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Improved Liquid Chromatography Methods for the Separation and Quantification of Biotin in NIST Standard Reference Material 3280: Multivitamin/Multielement Tablets

BRYANT C. NELSON,* KATHERINE E. SHARPLESS, AND LANE C. SANDER

National Institute of Standards and Technology, Analytical Chemistry Division, Gaithersburg, Maryland 20899-0001

Two independently developed liquid chromatography (LC) methods for the quantitative determination of biotin in multivitamin/multielement tablets (NIST Standard Reference Material 3280 (SRM 3280)) are described. The methods use distinctly different tablet extraction solvents (methanol vs 1.5% aqueous formic acid) and analyte detection principles (mass spectrometry (MS) versus evaporative light-scattering detection (ELSD)) to ensure quantitative reliability. The use of different extraction and detection procedures allows cross-validation of the methods and enhances confidence in the final quantitative results. Both methods yield highly comparable results for the mean level of biotin (LC/MS = 26.5 mg/kg ± 0.3 mg/kg (n = 12); LC/ELSD = 24.7 mg/kg ± 1.7 mg/kg (n = 12)) in SRM 3280, yet the methods differ considerably in their analytical characteristics. The isotope-dilution LC/MS method exhibits excellent linearity from 0.02 ng to 77 ng biotin on-column with a method limit of detection (LOD) and limit of quantification (LOQ) of 0.02 ng (S/N > 3) and 0.06 ng (S/N > 10) biotin on-column, respectively. The LC/ELSD method exhibits good linearity from 155 ng to 9900 ng biotin on-column with a method LOD and LOQ of 155 ng (S/N > 3) and 310 ng (S/N > 10) biotin on-column, respectively. Method performance data indicates that the LC/MS method is analytically superior to the LC/ELSD method; however, either method in combination with SRM 3280 should provide quality assurance, accuracy, and traceability for biotin levels in multivitamin/multielement dietary supplements.

KEYWORDS: Biotin; dietary supplements; evaporative light-scattering detection; liquid chromatography; mass spectrometry; multivitamin/multielement tablets; standard reference material

INTRODUCTION

Biotin is an essential micronutrient required for the metabolism of carbohydrates and decarboxylation of amino acids (**Figure 1A**). It is a water-soluble B-vitamin that is present in minute amounts in every living cell, and it is also found in a variety of foodstuffs, such as cheese, beans, chicken liver, and cooked eggs. Severe biotin deficiency is rare because of the vitamin's natural presence in the body and in foodstuffs; however, deficiency can occur in individuals undergoing total parenteral nutrition or anticonvulsant therapy (*1*−*3*). On the other hand, marginal biotin deficiency is a common occurrence (*4*). Marginal biotin deficiency is mainly observed in women during early pregnancy, and the overriding concern is that the deficiency may become teratogenic (*4*−*7*). Recent evidence has linked reduced biotin status to impaired glucose tolerance, and thus there also exists a possible association between marginal biotin deficiency and increased risk for diabetes (*8*, *9*).

Hence, biotin status is an increasingly important feature of adult nutritional health. Many adults (≈70%) in the United

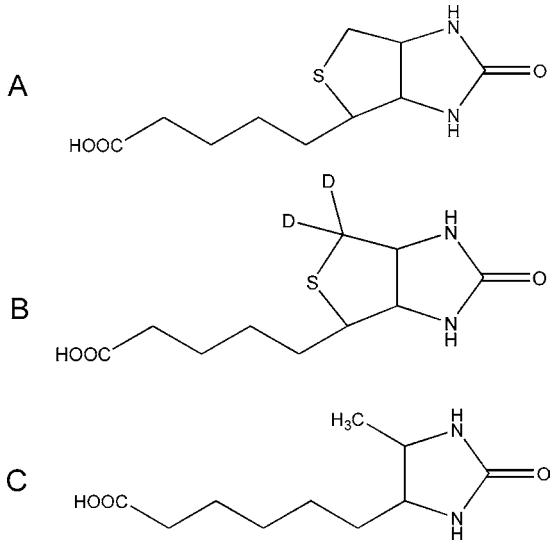

Figure 1. Chemical structures for (**A**) biotin (244.3 g/mol), (**B**) [$^2H_2$]-biotin (246.3 g/mol), and (**C**) desthiobiotin (214.3 g/mol).

* Author to whom correspondence should be addressed. Tel: 301-975-2517; fax: 301-977-0685; e-mail: bryant.nelson@nist.gov.

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States utilize dietary supplements (multivitamins) to improve their nutritional health status (*10*), and a large proportion of these supplements are formulated to provide 30 μg of dietary biotin; 30 μg is currently the daily recommended intake (DRI) (*11*). However, no suitable biotin reference methods or reference materials exist to verify commercial multivitamin label claims with respect to biotin levels. Additionally, no validated methods or natural-matrix materials exist for biotin for use in quality control or quality assurance.

The first methods for the quantitative determination of biotin were based on microbiological assays (*12*). Microbiological assays, as well as protein-binding assays and biosensor-based immunoassays, have been used for the determination of biotin in food matrices and in dietary supplements (*12*−*14*). The determination of biotin in food matrices is more challenging than the determination in dietary supplements because of the need to release protein-bound biotin in foods via acid hydrolysis or enzymatic digestion (*15*). Determination of biotin in dietary supplements, on the other hand, only requires appropriate solubilization of the free (non-protein-bound) biotin. The quantitative determination of biotin in a variety of multivitamin/multielement supplements has been successfully accomplished via affinity-binding methodology−solid-phase ^{125}I-avidin binding assay (*16*). However, the binding assay suffers from the need to use and dispose of a radioactive material (^{125}I), an inability to discriminate between biotin and biotin metabolites, and a lengthy (2-day) sample analysis (*16*, *17*). Quantification of biotin in multivitamin/multielement supplements has generally been approached via the application of physicochemical methods because of the need to separate trace levels (0.002% mass fraction) of biotin from other higher-level vitamins and elements (*12*). Thin-layer chromatography (TLC) methods were the initial methods applied to multivitamin supplements, but these methods suffer from both poor sensitivity and poor selectivity (*18*, *19*). The biotin molecule does not have significant UV absorbance nor appreciable native fluorescence (FL), yet LC methods based on either UV detection or FL detection replaced the TLC methods. LC/UV (*20*−*23*) methods require low wavelengths (200−230 nm), and LC/FL (*24*, *25*) methods require postcolumn derivatization with a fluorescent reagent to detect biotin. Consequently, LC/UV methods evince poor analyte selectivity while LC/FL methods are unduly laborious because of the need for analyte derivatization. LC with electrochemical detection has been applied to the qualitative determination of biotin in a multivitamin pharmaceutical (*26*). Unfortunately, data for the actual quantitative determination of biotin in the multivitamin pharmaceutical was not reported. At the time of writing, exhaustive review of the scientific literature revealed only two recent approaches utilizing mass spectrometry (*15*, *27*) and no approaches based on evaporative light-scattering detection for the quantification of biotin dietary supplements.

Two independent chromatography methods have been devised for the quantification of biotin in multivitamin/multielement tablets. One method is based on the use of isotope-dilution LC/MS, and the other method is based on LC/ELSD. Each method uses an independent tablet extraction procedure incorporating a stable internal standard compound for accurate quantification. Each method was developed, tested, and cross-validated through quantification of biotin in NIST SRM 3280.

EXPERIMENTAL PROCEDURES

Safety Considerations. The handling of organic solvents and organic acids should be regarded as potentially hazardous. Dimethylsulfoxide (DMSO) is a strong solvent and is an efficient carrier of other potentially harmful chemicals through the skin. Safe working conditions (use of safety goggles and disposable protective gloves) and solvent disposal procedures should be established before initiating work.

Reagents and Materials. D-Biotin (lot 073k07115, purity = 99%), desthiobiotin (lot 033K5007, purity = 99%), formic acid, and DMSO were obtained from Sigma Chemical Company (St. Louis, MO). [$^2H_2$]-biotin (lot SL32005147A1, purity = 98%) was obtained from Isosciences LLC (King of Prussia, PA). [$^2H_2$]-biotin (**Figure 1B**) contains two stable ^{2}H labels incorporated into its tetrahydrothiophene ring. The identity and purity of biotin, [$^2H_2$]-biotin, and desthiobiotin were confirmed by full-scan LC/MS and direct-infusion (*m*/*z* 50−500) analyses at NIST; no unidentified components greater than 1% (relative ion intensity) were detected in any of the reference compounds. HPLC grade methanol was obtained from J.T. Baker (Phillipsburg, NJ). Sep-Pak Vac 3 cm^3 solid-phase extraction (SPE) cartridges (C_{18}, 200 mg) were obtained from Waters Corporation (Milford, MA). SRM 3280 was obtained from the Standard Reference Materials Group at NIST. Each tablet was formulated as a single-unit daily dosage with a nominal tablet weight of 1500 mg. Each tablet contained 13 vitamins, including biotin, and 18 elements. SRM 3280 has been prepared as part of a collaborative effort between NIST and the National Institutes of Health's Office of Dietary Supplements (NIH/ODS) in support of the Dietary Supplement Ingredient Database that is being produced by the U.S. Department of Agriculture (*28*, *29*). SRM 3280 was prepared as a noncommercial batch of multivitamin/multielement tablets using a normal manufacturing procedure. Because some of the individual vitamins are coated or encapsulated to provide stability, and grinding would compromise this coating, the SRM will be provided in bottles containing 30 whole tablets. Because between-tablet homogeneity is not expected, it is necessary to grind (homogenize) several tablets and then remove a test portion for analysis. Although long-term storage of the ground tablets is not recommended, biotin levels in ground samples were observed to be stable for at least 24 h when the samples were stored at room temperature. Purified water (18 MΩ), prepared using a Millipore Milli-Q purification system, was used in all preparations. All other chemical reagents and solvents were ACS reagent-grade unless stated otherwise.

Reagent concentrations given in terms of percent (%) are to be considered as mass fractions (g/g) in all listed procedures. Preparation of analyte stocks/standards, samples, and calibrants were performed gravimetrically in all listed procedures, except where noted otherwise.

The LC methods described in this report for the quantification of biotin in multivitamin/multielement supplements are specific for supplements that do not contain yeast, yeast extracts, or liver extracts. Yeast, yeast extracts, and liver extracts contain small but significant amounts of biotin covalently bound to protein (*16*). Multivitamin/multielement supplements which contain yeast or liver must undergo alternative extraction procedures, for example, acid hydrolysis, to release the protein−biotin complex.

Methods. *Isotope-Dilution LC/MS.* (1) LC/MS Calibrants. Five separate biotin stock solutions (10 000 μg/mL) were prepared in DMSO. Each biotin stock solution was further diluted by a factor of 100 to 100 μg/mL with 1.5% formic acid solution. One stock solution (10 000 μg/mL) of the internal standard ([$^2H_2$]-biotin) was prepared in DMSO. The [$^2H_2$]-biotin stock solution was further diluted by a factor of 100 to 100 μg/mL with 1.5% formic acid solution. Individual calibrants were prepared by weighing discrete volumes of each of the five diluted biotin stock solutions and constant volumes of the diluted [$^2H_2$]-biotin stock solution into weighed portions (10 mL) of 1.5% formic acid solution. The nominal analyte concentrations corresponded to 3, 4, 5, 6, and 7 μg/mL for biotin. The nominal concentration of [$^2H_2$]-biotin in each of the calibrants was 3 μg/mL. The calibrants were stored at room temperature (25 °C) until needed.

(2) LC/MS Linearity Standards. An internal standard stock solution (10 mL) containing approximately 3 μg/mL [$^2H_2$]-biotin in 1.5% formic acid was prepared in a glass vial. A stock solution (1 mL) containing a biotin concentration of 495 μg/mL was prepared using the 3 μg/mL [$^2H_2$]-biotin solution as diluent. A set of 19 volumetric serial dilutions was prepared from the stock solution covering a biotin concentration range from 0.002 μg/mL to 495 μg/mL using the 3 μg/mL [$^2H_2$]-biotin stock solution as diluent. Each standard was analyzed using the LC/

Table 1. MS Instrument Parameters[a]

parameter	biotin/[$^2H_2$]-biotin
SIM ions (*m/z*)	245/247
fragmentor voltage (V)	120
capillary voltage (V)	4500
dwell time (ms/ion)	199
drying gas temperature (°C)	350
drying gas flow rate (L/min)	13
nebulizer pressure (kPa)	276

[a] Testing was conducted with SRM 3280.

MS method to estimate the method's linear range, limit of detection (LOD), and limit of quantification (LOQ).

(3) LC/MS Sample Analysis. A bottle of the SRM was opened, and half the content (15 tablets) was homogenized using an automatic mortar grinder. A 1500-mg sample was weighed into a 15-mL plastic centrifuge tube and was spiked with 300 μL of [$^2H_2$]-biotin (100 μg/mL). The sample was diluted with 10 mL of methanol, was vortex mixed, and was subjected to heated (60 °C) sonication for 5 min. The sample was then centrifuged at 3000*g* for 10 min, and the supernatant was removed and transferred to a clean 15-mL centrifuge tube. One milliliter of the supernatant was filtered through a regenerated cellulose filter (0.45-μm pore) directly into a sample vial.

Sample extracts and calibrants were injected (10 μL) onto the LC/MS system. Analyte/internal standard peak area ratios (area/area) and mass ratios (mg/mg) were subjected to linear least-squares regression analysis to produce calibration curves (*y*-intercept model) and calibration equations. Analytes in the sample extracts were quantified on the basis of the relevant calibration equation and the analyte/internal standard peak area response ratio detected in the sample extract. Samples and calibrants were injected (one time) and analyzed using the following analysis sequence: calibrant 1−5, sample, sample, sample, and so forth.

(4) LC/MS Instrumentation. Experiments were conducted on an HP1100 Series LC system coupled to an Agilent single quadrupole MS system operating in positive electrospray-ionization (ESI) mode. The LC system was outfitted with a binary pump, a variable wavelength UV absorbance detector, and an in-line mobile-phase vacuum degasser. Samples were analyzed using a Waters XTerra RP18 C_{18} analytical column (4.6 mm × 150 mm, 5-μm particle diameter) with an attached XTerra RP18 C_{18} guard column (3 mm × 20 mm, 5-μm particle diameter) held at 30 °C ± 1 °C. The isocratic LC elution conditions were as follows (solvent percentages are volume fractions): mobile phase A = 0.1% formic acid in water; mobile phase B = 0.1% formic acid in methanol; A/B = 77%/23% for 25 min; flow rate = 350 μL/min. Biotin and [$^2H_2$]-biotin were detected and quantified using selected ion monitoring (SIM) of the protonated analyte molecules $[M + H]^+$. MS operating parameters are summarized in **Table 1**.

LC/ELSD. (1) LC/ELSD Calibrants. For the LC/ELSD method, analyte (biotin) and internal standard (desthiobiotin, **Figure 1C**) stock and diluted stock solutions were prepared as previously described for the LC/MS stock solutions. Similarly, five individual calibrant solutions were prepared in 1.5% formic acid using appropriate dilutions of the biotin and desthiobiotin stock solutions. The nominal analyte concentrations corresponded to 10, 12, 16, 20, and 22 μg/mL for biotin. The nominal concentration of desthiobiotin in each of the calibrants was 12 μg/mL. The calibrants were stored at room temperature (25 °C) until needed.

(2) LC/ELSD Linearity Standards. The linear range, LOD, and LOQ of the LC/ELSD method were estimated using a serial dilution procedure similar to that previously described for the LC/MS method. The only differences in the procedures involved the use of desthiobiotin (12 μg/mL) instead of [$^2H_2$]-biotin as the internal standard diluent and the reduced breadth of the dilution range (12 volumetric serial dilutions covering a biotin concentration range from 0.242 μg/mL to 495 μg/mL).

(3) LC/ELSD Sample Analysis. A bottle of the SRM was opened, and half the content (15 tablets) was homogenized using an automatic mortar grinder. A 1500-mg sample was weighed into a 15-mL plastic centrifuge tube and was spiked with 400 μL of desthiobiotin (100 μg/mL). The sample was diluted with 10 mL of 1.5% formic acid, was vortex mixed, and was subjected to mechanical shaking for 30 min. The sample was centrifuged at 3000*g* for 10 min, and the supernatant was removed and transferred to a clean 15-mL centrifuge tube. The supernatant was immediately subjected to SPE.

Biotin was extracted from the supernatant using disposable C_{18} SPE cartridges as follows (all extractions were conducted with a manual vacuum-manifold system): (1) the cartridge was preconditioned by rinsing with 3 mL of each of the following in sequence: methanol, water, 1.5% formic acid solution; (2) the sample (3 mL) was applied to the cartridge and was pulled through under a light vacuum (2 min); (3) the cartridge was washed with 6 mL of 1.5% formic acid solution; and finally, (4) biotin/desthiobiotin were eluted by rinsing the cartridge with 1 mL of 1.5% formic acid in 50/50 water/methanol solution.

Sample extracts and calibrants were injected (40 μL) in duplicate onto the LC/ELSD system. Analyte/internal standard peak area ratios (log converted) and mass ratios (log converted) were subjected to linear least-squares regression analysis to produce the calibration curves and calibration equations. A zero-intercept calibration model was employed for all calculations. Because the light-scattering phenomenon is a nonlinear process and does not obey Beer's Law, the resulting data must be logarithmically transformed to produce a linear calibration curve. This type of ELSD data treatment is well established in the literature (*30*−*33*). Analytes in the sample extracts were quantified on the basis of the relevant calibration equation and the analyte/internal standard peak area response ratio detected in the sample extract.

Samples and calibrants were injected and analyzed on the basis of the following sequence: calibrant 1−5, sample, sample, sample, and so forth, calibrant 1−5, sample, sample, sample, and so forth.

(4) LC/ELSD Instrumentation. Experiments were conducted on a modular LC system coupled to an ELSD instrument. The LC system consisted of a Varian 9012 ternary LC pump and a BioRad AS-100 autosampler. The ELSD detector was a SEDEX 75 detector. Samples were analyzed using a Supelcosil LC-CN cyanopropyl analytical column (4.6 mm × 250 mm, 5-μm particle size) with an attached Supelcosil LC-CN guard column (3 mm × 20 mm, 5-μm particle size) operated at room temperature. The isocratic LC elution conditions were as follows (solvent percentages are volume fractions): mobile phase A = 0.1% formic acid in water; mobile phase B = 0.1% formic acid in methanol; A/B = 95%/5% for 15 min; flow rate = 1 mL/min. Biotin and desthiobiotin were detected using the following ELSD detector settings: gain = 7, temperature = 50 °C, nitrogen gas pressure = 300 kPa (3.0 bar).

RESULTS AND DISCUSSION

LC/MS Method Development. Biotin is not soluble in most common organic solvents and is only weakly soluble in water (≈220 μg/mL) and alcohols (≈800 μg/mL) (*34*). However, biotin is strongly soluble in the polar aprotic solvent, DMSO. Therefore, all biotin stock solutions were initially prepared in pure DMSO and then diluted, as required, with 0.1% formic acid in 50/50 water/methanol to prepare standards for ESI MS characterization. Flow injection analysis of biotin standards using positive-ion mode ESI was characterized by the presence of intense protonated molecules ($[M + H]^+$, *m/z* 245) in MS mode. Collision-induced dissociation of the protonated molecules in MS/MS mode produced weak fragment ions ($[M + H - H_2O]^+$, *m/z* 227) because of the nonspecific loss of water from the protonated molecules. MS sensitivity for biotin using negative-ion ESI was significantly reduced compared to the sensitivity observed for positive-ion mode ESI. On the basis of these results, a positive-ion mode ESI LC/MS method for biotin, along with the deuterated internal standard, [$^2H_2$]-biotin, was developed and optimized in SIM mode (see Experimental Procedures) using the abundant protonated molecules.

Extraction Optimization for LC/MS Analysis. Extraction of biotin from multivitamin/multielement tablets requires ap-

propriate selection and optimization of tablet solubilization conditions. Four different test solvents (water, 1.5% formic acid, methanol, ethanol) were selected, and their relative extraction efficiency for biotin was determined. The relative extraction efficiency of the test solvents was determined by adding a known amount of [$^2H_2$]-biotin to homogenized tablet samples and monitoring the resulting biotin/[$^2H_2$]-biotin area ratio in fully processed samples; the amount of [$^2H_2$]-biotin added to each sample was held constant. This extraction test procedure assumed 100% recovery of [$^2H_2$]-biotin. Test samples were extracted with each solvent using the conditions described in the Experimental Procedures section. Samples were extracted using the following sonication time periods: 0, 5, 10, 15, 30, 45, and 60 min. All sample extracts produced equivalent area ratios (within experimental error) during all sonication time periods indicating that the tested solvents were equally effective in extracting biotin from homogenized tablets. However, the water extract had a broad, unknown peak that eluted before biotin. This peak tended to grow larger and broader with extraction (sonication) time until the peak coeluted with the biotin peak. The ethanol extraction solvent also had limited utility because of the formation of skewed chromatographic peaks. On the basis of the limitations of water and ethanol, methanol and 1.5% formic acid were deemed more acceptable solvents for the extraction of biotin from the tablets. A time of 5 min was selected as the default time period for sample sonication.

Additional testing was conducted to verify complete extraction of biotin from tablets. Samples were continuously extracted with methanol and 1.5% formic acid for 45 h on a rotational mixer. There were no significant differences between the 5 min or 45 h biotin/[$^2H_2$]-biotin area ratios indicating that complete extraction of biotin was achieved within the nominal 5-min extraction period using either 1.5% aqueous formic acid or methanol. Methanol was selected as the optimal extraction solvent for the LC/MS method because of its volatility and inherent compatibility with ESI MS and also because it did not produce any interfering peaks. Aqueous formic acid was subsequently utilized as the comparative extraction solvent for the LC/ ESLD method (see below).

LC/ELSD Method Development. To establish an independent means for quantifying biotin levels in multivitamin/multielement tablets, an alternative chromatographic method based on the use of a cyanopropyl LC column coupled with ELSD was developed. Analytes amenable to sensitive detection by evaporative light scattering must be inherently less volatile than the LC mobile phase. Preliminary investigations utilizing the LC mobile phase formulated for the previously described LC/MS method along with biotin standards dissolved in the mobile phase showed this to be valid for biotin. However, the principle of isotope-dilution quantification is not applicable to ELSD detection, and hence it became necessary to screen a variety of biotin analogues to serve as potential internal standard compounds. Stable biotin analogues such as biocytin, biotin-4-fluorescein, biotin methyl ester, and desthiobiotin (**Figure 1C**) were tested using the preliminary LC/ELSD method, and the best response, as far as detection sensitivity and chromatographic separation were concerned, was observed for desthiobiotin. Desthiobiotin was thus selected as the biotin analogue for further method development. ELSD instrument conditions (gain setting, evaporator temperature, and gas flow rate) were optimized for both biotin and desthiobiotin at this point (see Experimental Procedures section); however, fine-tuning of the LC separation conditions was not attempted until after determination of the optimal tablet extraction/biotin purification conditions.

Extraction Optimization for LC/ELSD Analysis. To impart distinct analytical independence from the LC/MS extraction procedure, tablets for LC/ELSD biotin analysis were extracted using 1.5% formic acid instead of methanol. Further, the solubilized tablets were subjected to mechanical shaking instead of heated sonication during the extraction procedure. The appropriate length of time for extracting samples was determined via LC/MS using timed (0, 15, 30, 45, 60 min) extraction studies of samples spiked with [$^2H_2$]-biotin. The biotin/[$^2H_2$]-biotin extraction ratio for mechanical shaking extraction was maximal and stable from the 30-min time point onward; hence, 30 min was selected as the default extraction period.

However, direct LC/ELSD analysis of biotin in the solubilized tablets was not possible because of the presence of high levels of ELSD responsive compounds that coeluted with biotin. Cleanup of the tablet extracts was therefore required and efforts were focused on developing an efficient SPE procedure for this purpose. Five different SPE sorbents (NH_2, CN, C_{18}, C_8, diol) were tested using the elution conditions described in the Experimental Procedures section. The best results (minimal interfering peaks, good peak shape for biotin, strong ELSD response for biotin following sample elution) were achieved via sample purification with the C_{18} and C_8 sorbents. The NH_2, CN, and diol sorbents did not adequately retain biotin under the tested extraction conditions. The C_{18} sorbent was selected as the optimal sorbent for sample purification.

SPE recovery efficiencies for both biotin and desthiobiotin from solubilized tablets were individually determined on the basis of spiking experiments. Biotin and desthiobiotin were separately spiked into homogenized tablet samples, extracted, and analyzed via LC/ELSD according to the procedures described in the Experimental Procedures section. The amount of analyte (ng on-column) detected was determined by comparing the signal responses from the extracted samples to the analyte signal responses from independently prepared calibrants. The overall recovery (mean ± %RSD) of biotin added and extracted from tablet samples ($N = 3$) at three different levels (5, 10, 15 μg) was 108 ± 3%. The overall recovery (mean ± %RSD) of desthiobiotin added and extracted from tablet samples ($N = 3$) at three different levels (22, 27, 32 μg) was 121 ± 3%. The relative equivalency between the biotin (108%) and desthiobiotin (121%) recovery determinations indicates that the SPE of biotin and desthiobiotin from the tablet matrix is quantitative and equally selective for both analytes.

Complete extraction of biotin from tablets was verified via LC/MS experiments by comparing the biotin/[$^2H_2$]-biotin area ratio observed with 30 min of mechanical shaking in 1.5% formic acid against the area ratio observed with 45 h of continuous rotational mixing in 1.5% formic acid. There were no significant differences between the 30-min or 45-h biotin/[$^2H_2$]-biotin area ratios indicating that complete extraction of biotin from the tablet matrix was achieved within the nominal 30-min extraction period. The LC/ELSD method was thus finalized to include tablet extraction using 30 min of mechanical shaking and 1.5% formic acid.

Comparison of Method Performance Characteristics. The detection and quantification characteristics for each chromatographic method were independently established using serially prepared biotin linearity standards (see Experimental Procedures section). Results indicated (**Table 2**) that the two methods had substantially different analytical performance characteristics. The MS response for biotin was linear over more than 3 orders of magnitude while the ELSD response was only linear over 1.5 orders of magnitude. The ELSD linear response range is limited,

Table 2. Comparison of Method Performance Characteristics

analytical parameter	biotin (ng) by LC/MS	biotin (ng) by LC/ELSD
linear dynamic range[a]	0.02–77[b]	155–9900[c]
LOD[a,d]	0.02	155
LOQ[a,e]	0.06	310

[a] Determined via analysis of serially diluted calibrants as described in the Experimental Procedures section. All biotin values are masses of analyte injected on-column. [b] Calculated $r^2 = 0.998$, slope = 1.0559 (0.0151), *y*-intercept = 0.0177 (0.0125), SE estimate for regression line = 0.0395. [c] Calculated $r^2 = 0.996$, slope = 1.6794 (0.0465), *y*-intercept = –1.6293 (0.0747), SE estimate for regression line = 0.0741. Values in () signify the standard error (SE). [d] The LOD is the minimum detectable analyte signal that is at least 3 times the noise signal. [e] The LOQ is calculated by multiplying the LOD by a factor of 3 for the LC/MS method and by a factor of 2 for the LC/ELSD method. The S:N ratio for biotin at the listed LOQ is ≥10 for both analytical methods.

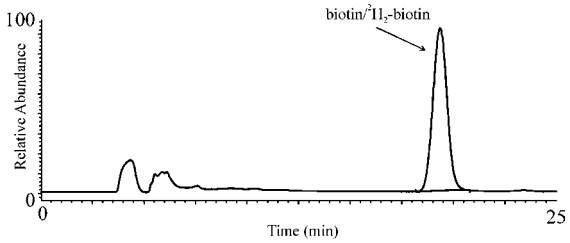

Figure 2. Representative total ion current chromatogram of biotin/[$^2H_2$]-biotin extracted from SRM 3280 multivitamin/multielement tablets using methanol extraction. The profile was collected using the LC/MS conditions described in the Experimental Procedures section.

as previously discussed, because of the inherent nonlinearity of the evaporative light-scattering process (*30*−*33*). Nevertheless, both methods have sufficient detection sensitivity to quantify microgram levels of biotin in multivitamin/multielement tablets. Detailed data regarding the analytical linear dynamic range, LOD, and LOQ sensitivity for each method is provided in **Table 2**.

Quantification of Biotin in NIST SRM 3280. The LC/MS method was cross-validated with the LC/ELSD method by applying each method individually to the quantification of biotin in SRM samples. Twelve randomly selected bottles of the SRM were opened and 15 tablets from each bottle were homogenized and extracted as described in the Experimental Procedures section (six bottles per method). The remaining 15 tablets from each bottle were homogenized on the following day. Two biotin measurements from each of the six bottles was conducted over a period of 2 days.

A characteristic total ion current (TIC) chromatogram from the LC/MS quantification of biotin in the SRM is shown in **Figure 2**. The profile shows all of the ions that were detected during MS analyses of the SRM, and it is clearly apparent that the ion signal from biotin/[$^2H_2$]-biotin is the predominant signal. A representative chromatogram from the LC/ELSD quantification of biotin in the SRM is shown in **Figure 3**. Biotin ($k' = 1.8$) and desthiobiotin ($k' = 2.0$) are both effectively retained and separated from potentially interfering components by the cyanopropyl LC column. Calibrants covering narrow analytical ranges for both the LC/MS (10−70 ng biotin on-column, 30 ng [$^2H_2$]-biotin on-column) and LC/ELSD (400−880 ng biotin on-column, 480 ng desthiobiotin on-column) methods were prepared and utilized for analyte quantification as described in the Experimental Procedures section. LC/MS calibrants demonstrated both good linearity ($r^2 = 0.999$, the relative standard error of the estimate for the regression line was 1.0%) and good analytical sensitivity (slope = 1.02). LC/ELSD calibrants also

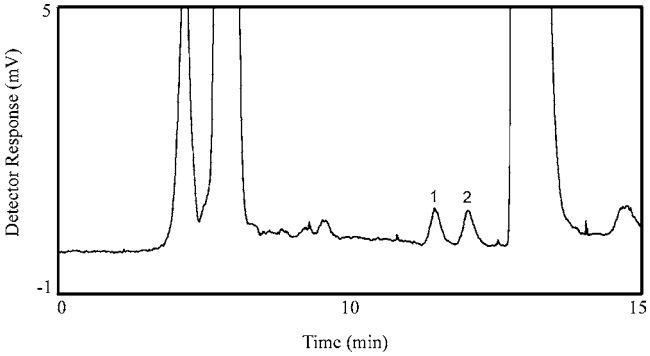

Figure 3. Representative chromatogram of (1) biotin and (2) desthiobiotin extracted from SRM 3280. The profile was collected using the LC/ELSD conditions described in the Experimental Procedures section.

Table 3. Quantification of Biotin in SRM 3280[a]

	bottle #	biotin (mg/kg) by LC/MS	bottle #	biotin (mg/kg) by LC/ELSD
	1	26.39	7	23.09
	2	26.34	8	25.97
	3	26.31	9	26.71
	4	26.26	10	24.83
	5	26.84	11	24.22
	6	26.57	12	23.63
overall mean		26.45		24.74
SD		0.29		1.7
RSD (%)		1.1		6.7
μg biotin/tablet[b]		40		37

[a] All values have been corrected for the consensus purity value (99%) for the d-biotin primary reference standard. Each reported value represents the mean determination from two independent sample preparations. [b] Calculated on the basis of a nominal tablet mass of 1500 mg.

demonstrated good linearity ($r^2 = 0.995$, the relative standard error of the estimate for the regression line was 5.4%) and good analytical sensitivity (slope = 1.73). A slope of 1.73 is not unusual for small molecule analytes using ELSD and is, in fact, concordant with the observed calibration slopes (1.7−1.8) reported by other researchers (*31*, *32*). The exponential relationship between peak area and analyte mass during the light-scattering detection process typically results in calibration slope values between 1 and 2; the theoretical basis for this slope range has been described by Stolyhwo and co-workers (*31*, *32*). Additionally, the calibration slope is usually not constant but depends on the intensity of the scattered light, on the analyte's diameter, and on the design of the light-scattering cell.

Detailed analytical results for the quantification of biotin in SRM 3280 using both chromatographic methods are given in **Table 3**. The precision of each method is excellent as shown by overall %RSDs of less than 7%. The mean biotin levels, 26 mg/kg and 25 mg/kg, determined by LC/MS and LC/ELSD, respectively, differ by approximately 8%. The difference between the two mean biotin levels is not significant at the 95% confidence level. Assessments of the biotin level in SRM 3280 were also conducted by collaborating laboratories utilizing LC/UV ($n = 2$) and LC/MS ($n = 2$) methods. The reported biotin levels (mean ± SD) were 26.5 mg/kg ± 0.71 mg/kg and 24.3 mg/kg ± 1.4 mg/kg for the LC/UV and LC/MS methods, respectively. Both collaborating laboratory determinations are highly concordant with the NIST biotin levels (**Table 3**). Additional assessment of the biotin level in SRM 3280 was conducted by the manufacturer of the tablets using microbiologic assay. The manufacturer's analysis returned a level of 37 μg biotin per tablet which correlates to a relative accuracy of 108%

and 100% for the LC/MS and LC/ELSD methods, respectively. The concordance among the NIST, the collaborating laboratories, and the SRM manufacturer's determinations supports the overall accuracy and reliability of the NIST LC/MS and LC/ELSD methods.

Conclusions. Two isocratic LC methods for the routine quantification of biotin in multivitamin/multielement tablets have been developed and cross-validated using SRM 3280. Results from these methods will be combined with results provided by collaborating laboratories to assign a certified value to biotin in the SRM. The methods represent substantial improvements in specificity over previously reported LC/UV methods utilizing low-wavelength detection and LC/FL methods utilizing post-column derivatization for the detection of biotin. Biotin is detected directly and specifically in tablet extracts on the basis of its electrospray-ionization and evaporative-light-scattering properties, respectively, via LC/MS and LC/ELSD. Additionally, the reported chromatographic methods incorporate the use of stable internal standard compounds throughout all extraction and analysis steps for improved biotin quantification. Analytical characterization of each method indicates that the LC/MS method is far superior to the LC/ELSD method in terms of quantitative sensitivity (LOD, LOQ), however, both methods produce similar quantitative results (**Table 3**). On the basis of the data described in this report, either method alone or both methods in combination could potentially be utilized as reference methods for the quantification of biotin in multivitamin/multielement tablets.

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LITERATURE CITED

(1) Mock, D. M.; Mock, N. I.; Nelson, R. P.; Lombard, K. A. Disturbances in biotin metabolism in children undergoing long-term anticonvulsant therapy. *J. Pediatr. Gastroenterol. Nutr.* **1998**, *26*, 245−250.

(2) Velazquez, A.; Zamudio, S.; Baez, A.; Murguia-Corral, R.; Rangel-Peniche, B.; Carrasco, A. Indicators of biotin status: a study of patients on prolonged total parenteral nutrition. *Eur. J. Clin. Nutr.* **1990**, *44*, 11−16.

(3) Zempleni, J.; Mock, D. M. Biotin biochemistry and human requirements. *J. Nutr. Biochem.* **1999**, *10*, 128−138.

(4) Zempleni, J.; Mock, D. M. Marginal biotin deficiency is teratogenic. *Proc. Soc. Exp. Biol. Med.* **2000**, *223*, 14−21.

(5) Mock, D. M. Marginal biotin deficiency is teratogenic in mice and perhaps humans: a review of biotin deficiency during human pregnancy and effects of biotin deficiency on gene expression and enzyme activities in mouse dam and fetus. *J. Nutr. Biochem.* **2005**, *16*, 435−437.

(6) Mock, D. M.; Henrich, C. L.; Carnell, N.; Mock, N. I. Indicators of marginal biotin deficiency and repletion in humans: validation of 3-hydroxyisovaleric acid excretion and a leucine challenge. *Am. J. Clin. Nutr.* **2002**, *76*, 1061−1068.

(7) Mock, D. M.; Quirk, J. G.; Mock, N. I. Marginal biotin deficiency during normal pregnancy. *Am. J. Clin. Nutr.* **2002**, *75*, 295−299.

(8) Fernandez-Mejia, C. Pharmacological effects of biotin. *J. Nutr. Biochem.* **2005**, *16*, 424−427.

(9) Cicero, A. F. G.; Derosa, G.; Gaddi, A. What do herbalists suggest to diabetic patients in order to improve glycemic control? Evaluation of scientific evidence and potential risks. *Acta Diabetol.* **2004**, *41*, 91−98.

(10) Department of Health and Human Services Office of the Inspector General. Dietary Supplement Labels: An Assessment. http://www.oig.hhs.gov/oei/reports/oei-01-01-00121.pdf, accessed May 2006.

(11) Yates, A. A.; Schlicker, S. A.; Suitor, C. W. Dietary reference intakes: the new basis for recommendations for calcium and related nutrients, B vitamins, and choline. *J. Am. Diet. Assoc.* **1998**, *98*, 699−706.

(12) Livaniou, E.; Costopoulou, D.; Vassiliadou, I.; Leondiadis, L.; Nyalala, J. O.; Ithakissios, D. S.; Evangelatos, G. P. Analytical techniques for determining biotin. *J. Chromatogr., A* **2000**, *881*, 331−343.

(13) Indyk, H. E.; Evans, E. A.; Caselunghe, M. C. B.; Persson, B. S.; Finglas, P. M.; Woollard, D. C.; Filonzi, E. L. Determination of biotin and folate in infant formula and milk by optical biosensor-based immunoassay. *J. AOAC Int.* **2000**, *83*, 1141−1148.

(14) Reyes, F. D.; Romero, J. M. F.; de Castro, L. Determination of biotin in foodstuffs and pharmaceutical preparations using a biosensing system based on the streptavidin-biotin interaction. *Anal. Chim. Acta* **2001**, *436*, 109−117.

(15) Holler, U.; Wachter, F.; Wehrli, C.; Fizet, C. Quantification of biotin in feed, food, tablets and premixes using HPLC/MS/MS. *J. Chromatogr., B* **2006**, *831*, 8−16.

(16) Tolaymat, N.; Mock, D. M. Biotin analysis of commercial vitamin and other nutritional supplements. *J. Nutr.* **1989**, *119*, 1357−1360.

(17) Mock, D. M.; Duboist, D. B. A sequential solid-phase assay for biotin in physiologic fluids that correlates with expected biotin status. *Anal. Biochem.* **1986**, *153*, 272−278.

(18) Groningsson, K.; Jansson, L. TLC determination of biotin in a lyophilized multivitamin preparation. *J. Pharm. Sci.* **1979**, *68*, 364−366.

(19) Postaire, E.; Cisse, M.; Hoang, L.; Pradeau, D. Simultaneous determination of water soluble vitamins by over pressure layer chromatography and photodensitometric detection. *J. Pharm. Sci.* **1991**, *80*, 368−370.

(20) Crivelli, S. L.; Quirk, P. F.; Steible, D. J.; Assenza, S. P. A reversed phase high performance liquid chromatographic (HPLC) assay for the determination of biotin in multivitamin-multimineral preparations. *Pharm. Res.* **1987**, *4*, 261−262.

(21) Ekpe, A. E.; Hazen, C. Liquid chromatographic determination of biotin in multivitamin-multimineral tablets. *J. Pharm. Biomed. Anal.* **1998**, *16*, 1311−1315.

(22) Heudi, O.; Kilinc, T.; Fontannaz, P. Separation of water-soluble vitamins by reversed-phase high performance liquid chromatography with ultra-violet detection: application to polyvitaminated premixes. *J. Chromatogr., A* **2005**, *1070*, 49−56.

(23) Hudson, T. S.; Subramanian, S.; Allen, R. J. Determination of pantothenic acid, biotin, and vitamin B12 in nutritional products. *J.—Assoc. Off. Anal. Chem.* **1984**, *67*, 994−998.

(24) Lahely, S.; Ndaw, S.; Arella, F.; Hasselmann, C. Determination of biotin in foods by high performance liquid chromatography with post-column derivatization and fluorimetric detection. *Food Chem.* **1999**, *65*, 253−258.

(25) Nojri, S.; Kamata, K.; Nishijima, M. Fluorescence detection of biotin using post-column derivatization with OPA in high performance liquid chromatography. *J. Pharm. Biomed. Anal.* **1998**, *16*, 1357−1362.

(26) Kamata, K.; Hagiwara, T.; Takahashi, M.; Uehara, S.; Nakayama, K.; Akiyama, K. Determination of biotin in multivitamin pharmaceutical preparations by high-performance liquid chromatography with electrochemical detection. *J. Chromatogr.* **1986**, *356*, 326−330.

(27) Chen, Z.; Chen, B.; Yao, S. High performance liquid chromatography/electrospray ionization mass spectrometry for simultaneous determination of taurine and 10 water-soluble vitamins in mulitivitamin tablets. *Anal. Chim. Acta* **2006**, *569*, 169−175.

(28) Dwyer, J. T.; Picciano, M. F.; Betz, J. M.; Fisher, K.; Saldanha, L. G.; Yetley, E. A.; Coates, P. M.; Radimer, K.; Bindewald, B.; Sharpless, K. E.; Holden, J.; Andrews, K.; Zhao, C.; Harnley, J.; Wolf, W.; Perry, C. Progress in development of dietary supplement ingredient databases at the NIH office of dietary supplements. *J. Food Compos. Anal.* **2006**, *19*, S108−S114.

(29) Holden, J. M.; Andrews, K. W.; Zhao, C.; Schweitzer, A. L.; Perry, C. R.; Harnly, J.; Wolf, W.; Dwyer, J. T.; Picciano, M. F.; Betz, J. M.; Saldanha, L. G.; Yetley, E.; Fisher, K.; Sharpless, K. E.; Radimer, K.; Bindewald, B. Dietary supplement ingredient database project: protocol development. *FASEB J.* **2005**, *19*, A86−A86.

(30) Elfakir, C.; Lafosse, M.; Dreux, M. Optimization of high performance liquid chromatographic analysis with UV detection: light scattering detection to establish the coelution of UV and non-UV absorbing constituents. *J. Chromatogr.* **1990**, *513*, 354−359.

(31) Stolyhwo, A.; Colin, H.; Guiochon, G. Use of light scattering as a detector principle in liquid chromatography. *J. Chromatogr.* **1983**, *265*, 1−18.

(32) Stolyhwo, A.; Colin, H.; Martin, M. J.; Guiochon, G. Study of the qualitative and quantitative properties of the light scattering detector. *J. Chromatogr.* **1984**, *288*, 253−275.

(33) Young, C. S.; Dolan, J. W. Success with evaporative light scattering detection. *LC−GC* **2003**, *21*, 120−128.

(34) Budavari, S. *The Merck Index*, 12th ed.; Merck & Co.: Whitehouse Station, NJ, 1996; p 810.

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