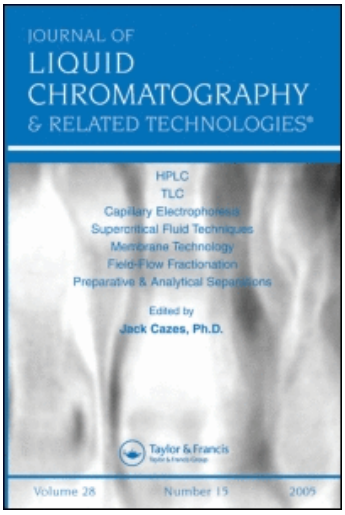


Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

High Performance Liquid Chromatographic Separation and Fluorescent Measurement of Taurine, a Key Amino Acid

James D. Stuart[a]; Terry D. Wilson[b]; Dennis W. Hill[c]; Frederick H. Walters[d]; Sung Y. Feng[e]
[a] Department of Chemistry U-60, University of Connecticut, Storrs, Connecticut [b] Department of Chemistry, Annhurst College, Woodstock, Connecticut [c] Microchemistry Laboratory, College of Agriculture and Natural Resources U-66, University of Connecticut, Storrs, Connecticut [d] Department of Chemistry and Physics, Quinnipiac College, Hamden, Connecticut [e] Marine Sciences Institute and Biological Sciences Group, University of Connecticut, Noank, Connecticut

To cite this Article Stuart, James D. , Wilson, Terry D. , Hill, Dennis W. , Walters, Frederick H. and Feng, Sung Y.(1979) 'High Performance Liquid Chromatographic Separation and Fluorescent Measurement of Taurine, a Key Amino Acid', Journal of Liquid Chromatography & Related Technologies, 2: 6, 809 — 821
To link to this Article: DOI: 10.1080/01483917908060105
URL: http://dx.doi.org/10.1080/01483917908060105

JOURNAL OF LIQUID CHROMATOGRAPHY, 2(6), 809-821 (1979)

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION AND FLUORESCENT MEASUREMENT OF TAURINE, A KEY AMINO ACID

James D. Stuart, Department of Chemistry U-60, University of Connecticut, Storrs, Connecticut 06268
Terry D. Wilson, Department of Chemistry, Annhurst College, Woodstock, Connecticut 06281
Dennis W. Hill, Microchemistry Laboratory, College of Agriculture and Natural Resources U-66, University of Connecticut, Storrs, Connecticut 06268
Frederick H. Walters, Department of Chemistry and Physics, Quinnipiac College, Hamden, Connecticut 06518
Sung Y. Feng, Marine Sciences Institute and Biological Sciences Group, University of Connecticut, Noank, Connecticut 06340

ABSTRACT

A rapid, sensitive method for the analysis of taurine in oyster hemolymph (blood) has been developed. Highly fluorescent, substituted isoindoles formed by the reaction of taurine and other amino acids with o-phthaldialdehyde/ethanethiol reagent were separated on a reverse phase, high performance liquid chromatographic column. It was necessary to carefully control the concentration of the sodium ion in the phosphate buffer in order to maintain an adequate resolution of both taurine and tyrosine from β-alanine and arginine. Calibration plots of the fluorescent taurine derivative were linear over 2.5 orders of magnitude with a limit of detection of 0.10 nanomoles per ml of oyster hemolymph.

INTRODUCTION

Recent reports have indicated that the amino acid, taurine (2-aminoethanesulphonic acid), plays a key role as a neuromodulator in the central nervous system of humans (1,2). Taurine is found in

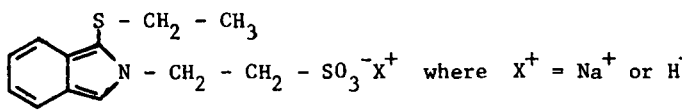
the retina, heart muscle and brain tissue of vertebrates. High concentrations of taurine in salt water invertebrates and its absence in fresh water organisms has led to the hypothesis that taurine plays an important role in the regulation of osmotic pressure (3). A co-author, S. Y. Feng (4), has studied the free amino acid composition in the hemolymph of normal and parasitized oysters. Significant increases in the levels of taurine, aspartic acid, glutamic acid, threonine and phosphoserine were observed in infected oysters. Feng, et al., proposed that the increases probably reflected the host's compensatory effort to replenish the depleted amino acid pools and to maintain osmotic regulation (4). We were interested in developing a rapid and sensitive method for determining taurine and the other amino acids that were reported by Feng, et al. (4), to be in the range of 50 to 400 nanomole per ml of oyster hemolymph.

Amino acid analysis on an amino acid analyzer using ion-exchange columns and post-column derivatization with ninhydrin has been well established (5). Of the many high performance liquid chromatographic analyses that have been reported, most have involved a prior separation of the free amino acid on an ion-exchange column followed by post-column derivatization. The ninhydrin/o-phenylacetaldehyde reaction (6), the fluorescamine reaction (7) and the o-phthaldialdehyde/2-mercaptoethanol reaction (8) have all proved useful for post-column derivatization. Comparison studies of the fluorescent characteristics of the fluorescamine, ninhydrin, o-diacetylbenzene and o-phthaldialdehyde/2-mercaptoethanol derivatives of amino acids have been reported (9-11).

High performance liquid chromotography, especially with reverse phase columns offers greater efficiency, ease of use and higher flow-rates over conventional ion-exchange columns. To provide for both an effective separation on either normal or reverse phase columns and an adequate detection, pre-column derivatizations to form dansyl (12, 13), or phenylthiohydantoin (PTH) (14) derivatives have been employed. Recent studies have involved the

pre-column derivatization with o-phthaldialdehyde/2-mercaptoethanol of gentamicin in serum (15) and biogenic amines in human plasma, urine and tissue (16).

Until recently, little was known about the structure of the highly fluorescent compounds formed in the reaction between primary amines and o-phthaldialdehyde/2-mercaptoethanol. Simons and Johnson have recently reported nuclear magnetic resonance and infra-red spectroscopic studies which showed that 1-alkylthio-2-alkyl substituted isoindoles were formed when alkylthiols and alkylamines (or amino acids) were condensed with o-phthaldialdehyde (17,18). The chemical structure of the proposed substituted isoindole formed by the reaction between taurine and o-phthaldialdehyde/ethanethiol appears below.



STRUCTURE 1

Simons and Johnson noted that the substituted isoindole formed by the reaction of propylamine and o-phthaldialdehyde/2-mercaptoethanol was unstable and degraded to an ethylene sulfide polymer and 2,3-dihydro-1-H-isoindol-1-one (17). By using ethanethiol, a thiol lacking the 2-hydroxyl group, in place of 2-mercaptoethanol, the stability of the substituted isoindole increased significantly. Also, when the ethanethiol derivative was first prepared in aqueous buffer and then transferred to a 95 percent ethanol solution, the relative fluorescence increased by 60 to 70 percent in contrast to the 1 to 6 percent increase for the 2-mercaptoethanol derivative (18). It was for these reasons, that in our studies ethanethiol, instead of 2-mercaptoethanol, was used, and the developed derivatization procedure called for the rapid mixing in aqueous buffer and dilution of the derivatized amino acids in methanol. A full report of our

developed method for the determination of many of the biological important amino acids in the picomole range will be appearing (19).

Noteworthy in this study was the fact that upon derivatization with o-phthaldialdehyde/ethanethiol, taurine was retained for a considerable period of time on a reverse phase column. Normally, this has not been the case (5,20). Taurine, due to its sulfonic acid group and high water solubility, was usually eluted in the dead volume or just after the dead-volume from most reverse phase and ion-exchange columns.

MATERIALS

Apparatus

Two different high performance liquid chromatographic systems were used. For the gradient elution studies, two Model 6000A pumps, controlled by a Model 660 Solvent Programmer and a U6K Universal Injector (Waters Associates, Milford, MA) were employed. The Model FS-970 Liquid Chromatographic Fluorometer (Schoeffel Instrument Corp., Westwood, N.J.) with the following settings and characteristics was used for detection: 5 µL flow-cell, excitation monochromator set at 229 nm, emission measured with a 470 nm cut-off filter, a sensitivity of 5.42, and a time constant of 0.5 sec. were used on the 1.0 µA full-scale, range setting.

For the isocratic elution studies, a single Model 6000A pump was employed which was equipped with a U6K Universal Injector (Waters Associates) and a filter fluorometric, liquid chromotographic detector (Fluorichrom, Varian Associates Inc, Palo Alto, CA). This fluorescent detector had the following characteristics: a 25 µL flow-cell, a 325 nm band excitation filter and a 385 nm cut-off emission filter. The chromatograms were recorded using a two pen, electronic integrating recorder (OmniScribe, Model 5213-15, Houston Instr., Austin, TX). For the separation, on either instrument, one 30 cm X 3.9 mm µ-Bondapak C_{18} column (Part No. 27324, Waters Associates) was used.

Chemicals

Methanol and acetonitrile were either distilled-in-glass (Burdick and Jackson, Muskegon, MI) or ACS Certified Reagent grade. The phosphoric, hydrochloric and boric acid, as well as the anhydrous disodium hydrogen phosphate, were ACS Certified grade (Fisher Scientific Co., Fairlawn, N.J.). Reagent grade water was prepared by running tap water through coupled Milli RO 4 and Milli-Q Systems (Millipore Corp., Bedford, MA). Taurine (No. 15,224-2 Aldrich Chemical Co.), amino acid standards (AMAC standard kit, No. 20065J Pierce Chemical Co.) and o-phthaldialdehyde (Fluoropa from Durrum Chemical Corp. available as No. 26010, Pierce Chemical Co., Rockford, IL.) were used. o-Phthaldialdehyde (No. P1378, Sigma Chemical Co., St. Louis, MO) was also used. Ethanethiol was used as received (No. E370-8 Aldrich Chemical Co., Milwaukee, WI). Prepackaged tetrabutylammonium phosphate was used for ion-pairing studies (PIC-A, Waters Associates, Milford, MA).

METHODS

Preparation of the Stock Phosphate Buffer

3.549 g of anhydrous Na_2HPO_4 were dissolved into about 400 ml of water and the pH adjusted to 7.2 with concentrated hydrochloric acid. The solution was then diluted to volume in a 500 ml volumetric flask resulting in a 0.0500 M solution. Diluted Phosphate Buffer for HPLC. Daily, 125 ml of the above stock solution were diluted to 500 ml and then filtered through a 0.45 μm membrane filter (Millipore Corp., Bedford, MA) immediately before use in the HPLC. This resulted in a 0.0125 M Na_2HPO_4 solution or a solution that was 0.0250 M in sodium ions.

Preparation of Borate Buffer

Enough boric acid was added with heat to 1.0 l of water to form a saturated solution. After cooling, the supernatant phase was filtered and adjusted to between pH 9.2 and 9.5 with sodium hydroxide.

Preparation of the o-Phthaldialdehyde/Ethanethiol Derivatizing Solution

Preferably in a 50 ml, amber volumetric flask, 50.0 mg of o-phthaldialdehyde were dissolved in the minimum amount of methanol, 50 µl of ethanethiol and 5.0 ml of the borate buffer were added, vortex mixed and the mixture was diluted to volume with methanol and again mixed. It is recommended that this solution be made daily and be protected from the light.

Preparation of the Amino Acid Standards

All of the amino acids, except for tryptophan, were made in aqueous solution at the level of 5.0 nanomoles/ml. For obtaining the calibration plots of peak height and area vs concentration, stock solutions of each amino acid standard at the 100.0 nanomole/ml level were made using an analytical balance weighing to the nearest 0.01 mg (Model H-54, Mettler Inst. Corp., Princeton, N.Y.). Tryptophan was made up in borate buffer.

Reconstitution of the Oyster Hemolymph Samples

Non-parasitized oysters taken during July 1978 from the waters near the University of Connecticut, Marine Research Laboratory, Noank, Connecticut, were exsanguinated via the adductor muscle. The clear hemolymph samples, without formed blood elements, were freeze dried and dessicated. Just prior to analysis, the freeze dried samples were weighed and reconstituted to their original volume with methanol. The resulting mixture was vortex mixed then centrifuged for about 5.0 min; the resulting clear supernatant phase was used for the analysis.

Derivatization of the Oyster Hemolymph Samples

To 0.100 ml of the clear supernatant phase was added 0.100 ml of the borate buffer, followed by 0.100 ml of the o-phthaldialdehyde/ethanethiol derivatizing reagent. The resulting solution was diluted to about 2.0 ml with methanol and 2.5 µl were injected for the gradient elution analyses. For the quantitative isocratic analyses, 0.100 ml of the clear supernatant phase was mixed with

0.500 ml of the o-phthaldialdehyde/ethanethiol reagent. The resulting solution was diluted with methanol in an appropriate volumetric flask and vortex mixed. A 10.0 µl aliquot was injected using a syringe with a Chaney Adapter (Model 701-NCH Hamilton Co., Reno, NV).

Derivatization Procedure for the Amino Acid Standards

To a 1, 5 or 10 ml volumetric flask, appropriate volumes of the concentrated amino acid standards were mixed with 0.050 µl of the o-phthaldialdehyde/ethanethiol solution. The resulting solutions were then diluted to volume with methanol and vortex mixed. 10.0 µl aliquots of derivatized β-alanine, tyrosine and taurine were injected using a syringe with a Chaney Adapter.

RESULTS AND DISCUSSION

Ion-pair chromatography was attempted on non-derivatized taurine with tetrabutylammonium phosphate (PIC-A). Various solvent ratios of acetonitrile/water and methanol/water were investigated but were not successful as taurine appeared to be eluted in the dead-volume of the µ-Bondapak C_{18} column. A recent application note has shown that the separation of amino acids as their dansyl derivatives can be enhanced by ion-pair chromatography (21).

Studies on the reproducibility of the amino acid profiles revealed that the sodium ion concentration in the pH 7.2 phosphate buffer influenced the retention behavior of several of the o-phthaldialdehyde/ethanethiol derivatized amino acids. Using a 30 percent acetonitrile/70 percent phosphate buffer solution, made by adjusting a 0.1 percent by volume solution of phosphoric acid to pH 7.2 with solid NaOH, the relative retention of taurine to arginine was 0.74. Keeping the pH of the buffer constant, the relative retention of taurine to arginine increased to 0.82 and to 1.17 when sodium chloride was added in the concentrations of 0.010 M and 0.10 M, respectively. Other studies, utilizing a 25 percent acetonitrile/75 percent phosphate buffer, prepared from phosphoric acid, and diluting the buffer 1 to 2 and 1 to 10 (pH unchanged on dilution) resulted in

decreases in the relative retention of taurine to arginine from 0.82 to 0.60 and to 0.29, respectively. Using the linear solvent program from 9 to 49 percent acetonitrile/pH 7.2, phosphate buffer, Table 1 summarizes still other data that showed that there existed a significant increase in the relative retentions of taurine to arginine and of tyrosine to arginine as a result of increasing the sodium ion concentration of the phosphate buffer from 0.010 to 0.025 M. The retention times of arginine stayed relatively constant (17.75 to 17.65 min) whereas the retention times of tyrosine changed from 13.8 to 15.9 min and the retention times of taurine changed from 14.2 to 16.7 min.

Of importance is the fact that the resolution between taurine and tyrosine increased from 0.85 to 1.66 as the sodium ion increased. These results indicated that the preparation of a reproducible phosphate buffer concentration was crucial or else irreproducible separations occured. Further studies are in progress in order to investigate the effect of retention times of the derivatized amino acids, such as depicted by Structure 1, with concentration of not only sodium but other ions.

TABLE 1

The Effect of Increasing the Sodium Ion Concentration on the Relative Retention Times of Taurine and Tyrosine.

Molar Concentration of Sodium ion	Relative Retention of Taurine/Arginine	Relative Retention of Tyrosine/Arginine	Apparent Resolution of Taurine/Tyrosine[a]
0.010	.797	.774	0.85
0.015	.862	.830	1.20
0.020	.908	.870	1.45
0.025	.946	.902	1.66

[a]Apparent resolution was calculated as $(R_{t(Tau)}-R_{t(Tyr)})/1/2(W_{(Tau)}+W_{(Tyr)})$

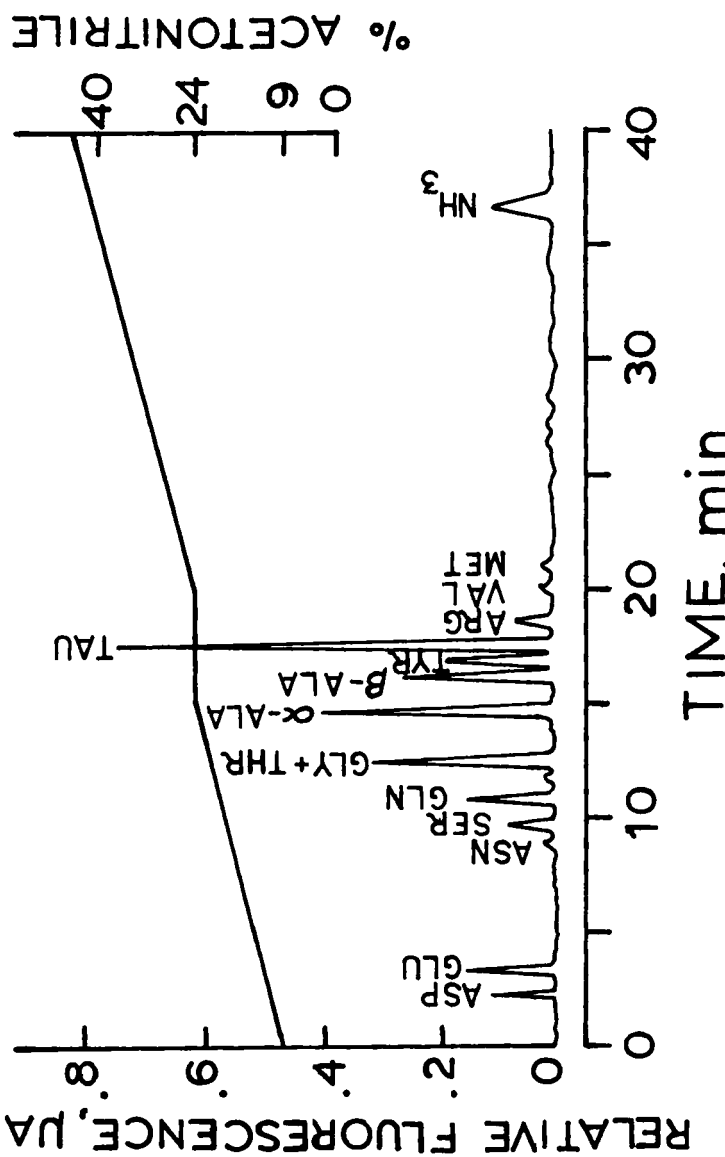


Figure 1. A Gradient Elution Profile of the o-phthaldialdehyde/ethanethiol Derivatized Free Amino Acids Extracted from Oyster hemolymph. Conditions: Column, μ-Bondapak C18, 30 cm x 3.9 mm; Solvent A - pH 7.2, 0.0125 M Na_2HPO_4 buffer, Solvent B - CH_3CN with a linear program from 9 to 49 percent (Solvent B/Solvent A), 40 min in duration with a 5 min isocratic hold at 15 min from the initiation of the program; flow-rate of 2.0 ml/min. Derivatization conditions, amounts injected and instrumental parameters described in the text.

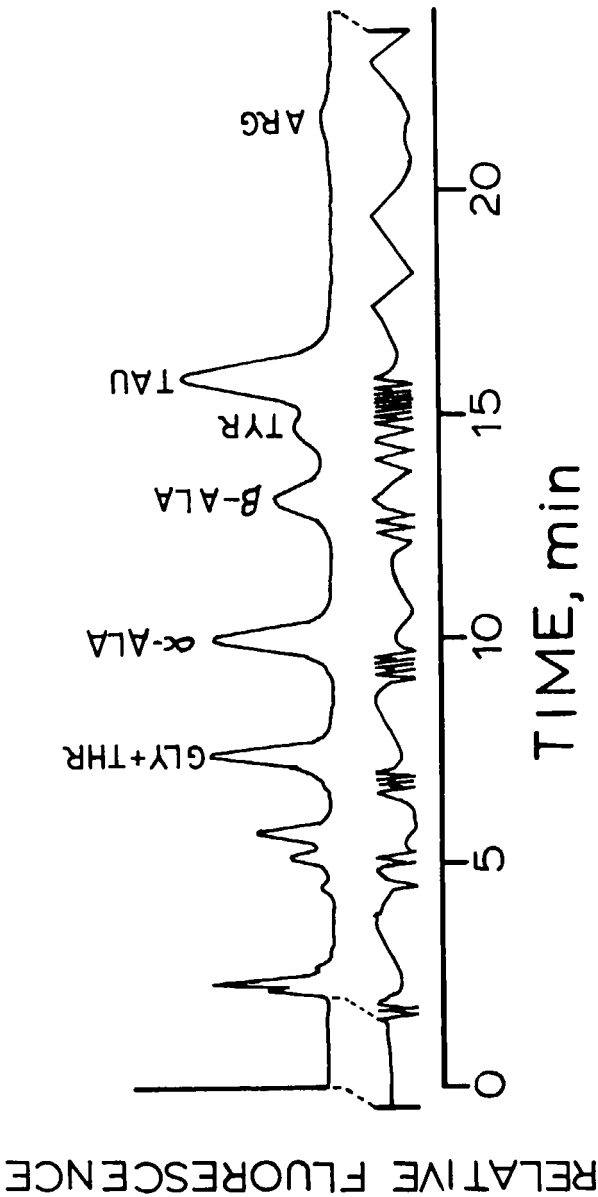


Figure 2. Isocratic Elution Profile of the Derivatized Free Amino Acids Extracted from the Same Oyster Hemolymph Shown in Figure 1. Conditions: Column, μ-Bondapak C_{18}, 30 cm x 3.9 mm. Solvent - 15 percent CH_3CN and 85 percent, pH 7.2, 0.0125 M Na_2HPO_4 buffer; flow-rate 1.2 ml/min. Derivatization conditions, amounts injected and instrumental parameters described in the text.

Figure 1 shows a gradient elution profile of the o-phthaldialdehyde/ethanethiol derivatized amino acids extracted from the hemolymph of a non-parasitized oyster. In agreement with Feng, et al. (4), high concentrations, above 100 nanomoles/ml, were found for glycine, alanine and taurine. In an attempt to better quantitate the levels of amino acids, especially taurine, extracted from oyster hemolymph, isocratic elution at a lower flow-rate was employed. Figure 2 shows a typical chromatogram. Unfortunately, under isocratic elution conditions with the less sophisticated liquid chromotographic instrument having the 25 µl fluorescent flow cell, but with the proper sodium ion concentration, the tyrosine peak is not adequately resolved from the larger taurine peak. However, using derivatized taurine standards, it was determined that a linear calibration was obtained over 2.5 orders of magnitude in the range from 1.0 to 200 nanomoles/ml. The limit of detection for taurine was 0.1 nanomoles/ml using the liquid chromatograph employed for the isocratic elution studies. Thus using the developed procedure and the available equipment, there is more than enough sensitivity to detect taurine, usually present in the 50 to 400 nanomole range per ml of oyster hemolymph. One needs merely to make the proper dilution of the reconstituted oyster plasma to work in the middle linear calibration range obtained for the standards. Further studies on the quantitative analyses of taurine and other amino acids not only in oyster hemolymph but also in other biological matrices are in progress. Derivatizing reagents similar to o-phthaldialdehyde/ethanethiol which should enhance the ultraviolet absorption of amines and amino acids are presently being investigated.

ACKNOWLEDGMENTS

We thank Dr. A. J. C. L. Hogarth for valuable discussions into the organic synthesis of other derivatizing reagents and Donna R. Allen for preliminary, liquid chromatographic work. We acknowledge

the loan of the Fluorichrom Fluorescent Detector from Mr. Robert Sterry, Varian Associates, Inc., Florham Park, N.J.

REFERENCES

1. King, J. S., book review in Clin. Chem., 24, 1430, 1978.
2. Barbeau, A. and Huxtable, R. J., eds., Taurine and Neurological Disorders, Raven Press, New York, 1978.
3. Jacobsen, J. G. and Smith, L. H., Physiol. Rev., 48, 424, 1968.
4. Feng, S. Y., Khairallah, E. A. and Canzonier, W. J., Comp. Biochem. Physiol., 34, 547, 1970.
5. Hamilton, P. B. and Anderson, R. A., Anal. Chem., 31, 1504, 1959.
6. Samejima, K., Dairman W., and Udenfriend, S., Anal. Biochem., 42, 222, 1971.
7. Weigele, M., DeBernardo, S. L., Tergi, J. P. and Leimgrubber, W., J. Amer. Chem. Soc., 94, 5927, 1972.
8. Roth, M., Anal. Chem., 43, 880, 1971.
9. Benson, J. R. and Hare, P. E., Proc. Nat. Acad. Sci. USA, 72, 619, 1975.
10. Schiltz, E., Schnackerz, K. D. and Gracy, R. W., Anal. Biochem., 79, 33, 1977.
11. Fourche, J., Jensen, H., and Neuzil, E., Anal. Chem., 48, 155, 1976.
12. Engelhardt, H., Asshauer, J., Neue, U. and Weigand, N., Anal. Chem., 46, 336, 1974.
13. Bayer, E., Grom, E., Kaltenegger, B. and Uhman, R., Anal. Chem., 48, 1106, 1976.
14. Haag, A. and Langer, K., Chromatographia, 7, 659, 1974.
15. Maitra, S. K., Yoshikawa, T. T., Hansen, J. L., Nilsson-Ehle, I., Palin, W. J., Schotz, M. C. and Guze, L. B., Clin. Chem., 23, 2275, 1977.
16. Davis, T. P., Gehrke, C. W., Gehrke, C. W., Jr., Cunningham, T. D., Kuo, K. C., Gerhardt, K. O., Johnson, H. D. and Williams, C. H., Clin. Chem., 24, 1317, 1978.

17. Simons, S. S., Jr. and Johnson, D. F., J. Amer. Chem. Soc., 98, 7098, 1976.
18. Simons, S. S., Jr. and Johnson, D. F., Anal. Biochem., 82, 250, 1977.
19. Hill, D. W., Walters, F. H., Wilson, T. D. and Stuart, J. D., Anal. Chem., in press.
20. Meek, J. L., Anal. Chem., 48, 375, 1976.
21. Cooke, N. H. C., Altex Chromatogram, 1, May 1978. (Altex Scientific, 1780 Fourth St., Berkeley, CA. 94710).