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Note

Response amplification of histidine in gas–liquid chromatographic analysis of amino acid mixtures

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World wide interest in the development and use of new protein sources for food and animal feeds has motivated research to improve methods for analyzing their amino acid compositions, which are commonly used as a criterion of nutritive quality. Amino acid analysis by gas–liquid chromatography (GLC) in recent years, has proved to be quantitatively accurate and as reliable as classical ion-exchange chromatography[1]. For GLC analysis, volatile derivatives of the amino acids, most often the N-trifluoroacetyl (N-TFA)–*n*-butyl esters[2], must be prepared.

One major weakness in the GLC analysis of N-TFA–*n*-butyl esters has been the poor quantitation of the histidine derivative. This difficulty has been reported[3–7] to be caused by: (a) the sensitivity required on the gas chromatograph for analysis of low levels of histidine, (b) the presence of both the monoacyl and diacyl derivatives, (c) the interaction of the derivatives with the stationary phase and (d) lack of separation from one or more other amino acids. Several methods have been reported to solve the histidine quantitation problem[3–7] but all have some weakness. The most recent method involves the use of a mixed phase, OV-17–OV-210, and a Sol-Vent™* device for injecting large volumes of the derivative[3].

In our work on determining the amino acid compositions of leaves and leaf protein concentrates (LPCs), we needed a method that would quantitate low levels of histidine in small samples, require no extra instrumentation and efficiently separate and quantitate the other amino acids. In trying various techniques[4–6,8–10] and column packings, we found other factors which affected the response of the detector to the histidine N-TFA–*n*-butyl ester. These findings should be of value in the development of improved chromatographic systems for the GLC analysis of amino acids from hydrolyzed protein.

EXPERIMENTAL

Apparatus

An Autolab System IVB Chromatography Data Analyzer (Spectra-Physics)

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was used with the following flame-ionization gas chromatographs: (a) a high efficiency Hewlett-Packard Model 7610A and (b) a Packard Becker Model 407. A fluidizer was used to remove solvent from column packings; a thermostat, to control oil baths for derivatization; and an ultrasonic bath, for mixing. Pyrex glass culture tubes with Teflon™-lined screw caps were used for hydrolysis (20 × 150 mm and 25 × 200 mm) and acylation (13 × 100 mm); and samples to be analyzed by GLC were held in microvials with open top caps equipped with Teflon septa.

Reagents

Amino acids were purchased from Mann Research Labs. (New York, N.Y., U.S.A.), or United States Biochemical Corporation (Cleveland, Ohio, U.S.A.). Technical grade *n*-butanol and methylene chloride were distilled in an all-glass system. The 3 *N* HCl–*n*-butanol for esterification was prepared with HCl gas bubbled through cooled *n*-butanol in a nitrogen-purged flask.

Sample preparation

Samples were prepared for chromatographic analyses by the macro method of Roach and Gehrke[11] with this modification: the esterification temperature was 115° instead of 100°. Derivatives of equal molar standard solutions were used for calculating relative molar responses (RMRs).

Columns

The following stationary phases were coated on 100–120 mesh Gas-Chrom Q: 1% SP-2401; 1.5% SP-2401–0.5% OV-7 and 0.5% OV-210–0.5% OV-17–0.4% OV-7. The support and stationary phases were from Applied Science Labs., except the SP-2401, which was from Supelco. All-glass 183 cm × 0.4 cm columns were used. The stationary phase(s) was dissolved in about 50 ml of acetone; and the solution was poured over the solid support, wetting it completely. More acetone was used for quantitative transfer and for making a slurry of the mixture. The slurry was then placed on a hot water bath (80–90°) and stirred with a gentle folding action until it became free flowing from loss of the solvent. Finally residual solvent was removed with a fluidizer heated to about 60° (ref. 12). For conditioning, the columns were heated slowly several times in the gas chromatograph. The column temperature was raised at 1–2.5°/min until it reached the desired upper limit; it was then maintained at that limit for 15 min. The upper limit temperature was established as 5° higher than the temperature at which the last amino acid derivative would be eluted from the column.

Chromatography

Conditions for chromatography included helium carrier gas flow at 60 ml/min, injection port temperatures of 210° and 245° with the SP-2401 column at 245° with the mixed phase columns (1.5% SP-2401–0.5% OV-7 and 0.5% OV-210–0.5% OV-17–0.4% OV-7), and flame-detector temperatures of 250° ± 5°. A 1-min isothermal post injection interval was used followed by programming at 8°/min. Experiments were conducted with different initial column temperatures from 80 to 160°.

Histidine was determined on the mixed phase 1.5% SP-2401–0.5% OV-7 column, by a modification of the technique used by Roach *et al.*[6]. Trifluoroacetyl

anhydride (TFAA) was post injected within 1 min after the N-TFA–*n*-butyl ester injection. On the 1% SP-2401 and the 0.5% OV-210–0.5% OV-17–0.4% OV-7 columns, histidine was determined by direct injection of the N-TFA–*n*-butyl esters. On all three columns the diacyl histidine derivative eluted between aspartic acid and glutamic acid.

RESULTS AND DISCUSSION

In assaying the amino acid compositions of several new protein food sources (leaf protein concentrates prepared at the Mayaguez Institute of Tropical Agriculture, Mayaguez, Puerto Rico), by GLC we found that the results of our GLC method agreed closely with values obtained by ion-exchange chromatography. The precision of the histidine determination was better by GLC than by the ion-exchange method but was still not thoroughly satisfactory. The small detector response of the histidine derivative on the SP-2401 column, and the fact that it sometimes would not separate from glutamic acid led us to explore other columns and methods.

On the SP-2401–OV-7 column the peak for the histidine diacyl derivative could be amplified by the post injection of TFAA. The TFAA caused the on-column acylation of the histidine monoacyl derivative, changing it to the diacyl derivative. The increase in the diacyl histidine response is illustrated in Fig. 1. Further experiments with this technique showed that the extent of the acylation depended upon the column temperature at the time of the TFAA injection. A linear regression plot of the histidine to glutamic acid RMR *versus* the column temperature at the time of TFAA injection is shown in Fig. 2. The coefficient of determination (r^2) for the variation of RMR was 0.98. The extent of acylation varied directly with column temperature between 80 and 160°. When experimental and chromatographic conditions were replicated exactly in standard and sample analyses, histidine could be quantitated

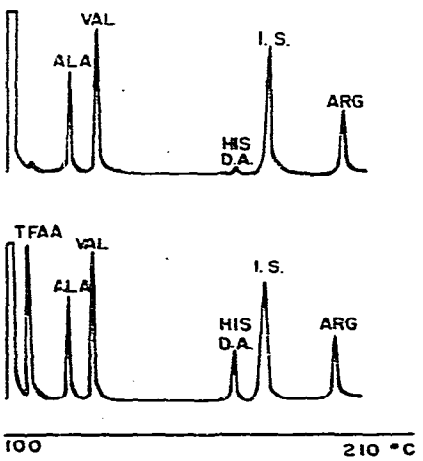

Fig. 1. Chromatograms showing the effect of post injection (20 μl) of TFAA on the response of the diacyl histidine derivative. Sample: N-TFA–*n*-butyl esters of a 2.5 m*M* solution of alanine, valine, histidine, tranexamic acid (I.S.) and arginine; 2 μg histidine injected. Column: 1.5% SP-2401–0.5% OV-7 on Gas-Chrom Q (100–120 mesh). Initial temperature: 100°; 1-min post injection interval programming: 8°/min.

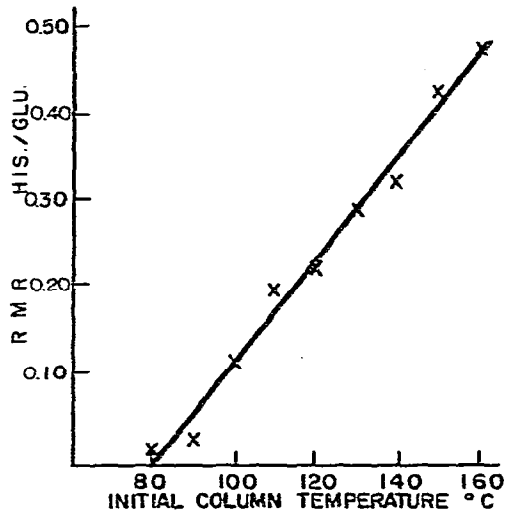

Fig. 2. Initial column temperature *versus* RMR of diacyl histidine N-TFA–*n*-butyl ester. Internal standard: glutamic acid. Each data point represents the mean of three determinations. Injection: 2 μg histidine represented plus post injection of 20 μl TFAA. Column: 1.5% SP-2401–0.5% OV-7.

when injected at any temperature at which a reasonable detector response was achieved, but better precision was achieved above 130°.

We wanted to simplify the technique by eliminating the post sample injection procedure, and therefore attempted to get a good histidine response on a column prepared with a newly selected mixed phase, 0.5% OV-210–0.5% OV-17–0.4% OV-7. On the basis of the previous test we selected an initial column temperature of 140°, and set the injection port at 245° $\pm$ 5°. We determined the smallest amount of histidine that could be reliably measured with that column. Using equal molar standards the RMR was found to be constant for amounts of histidine within the 0.5- to 2.0-μg range and dropped rapidly as the amount of histidine was reduced from 0.5- to 0.2 μg (Fig. 3).

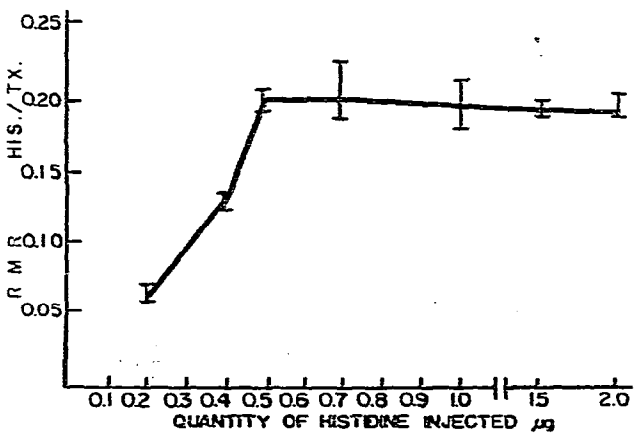

Fig. 3. Change in the RMR with decreasing concentration. Each data point represents the mean of three determinations. Column: 0.5% OV-210–0.5% OV-17–0.4% OV-7 on Gas-Chrom Q (100–120 mesh). Initial temperature 140°; injection port 245°; programming 8°/min.

Previous work[13] showed that the histidine contents of several types of leaf protein concentrates range from 1.1 to 4.3 g/100 g total amino acids recovered. A simulated LPC mixture containing 2% histidine was derivatized and analyzed with the OV-210–OV-17–OV-7 column. Recoveries of histidine were found to be 93 to 100%, confirming the capability of this column to quantitate small amounts of histidine by a standard injection procedure.

In order to test the applicability of the method to other columns, we tested the SP-2401 column with respect to the effect of column temperature and injection port temperature on detector response to histidine. A 2.5 m*M* standard solution of amino acids was derivatized and injected onto the column without post injection of TFAA. We found that for initial temperatures of 80 to 140°, the histidine assays were almost identical to the corresponding assays made with the SP-2401–OV-7 column using the post injection technique. This indicated that as long as excess TFAA was present in the sample, it was the effect of initial column temperature rather than the post injection of TFAA which increased the response. A high injection port temperature (245°) assured the volatilization of the derivative and excess acylating reagent (TFAA) present in the sample. At the initial column temperatures normally used for total amino acid analyses (about 100°) the TFAA is eluted before the column reaches the temperature that is optimum for acylation of the histidine monoacyl derivative to the diacyl derivative. Profiles showing the dramatic increase in the formation of the histidine diacyl derivative with increased temperatures are shown in Fig. 4.

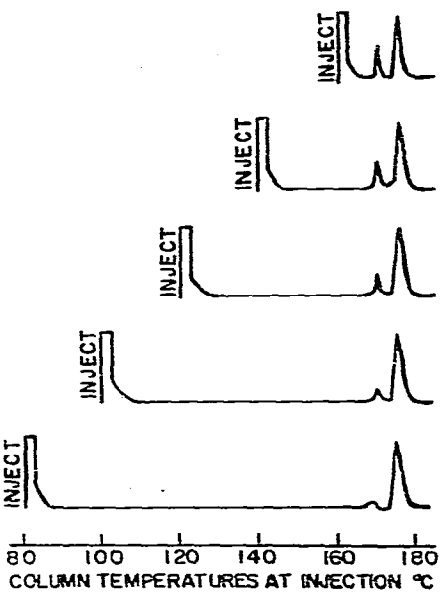

Fig. 4. Chromatograms showing the response of the diacyl histidine derivative at various initial column temperatures. Sample: N-TFA–*n*-butyl esters of histidine and tranexamic acid (I.S.) from a 2.5 m*M* solution; 2 μg histidine injected. Column: 1% SP-2401 on Gas-Chrom Q (100–120 mesh): 1 min post injection interval; programmed at 8°/min.

CONCLUSIONS

The detector response to the histidine N-TFA–*n*-butyl ester(s) can be improved on several GLC columns by providing conditions of chromatography that optimize the elution of the diacyl histidine derivative. The sample should be injected into an all-glass column with an excess of TFAA present, a high injection port temperature (*i.e.*, 245°), and a high column temperature (*e.g.*, 140°).

A method was developed which shows that from 0.5 μg to 2.0 μg of derivatized histidine can be accurately determined with an on-column injection onto a mixed phase 0.5% OV-210–0.5% OV-17–0.4% OV-7 column.

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