

Determination of Amino Acids in Wort and Beer by Reverse-Phase High-Performance Liquid Chromatography¹

H. Garza-Ulloa, R. Garza Cantú, and A. M. Canales Gajá, *Cervecería Cuauhtémoc, S.A., 64000 Monterrey, N.L., México*

ABSTRACT

An analysis of 19 amino acids in wort or beer was based on the precipitation of proteins followed by precolumn derivatization of the amino acids with *o*-phthaldialdehyde/2-mercaptoethanol. Amino acid separation was accomplished by means of a reverse-phase column. A detection limit at picomole level was reached through fluorescence. Variation coefficients for retention times and amino acid concentrations are reported. The results obtained by this method were evaluated in relation to those from a similar method where amino acid separation was accomplished by ion-exchange and postcolumn derivatization.

Key words: Amino acids analysis, Beer, High-performance liquid chromatography, Precolumn derivatization, Wort

An adequate amino acid content in wort is necessary to promote yeast budding and energetic fermentation. It also influences the biological stability and color of the wort and, to a certain extent, the content of higher alcohols and other volatiles that might cause flavor deviations or diminish the quality in beer (10).

Published papers reveal widespread interest in elucidating the complex mechanisms concerning selective absorption of the different amino acids present in wort by the yeast (19) and their relationships as precursors of a given volatile (3,6,18).

Determination of each of the amino acids in wort is of great importance, because yeast metabolizes each one of them in a different manner. Studies by Jones and Pierce classify amino acids according to the "essential" nature of their keto-analogues for yeast metabolism (11,12). Lysine, histidine, arginine, and leucine deficiencies in wort can bring about important changes in yeast-induced metabolism of nitrogen, lowering the ultimate quality of the beer (12,15).

Charalambous (2) reports a compilation of the diverse methods employed for selective determination of amino acids in beer. Most of them have such limitations as time-consuming analyses, complicated derivatization procedures, and costly and sophisticated instrumentation.

Among instrumental analysis techniques recently applied in the brewing lab, high-performance liquid chromatography (HPLC) represents an important contribution to the study of particular problems in beer production. However, despite its wide range of applications in brewing (4), references concerning analysis of amino acids are few (21), and although some preliminary studies on separation are reported, they include qualitative but not quantitative data.

The HPLC system has found its major application in the analysis of nonvolatile compounds such as amino acids, which are difficult to analyze effectively by other analytical systems. Many papers have been published on the application of the HPLC system to amino acid analysis in other fields. The two most widely used chromatography techniques are with precolumn derivatization and reverse-phase column or with postcolumn derivatization and ion-exchange column (20).

Under HPLC, amino acids are usually derivatized for easier detection (13), because for the most part they are weak chromogens in the UV-vis region; they are not detected in their normal concentration levels by the refraction index.

The same classic separation principle utilizing ion exchange (17), which has been applied for more than 25 years to amino acid analysis by chromatography of liquids, has been adapted to the HPLC system. Determination is done by means of ion-exchange chromatography conjointly with postcolumn derivatization, using ninhydrin (5) or a fluorogenic reagent (5,8).

Several precolumn derivatization techniques have been applied to HPLC systems, using a reverse-phase column for separation. Two of the most widely used procedures are formation of derivatives with dansyl (23), or *o*-phthaldialdehyde/2-mercaptoethanol (OPA/2-ME) (7,22). Nevertheless, dansyl derivatives do not show enough specificity because they generate multi-derivates for several amino acids, making quantitation difficult (7).

Precolumn derivatization with OPA/2-ME offers advantages in determining amino acids through the HPLC reverse-phase column system as it forms low-polarity products and reaction occurs in an aqueous medium, is quick at room temperature, is reproducible and quantitative, and generates highly fluorescent derivatives.

When 2-ME is present, OPA reacts with primary amino acids to form substituted isoindole products (Fig. 1). As OPA does not react with secondary amines, the amino acids proline and hydroxyproline are not detected directly and it becomes necessary to first oxidize them with hypochlorite (8).

The purpose of this study was to develop a simple and inexpensive method for the determination of free primary amino acids in wort and beer. The HPLC system utilizing precolumn derivatization with OPA/2-ME and reverse-phase column separation fulfilled this purpose.

Because the application of this method to the analysis of amino acids in wort and beer has not been described in the brewing literature, its evaluation for that purpose was deemed worthwhile.

EXPERIMENTAL

Equipment

The HPLC system by gradient was a 344 Beckman (Beckman Instruments Inc., Berkeley, CA 94710); the injector was an Altex 210 (Altex Scientific Inc., Berkeley, CA 94710), and the detector was a model 3301 Spectra/Glo fluorometer (Gilson Medical Electronic Inc., Middleton, WI 53562) equipped with 360-nm excitation filter and a 455-nm emission filter. A Varian 4270 (Varian, Palo Alto, CA 94303) was utilized as a data system. The column was 150 × 4.6 mm (i.d.), prepacked with a 5- μ m diameter ODS ultrasphere (Altex Scientific, Inc.) joined to a 50 × 2 mm precolumn packed with a 25–37 μ m diameter VYDAC-RP (The Separations Group, Hesperia, CA 92345).

Reagents and Chemicals

The solution of amino acid standards (2.5 μ mol/ml) was from Pierce Chemical Co. (Standard H., no. 20089, Pierce Chemical Co., Rockford, IL 61105).

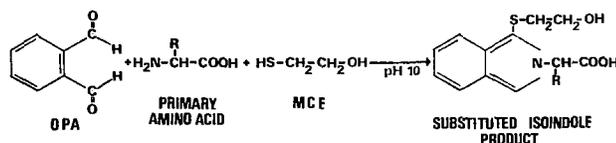


Fig. 1. Derivatization reaction of primary amino acids.

¹Presented at the 51st Annual Meeting, Milwaukee, WI, June 1985.

The amino acids asparagine, arginine, gamma-amino butyric acid, tryptophan, and homoserine were from the Sigma Chemical Co., St. Louis, MO 63178. All of the chemicals used were reagent grade (Productos Químicos, Monterrey, México). Water was double distilled and filtered, as were all of the solvents, through compatible membrane filters (Gelman Sciences, Inc., Ann Arbor, MI 58016) with a 0.45 μm porosity.

1) *Saturated sodium borate solution.* Water was saturated with decahydrated sodium tetraborate, and pH adjusted to 10.0 with an aqueous solution of 3*N* sodium hydroxide.

2) *Internal standard solution.* Homoserine (50 g) was measured, dissolved, and diluted to 5 ml with an aqueous solution of 0.1*N* hydrochloric acid.

3) *Standard amino acid solution.* Asparagine (150.0 mg), 50.0 mg of glutamine, 200.0 mg of gamma amino butyric acid, and 100.0 mg of tryptophan were weighed, dissolved, and diluted to 25 ml with an aqueous solution of 0.1*N* hydrochloric acid.

4) *Reagent solution.* *o*-Phthaldialdehyde (125 mg) was dissolved in 5 ml of methanol.

5) *Standard reagent solution.* To 1 ml of reagent solution, 1 ml of methanol and 50 μl of 2-ME were added and made up to 5 ml with saturated sodium borate solution.

6) *Precipitant solution.* A 0.3% solution of sulfosalicylic acid in isopropyl alcohol (w/v) was prepared.

7) *0.018*M* Buffer solution (pH 7.2).* Anhydrous disodium hydrogen phosphate (2.56 g) was dissolved in 1 L of 6×10^{-4} *M* sodium azide aqueous solution. The pH was adjusted to 7.2 with 3*N* hydrochloric acid, and the solution was filtered through a Gelman membrane filter (Gn-6, cat. no. 82082A).

8) *Eluant A.* Methanol, 0.018*M* buffer solution, and tetrahydrofuran were mixed in a 12:85:3 (v/v) ratio. The mixture was homogenized thoroughly and filtered through a Gelman membrane filter (Alpha 450, cat. no. 83414).

9) *Eluant B.* The same components as in eluant A were mixed, homogenized, and degassed by ultrasonic equipment (Bransonic B-32, Branson, Woburn, WA 01801) but in a volumetric proportion of 55:42:3.

Analysis Procedure

Sample preparation. Four milliliters of the sample was poured into a 5-ml volumetric flask, and 100 μl of solution 2 for wort analysis (or 15 μl of solution 2 for beer analysis) was added and made up to the mark with water. Solution 6 (270 μl) was mixed with

TABLE I
Solvent Gradient Composition^a

Time (min)	Function (eluant)	Value ^b (%)	Duration (min)
0	B	0	0
15	B	30	9
24	B	90	16
40	B	100	10
53	B	0	10

^aFlow rate: 1 ml/min; column temperature: 21°C.

^bTotal composition is formed by eluant A and eluant B.

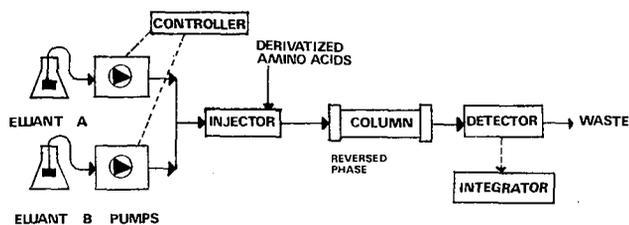


Fig. 2. Schematic diagram showing the high-performance liquid chromatography method using precolumn derivatization, reverse-phase column, and fluorescence detector.

30 μl of either of the previous solutions (for wort or beer analysis) in a 0.3- μl Pierce reacti-vial (cat. no. 13220, Pierce). This mixture was stirred for 1 min and centrifuged at $20,000 \times g$ for 1 min.

Derivatization. Seventy-five microliters of solution 5, 25 μl of water, and 25 μl of sample were poured into a 0.3-ml Pierce reacti-vial equipped with stirring rod. Chronometrical timing was started and magnetic stirring (mag Mix agitator, GCA Precision Scientific, Chicago, IL 60647) begun in position 5. Stirring was halted at 60 sec, and after 90 sec the injection valve was purged with 50 μl of reaction mixture. At 120 sec, the derivatized sample was injected into the column. The system is diagrammed in Figure 2.

Separation. Flow rate was 1 ml/min, and injection volume was 10 μl for wort and 20 μl for beer. Separation occurred at room temperature (approximately 21°C). The schedule used for eluants is shown in Table I.

Quantitation. Detector conditions were: photomultiplier switch, $\times 10$; exit to recorder, 10 mV; range, $\times 2$. A 16-fold attenuation was utilized for wort in the integrator, and fourfold attenuation for beer. The peak height of each amino acid was determined in relation to the height of the internal standard (homoserine), and each was multiplied by its corresponding calibration factor. Results were reported in milligrams per liter, carried out to one decimal.

Calibration

Calibration standards were prepared by diluting Standard H amino acids (Pierce) and solution 3, respectively, in water to 4 ml: for wort, 480 μl and 40 μl ; for beer, 96 μl and 8 μl . In accordance with the procedure described for sample analysis, calibration standards were analyzed until the height relationships between each amino acid and the internal standard were obtained. The calibration factor for each amino acid was determined by dividing its concentration (mg/L) by its corresponding height relationship.

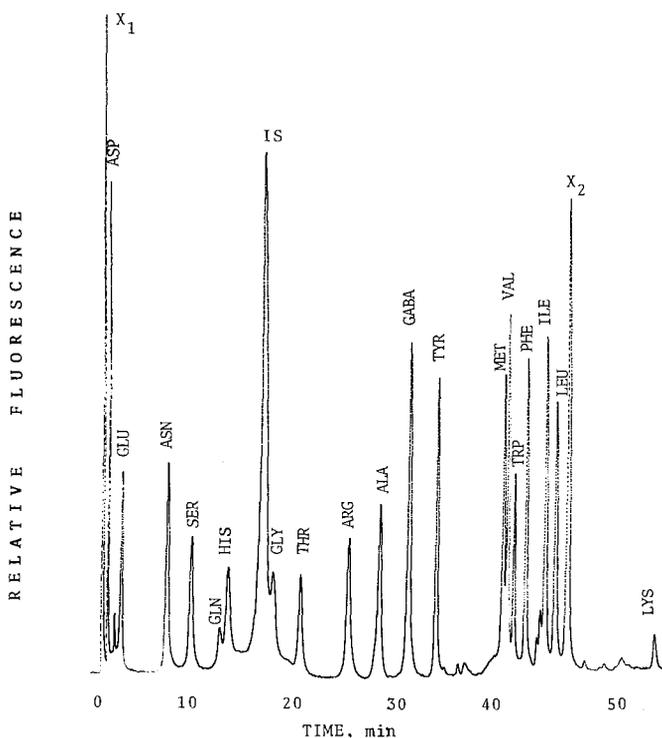


Fig. 3. Chromatogram of a standard mixture of amino acids: aspartic acid (Asp), glutamic acid (Glu), asparagine (Asn), serine (Ser), glutamine (Gln), histidine (His), homoserine (IS), glycine (Gly), threonine (Thr), arginine (Arg), alanine (Ala), gamma-amino butyric acid (Gaba), Tyrosine (Tyr), methionine (Met), valine (Val), tryptophan (Trp), phenylalanine (Phe), isoleucine (Ile), leucine (Leu), and lysine (Lys). Each was 48 pmol, except for Asn 73, Gln 22, Gaba 120, Trp 31, and IS were 126 pmol. Peaks labeled X₁ and X₂ are unidentified. Chromatographic conditions are described in text.

RESULTS AND DISCUSSION

Sample Preparation

Treatment of the sample consisted of adding the internal standard, eliminating interference (mainly proteins) by precipitation, and maintaining amino acid concentration as much as possible within its linear range of approximately 5–200 injected pmol (9,22). Centrifugation was preferred to filtration, which did not yield reproducible results, and because frequent losses of sample occurred due to excessive obstruction of filters (0.45 μ m porosity).

Derivatization

A typical chromatogram of a standard amino acid mixture after reaction with OPA/2-ME is shown in Figure 3. The elution sequence was as follows: aspartic acid, glutamic acid, asparagine, serine, glutamine, histidine, homoserine (internal standard), glycine, threonine, arginine, alanine, gamma-amino butyric acid, Tyrosine, methionine, valine, tryptophan, phenylalanine, isoleucine, leucine and lysine. The remaining peaks were not identified.

The use of OPA/2-ME as a derivatizing agent has the advantages already mentioned, such as formation of low-polarity substituted isoindole products, the reaction occurs in an aqueous medium and is quick at room temperature, is reproducible and quantitative, and generates highly fluorescent derivatives. However, because derivatives have a relatively short life span, it has its limitations. Therefore, the reproducibility and sensibility of the procedure are directly affected when reaction time and elapsed time before injection into the chromatographic column are not strictly controlled.

These limitations may be compensated for to a certain extent by using equipment that allows automatization of precolumn derivatization (20), but this adds to the cost. On the other hand, less expensive manual derivatization may be utilized. We tested several

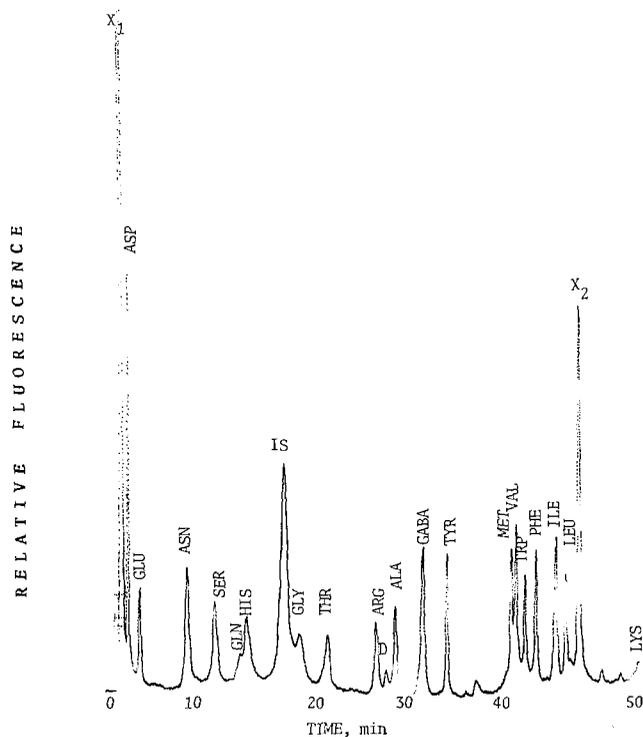


Fig. 4. Chromatogram of a standard mixture of amino acids, identical to Fig. 3 except that old derivatization reagent (*o*-phthaldialdehyde/2-mercaptoethanol) was used. Peak D corresponds to a product of the deteriorated derivatization reagent.

manual derivatization procedures and selected the one reported by Burbach and collaborators (1).

Reagent stability is another factor to consider. The OPA/2-ME reagent has been reported to be indefinitely stable at room temperature, requiring only infrequent additions of 2-ME (22). However, the convenience of periodic renewal has also been reported (7).

In our experiments, we found that reactivation by adding 2-ME caused turbidity (precipitation) and diminished reagent activity, so it should be discarded. Loss of reagent activity becomes apparent through a decrease in the fluorescent intensity of the derivatives and by the formation of a degradation product of the reagent, which in the chromatogram is located between the arginine and the alanine, as shown in Figure 4. A criterion for discarding the reagent is to measure the ratio of the peak heights of the degradation product (peak D in Fig. 4) against the internal standard. If this ratio is equal to or higher than 0.1, the reagent should be discarded.

Separation

The selection of the eluant composition to be used and its elution program was based on the results obtained under the experimental conditions reported by Hill et al (7), Jones et al (9), and Turnell and Cooper (22). The resolution obtained in the analysis of a 15° wort and a beer (O 13° P) are shown in Figures 5 and 6.

Besides the 19 amino acids, in both Figures 5 and 6 the internal standard peak (homoserine) may be perceived, as well as other peaks originated from sample (peak I), precipitant reagent (peak X₁), and buffer solution (peak X₂).



Fig. 5. Typical chromatogram of amino acids in wort (15° P): aspartic acid (Asp), glutamic acid (Glu), asparagine (Asn), serine (Ser), glutamine (Gln), histidine (His), homoserine (IS), glycine (Gly), threonine (Thr), arginine (Arg), alanine (Ala), gamma-amino butyric acid (Gaba), Tyrosine (Tyr), methionine (Met), valine (Val), tryptophan (Trp), phenylalanine (Phe), isoleucine (Ile), leucine (Leu), and lysine (Lys). Peaks labeled X₁, X₂, and I are unidentified. Chromatographic conditions are described in text.

Quantitation and Calibration

Precision (reproducibility). The coefficient of variation was utilized to indicate precision both in qualitative analysis (retention times) and in quantitative analysis (measurement of areas and height of the peaks).

Table II shows the average coefficient of variation of all amino acids present in a wort analysis, for retention times, areas, and peak heights, taking into consideration absolute and relative values, using asparagine, alanine, and phenylalanine as reference peaks for qualitative analysis and homoserine as internal standard in quantitative analysis.

In both instances, results of relative measurements are more valid; and lower variation coefficients were obtained when measuring heights than when integrating areas, which is consistent with recently published data (16). The variation coefficients obtained are similar to those reported by Turnell and Cooper (22).

Linearity Range

When amino acids were derivatized with OPA/2-ME, a linear relationship was obtained between the peak area and the number of picomoles injected, within a range of 0.4 to several hundred picomoles (9); this linearity was maintained in concentrations of up to 206 mg/L (22). However, when concentration levels lower than 5 pmol were injected, there were variations from linearity for some of the derivatives (9).

In light of these data, and the fact that the maximum concentration of amino acids to be analyzed in wort does not exceed 150 mg/L—corresponding to an injection of approximately 175 pmol in the method described—we proceeded to test linearity within a range of 5–200 pmol injected for each amino acid,

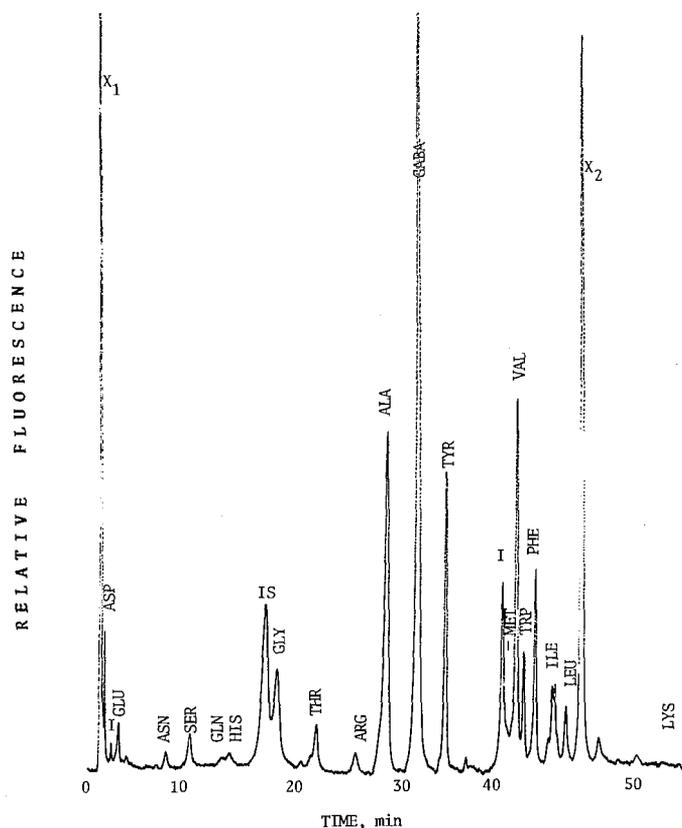


Fig. 6. Typical chromatogram of amino acids in beer (O 13° P): aspartic acid (Asp), glutamic acid (Glu), asparagine (Asn), serine (Ser), glutamine (Gln), histidine (His), homoserine (IS), glycine (Gly), threonine (Thr), arginine (Arg), alanine (Ala), γ -amino butyric acid (Gaba), Tyrosine (Tyr), methionine (Met), valine (Val), tryptophan (Trp), phenylalanine (Phe), isoleucine (Ile), leucine (Leu), and lysine (Lys). Peaks labeled X₁, X₂, and I are unidentified. Chromatographic conditions are described in text.

equivalent to approximately 4–170 mg/L for wort and 2–85 mg/L for beer.

Results obtained showed that, for most amino acids, a linear relationship is attained within the concentration interval tested. Exceptions were asparagine and lysine, which at concentrations higher than 100 pmol showed deviations from linearity. We tried to maintain the concentration of amino acids to be injected within the interval of linearity, either by varying dilution in the reaction mixture or by varying the injection volume.

Calibration Factors

Knowing that the analysis maintains its linearity within the concentration range tested, the system was calibrated with a single concentration value for each amino acid, which corresponded to the average concentration expected in the sample. Relative values were utilized for quantitation of peak heights.

Homocysteine, homoserine, and norvaline were tested as internal standards (22). Homoserine was selected as the most adequate, because homocysteine presented problems of resolution with glutamic acid, as did norvaline, which totally overlapped with tryptophan.

For better results, it is advisable to repeat calibration each time a new internal standard solution batch is prepared (solution 2), as well as when beginning analysis of a new batch of samples.

Quantitation

Analyses of samples of wort and beer were carried out using the method described. A blank determination was needed, where 4

TABLE II
Between-Run Precision of Amino Acid Estimation in Wort (15° P)^a

Measurement	Coefficient of Variation		Concentration, mg/L	
	Average	Range	Average	Range
Retention time				
Absolute	0.44	0.16–0.88	72.78	13.3–136.1
Relative ^b	0.36	0.00–1.53	72.78	13.3–136.1
Peak area				
Absolute	7.88	1.26–12.98	68.86	16.0–114.2
Relative ^c	6.59	2.10–14.30	59.46	25.0–100.0
Peak height				
Absolute	7.96	3.19–14.27	59.46	25.0–100.0
Relative ^c	4.58	1.70–10.20	59.46	25.0–100.0

^a n = 5.

^b References: asparagine, alanine, and phenylalanine.

^c Internal standard: homoserine.

TABLE III
Typical Analysis of Amino Acids in Wort and Beer (mg/L)

Amino Acid	Wort (15° P)	Beer (O 13° P)
Aspartic acid	49.9	12.9
Glutamic acid	38.7	3.3
Asparagine	63.2	2.1
Serine	43.3	3.3
Glutamine	12.3	Trace
Histidine	20.6	Trace
Glycine	28.2	10.3
Threonine	52.1	5.8
Arginine	61.2	1.8
Alanine	65.0	8.4
γ -Amino butyric acid	111.5	107.4
Tyrosine	62.1	5.5
Methionine	59.6	Trace
Valine	95.1	2.6
Tryptophan	28.3	6.5
Phenylalanine	92.1	3.0
Isoleucine	64.0	Trace
Leucine	122.5	4.1
Lysine	107.8	Trace

TABLE IV
High-Performance Liquid Chromatography (HPLC)
with Precolumn Derivatization and Reverse-Phase Column Compared
with HPLC Using Postcolumn Derivatization and Ion-Exchange Column^a

Variable	Method Precolumn	Method Postcolumn
Sample preparation		
Deproteinization	Desirable	Necessary
Derivatization		
o-Phthaldialdehyde (μg/analysis)	375	32,500
Equipment	Low cost	Expensive
Separation		
Column	Low cost	Expensive
Eluant	Known	Proprietary
Chromatographic run, min	55	65
Average retention time (C.V.)	0.36	1-4
Quantitation		
Medium peak heights (C.V.)	4.58	3.00
Analysis time, min	75	120

^aThe precolumn method is described in this paper; the postcolumn method is from Cunico and Schlabach (5).

ml of the sample was substituted for 4 ml of water when a series of analyses was initiated. From the blank determination results, we were able to correct possible interferences, and the condition of the reagent was evaluated.

A typical analysis of the amino acid profiles in wort (15° P) and beer (O 13° P), obtained by the method described, is shown in Table III.

Comparison of Some Characteristics of the HPLC Method Utilizing Precolumn and Postcolumn Derivatization

Some of the characteristics of the HPLC method, utilizing precolumn derivatization and reverse-phase column separation, are compared with data reported by Cunico and Schlabach (5) on a method utilizing postcolumn derivatization and ion-exchange column separation (Table IV). The coefficients of variation for the HPLC method described were obtained considering the average values of the 19 amino acids mentioned.

CONCLUSIONS

The HPLC method described is applicable to the analysis of 19 amino acids in wort and beer. The time required per chromatogram was 55 min; total analysis time was 75 min. The average precision

attained for all the amino acids was consistent with a variation coefficient of 0.36 for relative retention times, and a variation coefficient of 4.58 either for average peak heights or concentration in milligrams per liter.

The method yielded linear results for wort and beer analysis in concentrations of 5–200 pmol injected.

Cost per analysis was low, because the composition of the eluants was known, a minimal amount of OPA/2-ME derivatization reagent was required, and the column was inexpensive.

LITERATURE CITED

- Burbach, J. P. H., Prins, A., Lebouille, J. L. M., Verhoef, J., and Witter, A. *J. Chromatogr.* 237:339, 1982.
- Charalambous, G. Involatile constituents of beer. Page 205 in: *Brewing Science*. Vol. 2. J. R. A. Pollock, ed. Academic Press: New York, 1981.
- Chen, E. C.-H. *J. Am. Soc. Brew. Chem.* 36:39, 1978.
- Cieslak, M. E. *J. Am. Soc. Brew. Chem.* 41:10, 1983.
- Cunico, R. L., and Schlabach, T. *J. Chromatogr.* 266:461, 1983.
- Drawert, F., Hagen, W., and Veinoglou, F. *Brauwissenschaft* 35:297, 1982.
- Hill, D. W., Walters, F. H., Wilson, T. D., and Stuart, J. D. *Anal. Chem.* 51:1338, 1979.
- Ishida, Y., Fujita, T., and Asai, K. *J. Chromatogr.* 204:143, 1981.
- Jones, B. N., Pääbo, S., and Stein, S. *J. Liq. Chromatogr.* 4(4):565, 1981.
- Jones, M. *Eur. Brew. Conv. Wortsymposium, Monogr. 1* Zeist 1974, p. 90.
- Jones, M., and Pierce, J. S. *J. Inst. Brew.* 70:307, 1964.
- Jones, M., and Pierce, J. S. *Eur. Brew. Conv., Proc. Congr. 12th, Interlachen, 1969*, p. 151.
- Lawrence, J. F. *J. Chromatogr. Sci.* 17:147, 1979.
- Marinelli, L., Heckel, M., and Whitney, D. *Am. Soc. Brew. Chem., Proc., 1967*, p. 37.
- Meilgaard, M. C. *Tech. Q. Master Brew. Assoc. Am.* 13:78, 1976.
- McCoy, R. W., Aiken, R. L., Pauls, R. E., Ziegel, E. R., Wolf, T., Fritz, G. T., and Marmion, D. M. *J. Chromatogr. Sci.* 22:425, 1984.
- Moore, S., Spackman, D. H., and Stein, W. H. *Anal. Chem.* 30:1185, 1958.
- Nakatani, K., Takahashi, T., Nagami, K., and Kumada, J. *Tech. Q. Master Brew. Assoc. Am.* 21:73, 1984.
- Pierce, J. S. *J. Inst. Brew.* 88:228, 1982.
- Pfeifer, R., Karol, R., Korpi, J., Burgoyne, R., and McCourt, D. *Am. Lab.* 15(3):77, 1983.
- Schuster, R. *Anal. Chem.* 52:617, 1980.
- Turnell, D. C., and Cooper, J. D. H. *Clinical Chem.* 28:527, 1982.
- Weidmeier, V. T., Porterfield, S. P., and Hendrich, C. E. *J. Chromatogr.* 231:410, 1982.

[Received August 1, 1985. Accepted November 5, 1985.]