method. As shown in the table, there is no statistical difference between the methods with respect to the means and variances.

After the HCl–citric acid solution is added to the crushed tablet, a pH of 0.9–1 is observed. Slight effervescence occurs but subsides in less than 1–2 min. The extraction of the undissociated salicylic acid and aspirin by chloroform is then accomplished. The aspirin and salicylic acid are extracted from the chloroform solution by the buffer solution. The whole experiment takes about 10 min.

To ensure complete extraction of salicylic acid and aspirin into the chloroform solution, some of the aqueous phase was treated with 1 *N* NaOH and tested for the absence of salicylate fluorescence. Also, to be assured of complete extraction of salicylic acid from the chloroformic solution, some of the organic phase was run on the spectrophotometer to observe the absence of salicylic acid.

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Gas-Liquid Chromatography of *d*-Biotin

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Abstract □ A rapid and specific gas chromatographic method has been developed for the detection and determination of *d*-biotin. This technique has been found applicable to agricultural premixes and pharmaceutical injectable preparations. After suitable preliminary extraction, the biotin silyl ester is prepared using bis-(trimethylsilyl)acetamide (BSA) reagent. The silyl ester of *d*-biotin exhibited good peak symmetry and a linear response when utilizing a hydrogen flame ionization detector with a 2% OV-17 column operated at a temperature of 190°. The conditions for the assay of *d*-biotin in several preparations are described employing *n*-octacosane as the internal standard. The standard deviation of the developed procedure under the conditions studied was ±2.7%.

Keyphrases □ *d*-Biotin—determination □ Parenterals, agricultural premixes—*d*-biotin analysis □ *n*-Octacosane—internal standard □ GLC—analysis

d-Biotin is widely distributed in animals and plants and was first isolated from egg yolk (1). It is required in comparatively small amounts for the growth of bacteria, plants, and animals and appears to be related to the process of cell development. In 1940, György *et al.* (2) published their work on the identity of biotin. In the following 2 years, du Vigneaud *et al.* (3, 4) established the empirical and structural formulas. In 1943, Harris *et al.* (5) synthesized *d*-biotin (the naturally occurring form) and in 1949, Goldberg and Sternbach (6) patented their findings of a more economical synthesis.

While several microbiological techniques are currently employed for the determination of *d*-biotin, a comprehensive survey of the literature indicated that GLC had heretofore not been employed for quantitative analysis. A considerable number of satisfactory GLC procedures have been reported for various water-soluble vitamins. These procedures involve the preparation of the appropriate trimethylsilyl derivatives (ester or ether) prior to GLC analysis.

Carboxylic organic compounds, such as biotin, are not sufficiently volatile for direct analysis employing GLC. However, the chemical structure of *d*-biotin indicated the possibility of substituting the active hydrogen of the carboxyl group with a silyl group, thereby making the gas chromatographic analysis possible. Horning *et al.* (7) mentioned the qualitative response of *d*-biotin silyl ester in their study of urinary acids and related compounds.

This paper describes the quantitative GLC determination of *d*-biotin after the conversion to its silyl ester with bis-(trimethylsilyl)acetamide (BSA). The derivative is easily formed and serves very well for quantitative analysis. The linearity of detector response with concentration injected, reproducibility, recovery data, and procedures for handling several types of dosage forms are described.

EXPERIMENTAL

Operational Parameters—The instrument used for this work was a Varian Aerograph model 204 B, equipped with a hydrogen-flame ionization detector. The column used was a stainless steel coil, 121.9 cm. (4 ft.) long and 3 mm. o.d., packed with 2% OV-17 on diatomite (Chromosorb G), AW/HMDS treated, 70/80 mesh. Prior to use, the column was flow conditioned at 225° for 16–20 hr. with a stream of nitrogen. The temperatures were: column, 190° (or adjusted accordingly to obtain a retention time of approximately 19 min. for *n*-octacosane); injector port, 275° with a Pyrex insert; detector, 275°. The flow rates were: carrier gas—nitrogen, 60 ml./min.; detector gas—hydrogen, 30 ml./min.; and air, 300 ml./min. All injections were made using a 10-μl. Hamilton syringe with an injection volume of approximately 5 μl. The instrument was operated at a range of 10 and 32× attenuation (or sufficient to obtain a 50% peak response on the recorder scale). The recorder used was 0–1 mv. (Texas Instrument) with a pen response of 0.4 sec. and a chart speed of 12 in./hr. All peak areas were measured using a disk integrator. Under the conditions stated the relative retention time of *d*-biotin silyl ester is 0.68 with respect to the internal standard, *n*-octacosane, which has a specific retention time of approximately 19 min. A sample chromatogram is shown in Fig. 1.

Reagents and Chemicals—Liquid phase phenylmethylsilicone fluid, OV-17, *n*-octacosane, and bis-(trimethylsilyl)acetamide (BSA) were commercial grade (Applied Science Laboratories, Inc.). The solid support, diatomite (Chromosorb G), AW/HMDS treated, 70/80 mesh, was used as purchased (F & M Scientific Corp.). All solvents were analytical reagent grade, requiring no further purification.

Standard Preparation—*Internal Standard Solution*—Five hundred milligrams of *n*-octacosane, accurately weighed into a 50-ml. volumetric flask, was dissolved and diluted to volume with *n*-hexane.

Reference Standard Solution—An accurately weighed 50-mg. portion of standard *d*-biotin was placed in a 50-ml. volumetric flask and treated with 5.00 ml. of bis-(trimethylsilyl)acetamide reagent. The flask was stoppered and heated on a steam bath with frequent agitation until the reaction was complete and the solution clear (approximately 20–30 min.). The mixture was cooled, 5.00 ml. of internal standard solution added, and the solution diluted to volume with *n*-hexane.

Sample Preparation—*A.* d-*Biotin Premix: 1%*—An accurately weighed portion of premix (equivalent to about 10 mg. of *d*-biotin) was placed in a 50-ml. centrifuge tube. Fifteen milliliters of absolute ethanol was added and the mixture warmed on a steam bath for 10 min., with occasional agitation. The mixture was centrifuged and the clear supernatant liquid transferred to a 125-ml. conical flask without disturbing the residue. The extraction was repeated four more times and the extracts combined. The combined extracts were evaporated to dryness on a steam bath under a stream of nitrogen. Exactly 2.0 ml. of bis-(trimethylsilyl)acetamide reagent was added. The vessel was stoppered and heated on a steam bath for 20–30 min. with frequent agitation. The sample preparation was cooled and 1.00 ml. of internal standard solution (containing 10 mg. of *n*-octacosane) was added. Quantitative transfer of the entire mixture into a 10-ml. volumetric flask was attained with the aid of *n*-hexane and the solution was diluted to volume with *n*-hexane (working sample solution).

B. d-*Biotin Parenteral: 0.05%*—A 20.0-ml. portion of sample preparation was transferred to a 125-ml. conical flask. Concentrated hydrochloric acid, 0.5 ml., and 30 ml. of anhydrous methanol were added. The solution was evaporated to dryness on a steam bath under a stream of nitrogen. Exactly 2.0 ml. of bis-(trimethylsilyl)-acetamide reagent was added; the flask was stoppered and heated on a steam bath for 20–30 min. with frequent agitation. The solution was cooled and 1.00 ml. of internal standard solution (containing 10 mg. of *n*-octacosane) was added. The entire mixture was quantitatively transferred into a 10-ml. volumetric flask with the aid of *n*-hexane and diluted to volume with *n*-hexane.

Standard Calibration and Sample Analysis—Chromatographic preruns of several 5-μl. injections of working standard solution was carried out in order to condition the column to determine the instrument sensitivity and the peak retention times.

Five-microliter volumes of the working standard and sample solutions, equivalent to approximately 5 mcg. of *d*-biotin, were alternately injected into the instrument using the aforementioned conditions. After the elution of the *n*-octacosane peak, the instrument was ready for another injection. Duplicate samples and standards were chromatographed and the respective areas determined from the integrator trace.

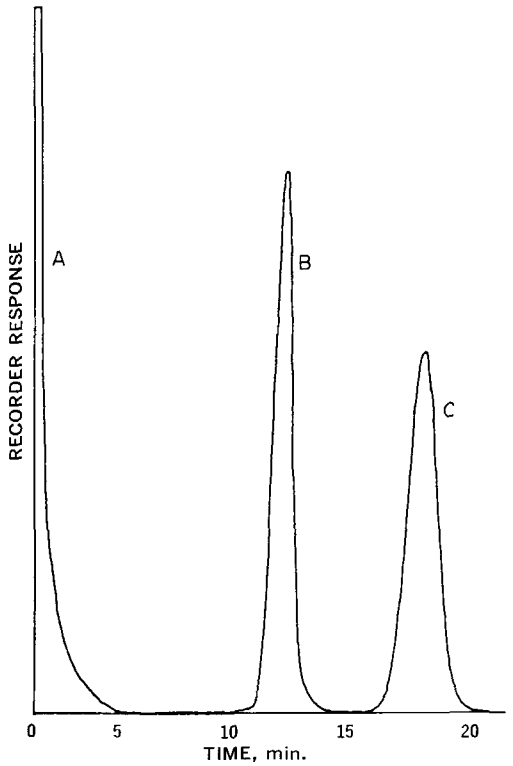


Figure 1—*Standard chromatogram showing relative retention times of biotin TMS derivative and internal standard. Key:* A, *solvent;* B, *biotin TMS; and* C, n-*octacosane.*

Calculations—*Response Factor for Biotin* (RF_B)—

$$RF_B = \frac{A_B\,(\text{std}) \times C_O}{A_O\,(\text{std}) \times C_B}$$

where A_B = peak area of biotin in the working standard, A_O = peak area of *n*-octacosane in the working standard, C_O = mg. of *n*-octacosane per ml. of working standard, and C_B = mg. of biotin per ml. of working standard.

Concentration of Biotin—

$$\frac{A_B\,(\text{spl}) \times C_O \times 10}{A_O\,(\text{spl}) \times RF_B \times \text{spl taken (g. or ml.)}} = \text{mg. biotin/g. (ml.)}$$

where *A* is area, *C* is concentration, and RF_B is the response factor for biotin; 10 = dilution factor.

RESULTS AND DISCUSSION

Hexamethyldisilazane and trimethylchlorosilane in the ratio of 2:1 was found unsuitable for the silylation of biotin, while bis-(trimethylsilyl)acetamide reagent was found satisfactory for complete and rapid silylation. This behavior was also reported by Horning (7). The selection of diatomaceous earth (Gas Chrom G), AW/HMDS treated, was arbitrary and any support properly

Table I—*d*-Biotin Premix Recovery Data

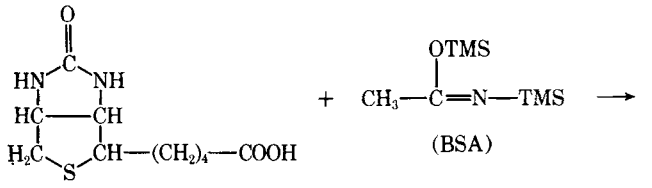
Sample No.	Amount Present, mg./g.	Experimentally Found, mg./g.	Recovery, %
1	10.00	10.05	100.5
2	11.05	10.85	98.2
3	12.05	12.02	99.8
4	13.05	13.25	101.5

Table II—Replicate Recoveries of *d*-Biotin

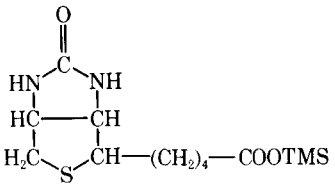
Run No.	Premix, mg./g. Found[a]	Injectable, mg./ml. Found[b]
1	10.37	0.532
2	10.27	0.543
3	9.87	0.547
4	10.23	0.567
5	10.58	0.556
6	10.64	0.573
Mean	10.33	0.553
σ	±0.28	±0.015
ts:95%	±0.71	±0.040

[a] Label claim: 10 mg./g. [b] Label claim: 0.500 mg./ml.

silanized may be suitable for good peak symmetry and quantitative recovery. Results obtained using either copper tubing or stainless steel tubing were equivalent. *d*-Biotin forms a TMS derivative when the carboxyl group exists in the acidic form. The sodium salt of *d*-biotin did not yield a TMS derivative. Therefore, it is assumed that reaction takes place at the carboxyl group to form the biotin silyl ester. The reaction apparent in the derivatization can then take the following form:



biotin



biotin silyl ester

Since the derivatization was shown to occur at the carboxyl center, concentrated hydrochloric acid was added to the parenteral sample prior to derivatization because the *d*-biotin present in the parenteral preparation is in a water-soluble salt form.

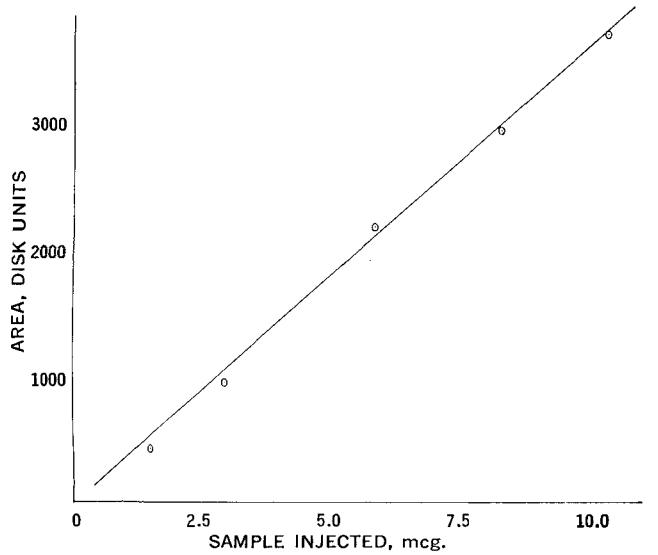


Figure 2—*Linearity of response with concentration. Column: 2% OV-17 on Chromosorb G, length 121.9 cm. (4 ft.); temperature 190°; 5 μl. injected; attenuation 32×. Micrograms of* d-*biotin injected as the silyl ester with the internal standard as a constant.*

Table III—Comparative Analyses of *d*-Biotin in Premix Samples[a,b]

Sample No.	Gas-Liquid Chromatography	Microbiological	Colorimetric (8)
1	9.5	11.7	9.2
2	11.1	11.6	10.8
3	10.9	11.9	10.4
4	10.9	10.4	10.2
5	10.1	10.9	10.2
6	10.2	10.0	9.2
7	10.8	10.4	10.8
8	10.4	10.3	10.5
9	10.7	11.2	10.7

[a] Label claim: 10 mg./g. [b] Results in mg./g.

Linearity data for *d*-biotin–TMS ester *versus* *n*-octacosane as the internal standard are shown in Fig. 2. The minimum detectable level for the *d*-biotin–TMS ester was approximately 0.3 mcg. at range 10, 1X. The recovery of known amounts of *d*-biotin added to a sample premix is given in Table I.

Initially, 5α-cholestane was chosen as the internal standard. However, it had a specific retention time of approximately 28 min. In order to reduce the chromatographic time, *n*-octacosane (C_{28} hydrocarbon), which had a specific retention time of approximately 19 min. under the prescribed conditions, was preferable. In addition, the latter proved more economical.

Six replicates of a premix containing 10 mg. of biotin per gram showed a mean of 10.33 with a relative standard deviation from the mean of ±0.28. The precision (*ts*:95%) was ±0.71 (Table II).

Six replicates of a sample of injectable containing 0.500 mg. of *d*-biotin per milliliter showed a mean of 0.553 mg. with a relative standard deviation from the mean of ±0.015. The precision (*ts*: 95%) was ±0.040 (Table II).

Table III shows the results of analyses of nine samples of *d*-biotin premix by the described GLC method compared to the microbiological assay and to the colorimetric procedure previously reported (8).

Investigation is still being carried out on the application of this procedure towards the determination of *d*-biotin, present in microgram levels, in multivitamin preparations. In these preparations, as well as certain types of premixes, additional suitable isolation techniques may be required in order to remove interfering components.

SUMMARY

A rapid and specific GLC technique has been developed for the detection and determination of *d*-biotin. The method involves the preparation of the TMS ester, addition of *n*-octacosane as an internal standard, and analysis employing a hydrogen-flame ionization detector. Conditions are outlined for the determination of *d*-biotin in premixes and in parenteral preparations.

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