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Quantification of biotin in feed, food, tablets, and premixes using HPLC–MS/MS

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Abstract

Two sensitive and specific methods for quantification of biotin in feed, food, tablets, and premixes based on HPLC–MS/MS have been developed and validated. Depending on sample matrix and biotin content different extraction procedures and HPLC conditions were applied. Key steps in sample preparation were an alkaline extraction or a hydrolysis with sulphuric acid followed by enzymatic digest with papain. For many samples with low biotin content the latter combination of extraction steps was shown to be necessary for an optimal release of biotin from the matrix. The first time synthesis of deuterated biotin for use as internal standard allowed the compensation of losses during sample work-up and ion suppression during HPLC–MS/MS analysis. The new methods are faster than the commonly used microbiological assay using *Lactobacillus plantarum*. Additionally, they have a higher specificity as results for biotin are based on determination of a chemically defined compound, and not of a biological activity. Quantification is applicable to samples with a biotin content >100 μg/kg. Results obtained with the new methods have been compared with those of the microbiological assay, and were in good agreement.
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1. Introduction

Biotin is an important vitamin functioning as coenzyme in several carboxylase-mediated metabolic reactions, including gluconeogenesis, biosynthesis of fatty acids, and metabolism of amino acids. Amongst others, a sufficiently high intake is important for healthy skin and hair. The natural biotin content of most feed and food is low, and typically in the range of few μg/kg in many vegetables to several hundred μg/kg in pork liver and egg yolk [1]. Additional to biotin, several derivatives such as biotinsulfoxide and α-dehydrobiotin, and chemically bound forms such as biocytin occur naturally. These compounds possess varying vitamin activity for selected organisms, mainly microorganisms, but often not for animals or humans. The existence of strong biotin-protein complexes such as biotin–avidin can reduce its bioavailability, and also complicates analytical investigations. All these facts pose high demands on an analytical method for quantification of biotin. Sample preparation must free the vitamin from complexes and chemical bonds. The determination itself must be very sensitive due to the low natural contents, and very specific due to the occurrence of derivatives. Probably the most widely used method for quantification of biotin in low concentrations is a microbiological assay using *Lactobacillus plantarum*, e.g. [2,3]. The microorganism, which requires biotin for growth and reproduction, is incubated with diluted sample extracts. The resulting increased turbidity of the extract is measured, and correlates with the biotin content of the sample. These assays are very sensitive, but they can lack specificity, as a biological activity is determined, which does not necessarily reflect a single defined chemical compound. In practice, this biological activity is rather due to the growth response of the microorganism towards all growth promoting or inhibiting compounds in the sample extract tested. For example, it also responds to selected biotin derivatives, and may be inhibited when antibiotics or heavy metals are present. A detailed investigation of all these influences is very difficult and not feasible during routine analysis when dealing with samples of unknown composition. Recently, published work using a more specific

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detection technique (HPLC/avidin binding) [4] indicates that many literature values for the biotin content of food may have to be reinvestigated using a method as the described. The microbiological assay is also time-consuming, as it involves incubation of the test organism with the sample extract for 19 h.

In order to overcome the drawbacks of the microbiological assay, a number of different analytical techniques have been used for analysis of biotin. The physical properties of biotin, e.g., the absence of a chromophor suitable for sensitive UV detection, and a high polarity of the molecule making it unsuitable for GC analysis, complicate the analysis in low concentrations. Published alternative methods include HPLC with fluorescence detection after post-column derivatisation [5], HPLC–MS [6,7], and various assays based on protein-binding, mainly using the strong biotin–avidin interaction [4,8–12]. In our laboratory, a microbiological assay [3] has been applied routinely for more than three decades. The goal was now to develop a new method having prominent advantages over the microbiological assay, mainly a shorter analysis time, and an increased specificity. It should be applicable to samples with both natural and added biotin content, mainly feed, food, tablets, and premixes, covering a broad content range. HPLC–MS/MS was the technique of choice for this purpose. The synthesis of isotopically labelled biotin as internal standard was prerequisite for method development. Different sample matrices and biotin contents resulted in the development of two adapted specific methods. Their development and validation with a special focus on sample preparation and comparison with the microbiological assay is presented.

2. Experimental

2.1. Materials, reagents, and solvents

An Agilent 1100 HPLC system coupled to a Sciex API 2000 tandem mass spectrometer equipped with turbo ion spray source and Analyst software was used for analysis of sample extracts. The HPLC system consisted of two gradient pumps with degasser, thermostated auto sampler and column department, and two 6-port switching valves. Analytical column 1 was a Symmetry Shield C18 column (2.1 mm × 50 mm, 3.5 μm) with pre-column (2.1 mm × 3 mm) from Waters. Analytical column 2 was a Vydac 218TP5215 (2 mm × 150 mm, 5 μm) from Grace Vydac. Hydrolysis of samples with sulphuric acid was performed in an autoclave at 120 °C and 2 bar. NMR spectra were recorded on a Bruker Avance 300 spectrometer equipped with 5 mm BBO BB-1H probe head. Biotin (USP Standard) was used as reference compound. Biotin-d_7 was synthesised as detailed in Section 2.2. Methanol (HPLC grade) and formic acid (Suprapur) were supplied by Merck. For all preparations Milli-Q water was used. All other chemicals were of analytical grade. Citrate-buffer pH 5.7 was prepared as following: 0.924 g citric acid and 2.1 g $Na_2HPO_4 \cdot 2H_2O$ were dissolved in 500 mL water. The pH of the solution was adjusted to 5.7 using phosphoric acid or sodium hydroxide solution, and made up to 1 L with water. Papain solution was prepared by dissolving 500 mg papain (EC 3.4.22.2; 30,000 U/mg; Merck 7144) in 50 mL citrate-buffer.

2.2. Synthesis of deuterated biotin

Deuterated biotin was synthesised as shown in Fig. 1 [13–15]. 1,4-Dichloro-octa-deutero-butane was purchased from Aldrich, the D-thiolactone **1** was obtained according to published methods [16]. The final product was obtained as a mixture of biotin-d_5, biotin-d_6, and biotin-d_7 in a ratio of 38/47/15 (m/m/m) as determined by quantitative ^{1}H NMR, overall yield of deuterated biotin 55%, for ^{1}H NMR and ^{13}C NMR spectral data see Table 1.

The mixture of biotin-d_5, biotin-d_6, and biotin-d_7 has been directly used for preparation of internal standard solutions. The most abundant component, biotin-d_6, was used for quantification by HPLC–MS/MS.

2.3. Samples used for method validation

Samples used for method validation are specified below. Those with high biotin contents have been denoted with capital letters, those with low contents with small letters. Most samples were originally received for routine analysis of different vitamins, some were bought in local grocery stores, and two samples were obtained from NIST. Label claims (if given) usually equal the vitamin content guaranteed by the producer during the shelf live time of the product, and are given for orientation only. They are often based solely on the added biotin, and do not include the natural biotin content of the samples.

Sample A: film-coated multivitamin multimineral tablet, claim 30 μg/tablet; sample B: effervescent multivitamin multimineral tablet, claim 150 μg/tablet; sample C: premix for feed, claim 35 mg/kg; sample D: pelleted feed, claim 400 mg/kg; sample E: premix for feed, claim 30 mg/kg; sample F: effervescent multivitamin multimineral tablet, claim 150 μg/tablet;

Table 1
^{1}H and ^{13}C NMR data of biotin-d_6 (δ in ppm, J in Hz)[a] used as internal standard for quantification of biotin with HPLC–MS/MS

Position	^{1}H[b]	^{13}C[c]
1	6.36 (brs)	–
2	–	162.7 (C)
3	6.44 (brs)	–
3a	4.13 (ddd, J = 7.4, 4.5, 1.7)	61.0 (CH)
4	3.09 (dd, J = 6.1, 4.5)	55.3 (CH)
6	α 2.82 (dd, J = 12.5, 5.1)	39.8 (CH_2)
	β 2.58 (d, J = 12.5)	
6a	4.30 (dd, J = 7.4, 5.1)	59.2 (CH)
δ	1.57 (d, J = 6.1)	27.4 (t, J = 19, *CHD*)
γ	–	27.0 (m, CD_2)
β	–	23.4 (m, CD_2)
α	2.17 (s, CHD)	32.9 (t, J = 19, *CHD*)
	2.18 (s, CH_2)[d]	33.2 (s, CH_2)[d]
COOH	11.96 (brs)	174.4 (C)

[a] All assignments are based on extensive 1D and 2D NMR measurements (HMBC, HSQC, COSY) in comparison with biotin.
[b] DMSO-d_6, 300 MHz, C*H*D_2SOCD_3 = 2.50 ppm.
[c] DMSO-d_6, 75 MHz, (CD_3)$_2$SO = 39.5 ppm, broadband ^{1}H decoupled, multiplicity given for C–D coupling.
[d] Assignments for biotin-d_5.

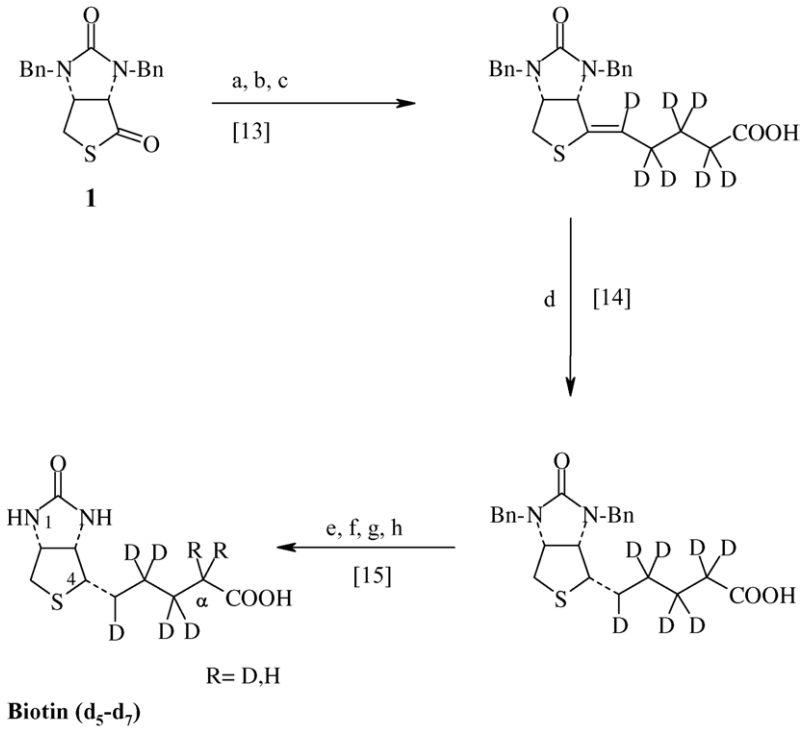

Fig. 1. Synthesis of deuterated biotin as internal standard. During the last steps of the synthesis hydrogen partly exchanged with deuterium at the α-position. According to the number of remaining deuterium atoms in the molecule, the resulting compounds are referred to as biotin-d_7, biotin-d_6, and biotin-d_5. (a) $Cl(CD_2)_4$-Cl, Mg; (b) CO_2; (c) dilute H_2SO_4 refl.; (d) Pd/H_2; (e) 48% HBr refl.; (f) NaOH/ $COCl_2$; (g) NaOH, refl. (h) dilute H_2SO_4.

sample G: film-coated multivitamin multimineral tablet, claim 15 μg/tablet.

Samples a and b: cow milk; sample c: multi-vitamin fruit juice, label claim 230 μg/L; sample d: bird feed, label claim 57 μg/kg; sample e: rabbit feed, label claim 250 μg/kg; sample f: feed without claim; samples g and h: baby food (instant milk), label claims 110 and 120 μg/kg, respectively; samples i and j: homogenated eggs; samples k and l: baby food (instant milk), label claims 150 μg/kg; samples m–t: feed without claim; sample u: eggs; NIST 8435: milk powder, claim 150–160 μg/kg; NIST 2383: baby food, claim 54 ± 12 μg/kg; sample v: milk; sample w: milk.

2.4. Sample extraction procedures

Depending on biotin content and sample matrix, three different extraction procedures have been applied, either alone or in combination (see Section 3.2 for details). They are referred to as alkaline extraction (Section 2.4.1), extraction using hydrolysis with sulphuric acid (Section 2.4.2), and extraction using enzymatic digest with papain (Section 2.4.3).

2.4.1. Alkaline extraction

5.0 g of homogenised sample or one tablet were weighted into a 500 mL volumetric flask. Three grams of $Na_2S_2O_3 \cdot 9H_2O$, 10 mL ammonium hydroxide solution 2% and 250 mL water were added, and the flask was gently shaken for 20 min on a horizontal shaker. After making up to volume with water an aliquot was filtered through a paper filter and diluted with water to obtain a biotin concentration in the range of 0.01–1 μg/mL. This solution was further filtered (0.45 μm PVDF filter). 1.0 mL filtrate and 100 μL internal standard solution (biotin-d_6 1 μg/mL in methanol) were mixed and used for HPLC–MS/MS analysis.

2.4.2. Extraction using hydrolysis with sulphuric acid

Ten grams of sample, 100 mL sulphuric acid 2N, and 1 mL internal standard solution (biotin-d_6 40 μg/mL) were given into an Erlenmeyer flask. The flask was gently shaken until all sample material was wet and equally dispersed. It was then autoclaved for 30 min at 120 °C and 2 bar. After cooling to room temperature the pH of the extract was adjusted to 5.7 using sodium hydroxide solution. The extract was then made up to 250 mL with water. An aliquot was filtered through a paper filter, and if necessary, diluted to give a biotin concentration in the range of 0.5–50 ng/mL. This solution was further filtered (0.45 μm PVDF filter), and used for HPLC–MS/MS analysis.

2.4.3. Extraction using enzymatic digest with papain

Enzymatic digest was usually applied as second extraction step following hydrolysis with sulphuric acid (Section 2.4.2). In this case, 600 μL glutathione solution (10 mg/mL), 600 μL Na-EDTA solution (10 mg/mL), 6 mL papain solution, and 60 mL citrate-buffer were added to the extract directly after adjusting the pH to 5.7. If applied to samples without prior hydrolysis, 10 g

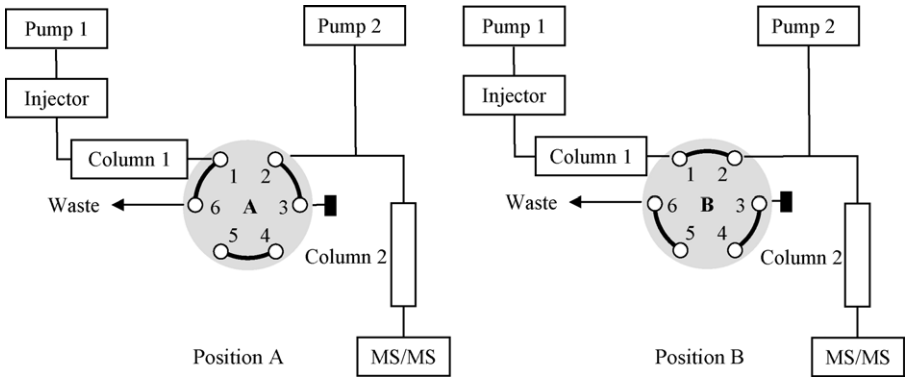

Fig. 2. Heart-cut column switching technique used for HPLC separation of sample extracts with low biotin concentrations. Position A: chromatography of extract on column 1 or chromatography of cut fraction on column 2; position B: transfer of the biotin containing fraction from column 1 to column 2; see Section 2.5.1 for details.

sample, 1 mL internal standard solution (biotin-d_6 40 μg/mL), and 100 mL water were mixed before adding the solutions for the digest as detailed above. In either case, the resulting mixture was then incubated at 37 °C under gentle shaking on a horizontal shaker for 16 h. After cooling to room temperature, the extract was made up to 250 mL with water. An aliquot was filtered through a paper filter, and if necessary, diluted to give a biotin concentration in the range of 0.5–50 ng/mL. This solution was further filtered (0.45 μm PVDF filter), and used for HPLC–MS/MS analysis.

2.5. HPLC–MS/MS analysis

2.5.1. HPLC conditions

Depending on the extraction method applied and on the expected biotin concentration, two different HPLC conditions have been used. They are referred to as HPLC conditions A and B. HPLC condition A was used for analysis of extracts with biotin concentrations in the range of 0.01–1 μg/mL. Usually, these extracts were obtained using alkaline extraction (Section 2.4.1). A Symmetry Shield C18 column (2.1 mm × 50 mm, 3.5 μm) with pre-column (2.1 mm × 3 mm) thermostated at 20 °C was used for chromatography. Extracts were kept at 8 °C, and 10 μL were injected for analysis. Eluents were water (A) and methanol (B) each containing 0.1% formic acid. A gradient program was used at a flow of 0.25 mL/min: 0.0–2.5 min, isocratic, 5%B; 2.5–7.0 min, linear gradient up to 75%B; 7.0–9.9 min, isocratic, 75%B; 9.9–10.0 min, linear gradient down to 5%B; 10.0–12.0 min, isocratic, 5%B. The retention time of biotin was approximately 7.8 min. HPLC condition B was used for analysis of extracts with biotin concentrations in the range of 0.5–50 ng/mL (Table 2). It based on HPLC condition A, and additionally included a heart-cut column switching technique with a second analytical column for improved separation (Fig. 2).

The heart-cut column switching technique transfers the biotin containing fraction from column 1 onto column 2 during a time frame of 1.5 min around the retention time of biotin on column 1. Column 2 was a Vydac 218TP5215 (2 mm × 150 mm, 5 μm). Injection volume was 100 μL. The gradient program for pump 1 operated with a flow of 0.25 mL/min, the flow gradient for pump 2 is given in Table 2. Final retention time of biotin was approximately 15.5 min. In order to decrease contamination of the ion source of the MS/MS the eluat was transferred to the MS/MS only during a time frame of 4 min around the expected retention time of biotin.

2.5.2. Mass spectrometry

A Sciex API 2000 tandem mass spectrometer was operated with a turbo ion spray source in positive mode at unit resolution. Biotin and biotin-d_6 were detected by selected reaction monitoring (SRM) in the multi reaction monitoring (MRM) mode of the instrument using the following software settings: biotin Q1 mass 245.10 amu, Q3 mass 227.10 amu; biotin-d_6 Q1 mass 251.20 amu, Q3 mass 233.20 amu, dwell time 200 msec each. Ion source and other instrument parameters were optimized for these transitions by infusion of biotin solution (10 μg/mL). The following settings were used (instrument parameter): curtain gas (CUR) 35 psi; ion spray voltage (IS) 4750 V; temperatur of the

Table 2
Column-switching parameters and gradient programs for HPLC condition B

Time (min)	%B (pump 1)	%B (pump 2)	Flow pump 2 (mL/min)	Valve position
0.0	5	5	0.25	A
2.5	5			
6.9		5		
7.0	75	0		B
8.5				A
8.6		0	0.25	
8.7		5	0.50	
9.5		5		
9.9	75			
10.0	5			
25.0		55		
25.1		75		
27.5		75		
27.6		5		
29.9			0.50	
30.0	5	5	0.25	

Remaining parameters and settings are given in Section 2.5.1.

heater gas (TEM) 475 °C; nebuliser gas (GS1) 65 psi; heater gas (GS2) 75 psi; collision gas N_2 (CAD) 6. For quantification, biotin standard solutions covering the respective concentration range were analysed in duplicate with each analytical series. Solutions contained biotin-d_6 in concentrations identical with those theoretically present in the sample extracts. The ratio of (area counts biotin) to (area counts biotin-d_6) was plotted against the corresponding concentration ratio. A linear regression using a $1/x$ weighting was calculated from these data for determination of biotin concentration in sample extracts.

2.6. Microbiological assay

The microbiological assay of biotin was performed using *L. plantarum* ATCC 8014 as published in [3].

3. Results and discussion

The main parts of the method development work included the following tasks: choice and synthesis of an isotopically labelled biotin as internal standard for sample extraction and MS/MS analysis, development of a broadly applicable sample extraction procedure, and development of an HPLC system combining MS/MS compatibility with high separation efficiency. This resulted in development of two new methods, which were then validated and compared with the microbiological assay.

3.1. HPLC–MS/MS analysis

3.1.1. Choice and synthesis of isotopically labelled biotin as internal standard

In order to compensate suppression of ionisation of biotin in the electro spray ion source, and of possible losses during sample extraction, the use of an internal standard was compulsory. Unfortunately, an isotopically labelled biotin was not commercially available. Therefore, a suitable compound first had to be synthesised. The isotopic distribution for biotin is: amu (relative abundance) 244 (100), 245 (12.7), 246 (5.9), 247 (0.6), and 248 (<0.1). Thus, the labelled biotin preferably should have a molecular weight of 248 or more. Considering the availability of intermediates from biotin synthesis and of commercially available labelled compounds, a synthesis leading to biotin-d_7 was envisaged (Fig. 1). Unfortunately, during the final steps of the synthesis some deuterium at the α-methylene group was exchanged with hydrogen as evident from ^{1}H NMR data (Table 1). The final product was a mixture of biotin-d_7, biotin-d_6, and biotin-d_5 in a ratio of 38/47/15 (m/m/m). Consequently, the most abundant component, biotin-d_6, was chosen as internal standard for quantification.

3.1.2. Stability of the deuterium labels of biotin during hydrolysis with sulphuric acid

Extraction of samples with a low biotin content involved hydrolysis with sulphuric acid 2N at 120 °C for 30 min (Section 2.4.2). Thus, the stability of the deuterium labels under these conditions had to be investigated before biotin-d_6 could be used as internal standard. As biotin-d_7 had lost some of its deuterium labels at the α-methylene group during synthesis of the material, the possibility of a similar loss during the hydrolysis step of the sample extraction had to be excluded. Therefore, the relative abundance of biotin-d_5, biotin-d_6, and biotin-d_7 in selected solutions was measured. MS/MS parameters were comparable with those given in Section 2.5.2. Additionally, transitions of biotin-d_7 (m/z 252 → 234) and biotin-d_5 (m/z 250 → 232) were monitored. A loss of deuterium at the α-methylene group would have been indicated by a relative increase of biotin-d_5 compared to the other two species. The relative peak area ratio biotin-d_5/biotin-d_6/biotin-d_7 was determined in three solutions, and proved the stability of the label: internal standard solution, no hydrolysis 85.6/100/47.3; internal standard solution after hydrolysis 83.1/100/47.7, milk sample with added internal standard solution after hydrolysis 84.5/100/48.7.

3.1.3. HPLC–MS/MS conditions

The availability of deuterated biotin as internal standard made a very specific and sensitive quantification of biotin by HPLC–MS/MS possible. Using water–methanol–formic acid mixtures as eluent, electro spray with positive ionisation proved to be the most sensitive ionisation method. Two prominent SRM transitions were observed for biotin, m/z 245 → 227, and 245 → 114. Only the first one was intense enough for quantification purposes. The corresponding transition, m/z 251 → 233, was used for biotin-d_6. The different sample extraction procedures (Section 2.4) made the development of two different HPLC conditions necessary (A and B, Section 2.5.1). HPLC condition A was primarily used for analysis of sample extracts resulting from alkaline extraction (Section 2.4.1) with biotin concentrations in the range of 10–1000 ng/mL. Using a water–methanol–formic acid gradient system on a Symmetry Shield C18 column, sufficient separation could be achieved injecting 10 μL extract with a run time of 12 min. HPLC condition B was primarily used for analysis of sample extracts resulting after acidic hydrolysis (Section 2.4.2) and enzymatic digest (Section 2.4.3) with biotin concentrations below 50 ng/mL. In order to reach a sufficient sensitivity of detection 100 μL sample extract had to be injected. Unfortunately, these extracts contained high amounts of dissolved organic compounds, mostly papain and peptide fragments from the enzymatic digest. This resulted in strong ion suppression, and rapid pollution of the ion source. Influences from the different sample matrices were much smaller by comparison, and have not been investigated separately. Attempts to clean the extracts using trapping columns or off-line SPE were not successful, most probably because the peptide fragments from the digest had similar physical properties as biotin. The use of avidin for a specific extraction of biotin from the solution was excluded due to its high cost for routine analysis. Finally, a heart-cut column switching technique was applied, transferring the biotin containing fraction from the first analytical column onto a second analytical column for an improved separation (Fig. 2). A flow of 0.5 mL/min on column 2 compared to 0.25 mL/min on column 1 allowed a dilution of the biotin fraction with 0.25 mL/min water during the transfer. This reduced the elution strength of the fraction, and enabled a refocusing of the biotin peak on column 2. Although still significant

Table 3
Analytical characteristics of the two HPLC–MS/MS methods (Section 2.5) used for quantification of biotin in sample extracts

HPLC condition (Section 2.5.1)	A	B
Concentration range	0.5–50 ng/mL	10–1000 ng/mL
Number of calibration levels	8	8
Weighting	$1/x$	$1/x$
Intercept	-4.95×10^{-6}	3.02×10^{-4}
Slope	4.57	0.276
R	0.9998	0.9967
Accuracy[a]	$100 \pm 5\%$	$100 \pm 5\%$

[a] Accuracy was calculated as experimental value relative to calculated fit for each concentration level (%).

ion suppression was observed, this technique allowed reaching a lower limit of quantification of 0.5 ng/mL. The analytical characteristics of the two HPLC–MS/MS methods are summarised in Table 3.

3.2. Sample extraction

Biotin must be extracted into aqueous solution prior to HPLC–MS/MS analysis. This is a difficult task as both naturally occurring and added biotin, various matrices, and physical and chemical bonds have to be considered. In case of samples fortified with relatively high amounts of biotin, such as tablets and premixes, the natural biotin content can be neglected. This simplifies the sample preparation, and an alkaline extraction using diluted ammonium hydroxide solution is sufficient (Section 2.4.1). This procedure is essentially the same which had been successfully applied in our laboratory for many years as sample preparation for the microbiological biotin assay. Therefore, biotin-d_6 as internal standard has been used only for correction of ion suppression in the MS/MS. It was added directly to the sample extract prior to HPLC–MS/MS analysis.

Quantifying naturally occurring biotin in feed and food samples requires more drastic extraction conditions. Here, the vitamin has to be released from its numerous physical and chemical bonds, including tissue, protein complexes, and peptide bonds. The most widely applied methods are an acidic hydrolysis, and an enzymatic digest using papain [3,4,10].

Hydrolysis with sulphuric acid under elevated temperature releases biotin from tissue, protein complexes, and even frees chemical bonded biotin, e.g., from biocytin. However, such a hydrolysis has been described to lead to a partial degradation of biotin, depending on acid concentration, time and temperature of the experimental conditions [10]. Nevertheless, for selected samples, e.g., peanuts, a possible loss was obviously more than compensated by an increased extraction of biotin. Losses can be minimised using a protocol with hydrochloric acid [4]. However, based on our experience, acidic hydrolysis may not be sufficient for samples with high protein contents such as milk and eggs. Here, remaining protein fragments might still be able to complex biotin.

This can be overcome by an enzymatic digest with papain which is often used as a second or even as the sole extraction step [3,10]. Such a digest is complicated by varying activity and purity of commercially available papain preparations. When using high amounts of papain, a possible biotin content of the enzyme also has to be considered. In the way the enzymatic digest is often used [3], it has two major drawbacks: a long analysis time due to incubation for up to 16 h, and the introduction of large amounts of peptide fragments, resulting from the digest and the enzyme itself, into the extract. While the influence of these fragments on the microbiological assay is limited, they interfere with MS/MS analysis by causing ion suppression and a rapid pollution of the ion source. Therefore, we altered our original procedure [3] by using a more active and pure preparation of papain (EC 3.4.22.2) with an activity of 30,000 USP-U/mg. This way, the amount of enzyme present in the sample extracts could be reduced from 9 mg/mL in the microbiological assay [3] to only 0.24 mg/mL. Although still causing major problems with the MS/MS analysis, the interference could be reduced by using a heart-cut column switching technique (Section 2.5.1) with two analytical columns for an improved separation.

As literature data and our own experience indicated that both acidic hydrolysis and enzymatic digest would be necessary for a complete extraction of natural occurring biotin from many samples [3,10], we investigated this topic in detail. If one extraction method alone could be shown to be effective enough for complete release of biotin, this would offer the advantage of large time savings during routine analysis. Investigation of hydrolysis procedures is complicated by the fact that biotin may not be completely stable under these conditions [10]. Selected degradation products may even possess some vitamin activity. By using a relatively unspecific quantification method, such as microbiology, influences on the analytical result cannot be excluded. The availability of isotopically labelled biotin, which was added to the samples prior to extraction in order to compensate possible losses, and the use of MS/MS as a more specific analytical method solved these problems.

For the comparison of the extraction efficiency, acidic hydrolysis (Section 2.4.2) and enzymatic digest (Section 2.4.3) were applied alone or in combination for extraction of selected samples. The biotin concentrations of the extracts were quantified by HPLC–MS/MS using condition B (Section 2.5.1), and results were compared.

In a first set of experiments, samples were extracted using the enzymatic digest with papain with or without prior hydrolysis using sulphuric acid (Table 4).

Biotin contents analysed using the additional acidic hydrolysis step for extraction were always higher than those obtained after enzymatic digest alone. For half of the samples results were even higher by more than 20%. Differences seemed to depend on the sample matrix. For milk and fruit juice relatively small differences (<20%) were obtained. In these samples, biotin occurs mainly free or protein-bond, making enzymatic digest a suitable extraction method. The largest differences (>20%) were obtained for feed samples. Here, most biotin was probably contained within tissue, and could not be released by enzymatic digest, but by acidic hydrolysis. From these results, it was obvious that for most sample types enzymatic digest alone is not

Table 4
Comparison of biotin contents analysed in eight selected samples with HPLC–MS/MS using enzymatic digest, or acidic hydrolysis and enzymatic digest as extraction method ($n = 2$)

Sample (type)	Biotin content, extraction using enzymatic digest (μg/kg)	Biotin content, extraction using acidic hydrolysis and enzymatic digest	
		(μg/kg)	(%) of content using enzymatic digest
a (milk)	90	99	110
b (milk)	35	41	117
c (fruit juice)	260	265	102
d (bird feed)	61	157	257
e (rabbit feed)	216	308	143
f (feed)	135	207	153
g (baby food)	126	136	108
h (baby food)	122	151	124

Table 5
Comparison of biotin contents analysed in eight selected samples with HPLC–MS/MS using acidic hydrolysis, or acidic hydrolysis and enzymatic digest as extraction method ($n = 2$)

Sample (type)	Biotin content, extraction using acidic hydrolysis (μg/kg)	Biotin content, extraction using acidic hydrolysis and enzymatic digest	
		μg/kg	(%) of content using acidic hydrolysis
i (egg)	368	468	127
j (egg)	140	177	126
k (baby food)	354	345	97
l (baby food)	222	291	131
m (feed)	147	151	103
n (feed)	102	89	87
NIST 8435 (milk powder)	152	286	188
NIST 2383 (baby food)	69	67	97

sufficient as sample preparation, and analytical results obtained will not reflect the true biotin content.

In a second set of experiments, it has been evaluated whether acidic hydrolysis alone would be enough for a complete release of biotin or whether additional enzymatic digest would be necessary. As this was expected to be the case for protein rich samples like eggs or milk powder, a different selection of samples was chosen for the experiments (Table 5).

Indeed, for most protein rich samples (samples i, j, l and NIST 8435) biotin contents analysed were more than 25% higher when an additional digest with papain had been applied. In comparison, in feed or food samples with a low protein content (samples m, n, NIST 2383) comparable biotin contents (±15%) were obtained with both extraction procedures. Therefore, acidic hydrolysis alone seems suitable as sole extraction step for biotin analysis only for certain matrices with low protein content where its efficiency has been proven.

3.3. Validation results of the method for samples with high biotin content (>10 mg/kg)

Alkaline extraction (Section 2.4.1) and HPLC–MS/MS analysis according to condition A (Section 2.5) formed the method of choice for samples with high biotin content (>10 mg/kg). Method validation included determination of accuracy by recovery, precision, and comparison of quantitative results with those obtained with the microbiological assay. Typical samples encountered during routine analyses were chosen for the experiments (Section 2.3). For determination of accuracy by recovery and precision selected samples were analysed six fold each with and without prior addition of biotin standard solution (Table 6).

For all samples a recovery higher than 92% was obtained. Relative standard deviations were in the range of 2–6%. Taken into account the inhomogeneity of the sample material, the precision of the HPLC–MS/MS determination itself was estimated to be even better.

In order to compare the biotin contents obtained by HPLC–MS/MS and microbiology, selected samples were analysed with both methods (Table 7). Determinations were carried out starting from identical alkaline sample extracts, which then were diluted as required for each of the quantification methods.

The comparison showed a very good agreement of the results of both methods. Differences for most samples were less than 5%. Only for one premix the HPLC–MS/MS analysis gave a content which was by more than 10% lower than the result obtained with the microbiological assay. A growth promoting substance other than biotin present in this particular premix might be responsible for this finding, as a recovery of 95.5% was determined for the same sample using HPLC–MS/MS (Table 6).

3.4. Validation results for the method for samples with low biotin content (<10 mg/kg)

Acidic hydrolysis (Section 2.4.2) followed by enzymatic digest (Section 2.4.3) for extraction and HPLC–MS/MS

Table 6
Accuracy by recovery and precision (R.S.D.; $n = 6$) for four selected samples using the method for high biotin contents (alkaline extraction and HPLC condition A)

Sample (type)	Biotin content[a]	R.S.D. (%)	Biotin content with spike[a,b]	R.S.D. (%)	Recovery (%)
A (film-coated tablet)	34.8 μg	2.8	48.8 μg	2.2	92.8
B (effervescent tablet)	165.5 μg	4.0	266.6 μg	3.7	101.1
C (premix)	23.3 mg	5.6	42.3 mg	2.6	95.2
D (feed)	357.8 mg	2.3	553.3 mg	2.5	92.2

[a] Values per tablet or kg, respectively.
[b] Spiked amounts (diluted in water) were: sample A, 15 μg; sample B, 100 μg; sample C, 20 mg; sample D, 212 mg.

Table 7
Comparison of HPLC–MS/MS and microbiology for quantification of biotin in six samples with high contents

Sample (type)	Biotin content using HPLC–MS/MS (mg/kg or μg/tablet)	Biotin content using microbiology (mg/kg or μg/tablet)	HPLC–MS/MS vs. microbiology (%)
A (tablet)[a]	34.8	35.7	97.5
B (tablet)[a]	165.5	161.0	102.8
C (premix)[a]	23.3	27.2	85.7
D (feed)[a]	357.8	325.6	109.9
E (premix)[b]	29.2	30.0	97.3
F (tablet)[b]	161.3	155.5	103.7
G (tablet)[b]	16.2	16.2	100.0

See also Table 6.
[a] $n = 6$.
[b] $n = 2$.

condition B for quantification (Section 2.5) formed the method of choice for samples with low biotin content (<10 mg/kg). For determination of accuracy by recovery and precision selected samples were analysed six fold each with and without addition of biotin standard solution (Table 8).

Only for the sample with the lowest biotin content (sample v), the recovery was below 90%. Precision was characterised by relative standard deviations in the range of 2–9%. Results for sample v also had the lowest precision, suggesting that the method is best applicable to samples with a biotin content >100 μg/kg.

A direct comparison of HPLC–MS/MS and microbiology for quantification of biotin in sample extracts with low concentrations was not possible. They contained high amounts of biotin-d_6 as internal standard (up to 80 times the biotin content) for correction of losses during extraction, and *L. plantarum* was able to use the biotin-d_6 instead of biotin for growth (data not shown). Therefore, extraction of the samples for the microbiological assay was performed as described in [3]. The two major differences to the extraction for quantification using HPLC–MS/MS were a higher concentration of a different papain (papain 3 U/mg, Fluka 76220) used for enzymatic digest, and the lack of an internal standard. Thus, possible losses of biotin during extraction cannot be compensated in the microbiological assay. For comparison of the new HPLC–MS/MS method with the microbiological assay 15 samples have been analysed in duplicate according to the two procedures (Table 9).

Additionally, two reference materials obtained from NIST have been analysed only with the new HPLC–MS/MS method. Out of 17 samples investigated, results for 9 samples differed by less than ±15%, and in four cases each the HPLC–MS/MS results were higher or lower by more than 15% than those from the microbiological assay. Higher results obtained with quantification by HPLC–MS/MS might be due to a sample matrix dependent biotin loss during hydrolysis which was compensated by the internal standard, but could not be compensated in the microbiological assay. Additionally, growth inhibiting substances such as antibiotics might have been present in those samples. This led to a partial inhibition of *L. plantarum*, and thus to lower biotin contents obtained with the microbiological assay. Lower results obtained with quantification by HPLC–MS/MS with differences up to 35% might be explained by the different

Table 8
Accuracy by recovery and precision (R.S.D.; $n = 6$) for five selected samples using the method for low biotin contents (acidic hydrolysis and enzymatic digest, HPLC condition B)

Sample (type)	Biotin content (μg/kg)	R.S.D. (%)	Biotin content with spike[a] (μg/kg)	R.S.D. (%)	Recovery (%)
d (bird feed)	137	5.1	322	5.8	92.5
e (rabbit feed)	305	2.3	484	4.0	89.5
g (baby food)	134	3.8	317	3.9	91.5
v (milk)	52	9.2	90	5.6	76.0
w (milk)	93	5.7	189	2.8	96.0

[a] Spiked amounts (diluted in water) corresponded to: samples d, e, and g, 200 μg/kg; sample v, 50 μg/kg; sample w, 100 μg/kg.

Table 9
Comparison of biotin content analysed in 17 selected samples using HPLC–MS/MS or microbiology, methods for low contents with acidic hydrolysis and enzymatic digest ($n = 2$)

Sample (type)	Biotin content using HPLC–MS/MS (μg/kg)	Biotin content using microbiology (μg/kg)	HPLC–MS/MS vs. microbiology (%)
a (milk)	99	129	76.6
b (milk)	41	55	74.5
c (fruit juice)	265	298	88.9
d (bird feed)	157	161	97.5
e (rabbit feed)	306	350	87.5
f (feed)	207	313	66.1
g (baby food)	136	103	132.0
h (baby food)	151	139	108.6
o (feed)	320	128	250.0
p (feed)	285	323	88.2
q (feed)	966	965	100.1
r (feed)	127	137	92.7
s (feed)	114	127	89.8
t (feed)	366	543	67.4
u (egg)	1031	1010	102.1
NIST 8435 (milk powder)	286	150–160[a]	184.7
NIST 2383 (baby food)	67	54 ± 12[a]	124.1

[a] Contents specified by NIST.

specificity of the two methods. It seemed likely that in some samples growth promoting compounds other than biotin were present which led to these results. This was most likely the case for samples which were not fortified with biotin, such as the milk samples a and b. Considering the good recoveries obtained using the HPLC–MS/MS method (Table 8), and the high specificity of MS/MS detection, biotin contents analysed using HPLC–MS/MS with internal standard are suggested being more accurate than those of the microbiological assay. Thus, published biotin contents of some food and feed obtained microbiologically may need reinvestigation with a more specific analytical method.

4. Conclusions

HPLC–MS/MS using biotin-d_6 as internal standard could be shown to be an accurate and precise method for analysis of biotin in extracts from feed, food, tablets, and premixes. The right sample extraction procedure proved to be crucial for the biotin contents analysed. While alkaline extraction could be used for samples with high biotin content (>10 mg/kg), consecutive hydrolysis with sulphuric acid and enzymatic digest with papain were necessary for an optimal release of biotin from most samples with low biotin contents. Hydrolysis or enzymatic digest applied alone often yielded lower biotin contents, and were sufficient only for extraction of selected sample types. The new methods are faster than a microbiological assay, and offer a higher specificity as a defined chemical compound, and not a biological activity is determined. Therefore, they are ideally suited for determination of biotin in samples with both natural and added vitamin content in routine analysis.

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