase
HABA	*p*-hydroxyazobenzene-2'-carboxylic acid
HPLC	high-performance liquid chromatography
HRP	horseradish peroxidase
IgG	immunoglobulin G
MCD	Multiple Carboxylase Deficiency
Mmc	methylmethoxycoumarin
MS	mass spectrometry
SIDS	Sudden Infant Death Syndrome

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