Journal of Pharmaceutical & Biomedical Analysis
Vol. 8, Nos 8–12, pp. 825–830, 1990
Printed in Great Britain

0731–7085/90 $3.00 + 0.00

Liquid chromatographic separation and stereoselective detection of L- and D-amino acids with catalytic reaction detection using immobilized enzymes*

E. DOMÍNGUEZ,†‡ G. MARKO-VARGA,§ M. CARLSSON§ and L. GORTON§

‡*Department of Applied Microbiology and* §*Department of Analytical Chemistry, University of Lund, P.O. Box 124, S-221 00 Lund, Sweden*

Abstract: A post-column LC detection system is described for the stereoselective detection of L- and D-amino acids. The effluent of the LC column passes an immobilized enzyme reactor (IMER) containing either L- or D-amino acid oxidase. The hydrogen peroxide formed in this reactor by the oxidation of the amino acids is then transported to a second IMER containing horse-radish peroxidase. With the addition of 4-aminophenazone and dichlorophenolsulphonyl chloride to the carrier, the hydrogen peroxide is reacted to form a red-coloured complex which is detected in a flow-through photometric cell at 514 nm. Applications to the analysis of amino acids in bovine and human sera are described.

Keywords: *D-Amino acid oxidase; L-amino acid oxidase; immobilized enzyme reactor; liquid chromatography; D- and L-amino acids, serum.*

Introduction

The analysis of amino acids is of great importance in the biomedical and biochemical fields. Several severe diseases are associated with defects in amino acid metabolism [1]. A variety of chromatographic methods has been developed to determine amino acids. In particular, reversed-phase liquid chromatography (LC) has become the most widespread technique [2]. Like carbohydrates, amino acids lack physical and chemical properties that make them easily and selectively detectable in complex solutions such as physiological fluids. For the last three decades, most of the methods proposed for the analysis of amino acids in biological materials, have relied on pre- or post-column derivatization to generate adducts detectable by spectroscopy [3–6], electrochemistry [7] or by chemiluminescence [8, 9].

D-Amino acids are not used for protein synthesis and have therefore been called "unnatural" or "non-physiological" amino acids. However, they have been found as constituents of fermented food [10], peptidoglycan of bacterial cell walls [11] and peptide antibiotics [12], as well as in certain animals [13]. Because D-amino acids are not synthesized by mammals, these compounds offer a potential use, as markers, for the fast detection of bacterial infection in food [14].

D- and L-amino acids can be resolved by LC by the use of various chiral stationary phases [15, 16], or by achiral columns with chiral mobile phase additives [17]. Enantioselectivity may be introduced in the detection step by the use of amino acid oxidases (AAO) [18]. These enzymes, L-amino acid oxidase (L-AAO) and D-amino acid oxidase (D-AAO), exhibit stereoselectivity for the different L- and D-isomers, but catalyse the deamination of a considerable number of amino acids. The use of immobilized enzyme reactors (IMER) in the post-column mode, in conjunction with LC, has been applied in practical analytical procedures [19, 20] and has been shown to increase the selectivity of classical detection systems [21–23]. The present work reports on the use of LC with enzymatic post-column derivatization for the stereoselective determination of L-amino acids and D-amino acids in serum samples. This work has been extended to preliminary studies on the immobilization of L-AAO and D-AAO.

*Presented at the "Second International Symposium on Pharmaceutical and Biomedical Analysis", April 1990, York, UK.

†Author to whom correspondence should be addressed. On leave from the Department of Pharmacy, Nutrition and Food Analysis. University of Alcalá de Henares, E-28871 Alcalá de Henares, Madrid, Spain.

Experimental

Reagents

The amino acids (Sigma, St Louis, MO, USA) were used as received. Millipore/Milli-Q water was used to prepare solutions and the mobile phase. 2,4-Dichlorophenol-6-sulphonyl chloride (DCPS) and 2,4-dichlorophenol (DCP) were obtained from Janssen (Beerse, Belgium). 4-Amino-1,2-dihydro-1,5-dimethyl-2-phenyl-3H-pyrazole-3-one "4-aminophenazone" (4-AP) was supplied by BDH (Dorset, UK). All other reagents were of analytical grade.

Enzymes and immobilization procedures

L-AAO (EC 1.4.3.2) from *Crotalus adamanteus* venom (Sigma, Type IV, 8.9 U mg^{-1} protein) was used as received. D-AAO (EC 1.4.3.3) from porcine kidney (Sigma, Type X, 9.8 U mg^{-1} protein) was dialysed overnight against 0.1 M phosphate buffer at pH 7.0 when immobilized with glutaraldehyde coupling. Horse-radish peroxidase, HRP (EC 1.11.1.7) (Sigma, Type VI, 310 U mg^{-1} solid) was used as received. The units are specified according to the manufacturer's specifications. L-AAO and D-AAO were immobilized on alkylamino- as well as on arylamino-derivatized controlled pore glass, CPG. HRP was immobilized on arylamino-derivatized CPG [24].

Glutaraldehyde coupling to alkylamino glass. CPG-10 (Serva cat. no. 44762) pore diameter 47 nm, particle size 37–74 μm, was washed, dried, silanized with 3-aminopropyltriethoxysilane, and activated with glutaraldehyde (2.5% solution in 0.1 M phosphate buffer at pH 7.0) according to previously reported procedures [25]. 133 mg (1120 units) of dialysed D-AAO were added per gram of the moist and activated CPG and the reaction was allowed to proceed overnight at 4°C, initially at reduced pressure. The same procedure, avoiding the dialysis step, was used for the immobilization of L-AAO charging 6.3 mg (561 units) per gram of CPG. Each of the steps above was followed by extensive washing on a G3 glass filter.

Azo coupling to arylamino silica. A commercial organically aminated arylamino silica was used, Si 500, pore diameter 50 nm and particle size 30 μm (Serva cat. no. 43668). The same charges of D-AAO and L-AAO as those mentioned above for the glutaraldehyde coupling were used. In this case D-AAO was not dialysed but dissolved in 0.1 M phosphate buffer pH 7.0. 20 mg (6200 units) of HPR were dissolved in 0.1 M phosphate buffer pH 8.5 [24] and this quantity was charged per gram of activated silica. Each of the steps above was followed by extensive washing on a G3 glass filter. The immobilized enzymes were packed into reactors having a low dead volume [26]. When not in use all reactors were kept in 0.1 M phosphate buffer pH 7.0 at 4°C.

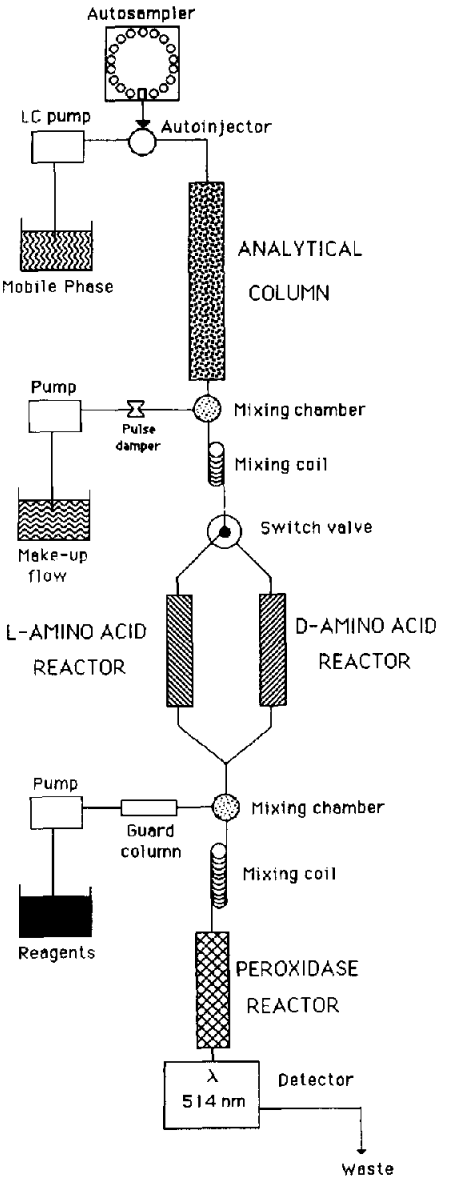

Figure 1
Schematic diagram of the LC post-column IMER system for the stereoselective determination of D- and L-amino acids. For details, see text.

Equipment

The LC system used in this work is shown in Fig. 1. It consists of an LC pump (Waters, Millipore, USA, Model 590), a thermostatted microsampler and autoinjector (Carnegie Medicin AB, Sweden, Model CMA/200) and a Micropak® analytical column (15 × 0.41 cm i.d.) (Varian, USA). The effluent of the column was mixed with a make-up flow delivered by a Gilson Minipuls 2 peristaltic pump connected to a pulse dampener. The combined flows passed through a knotted reactor (75 cm, 0.51 mm i.d.) to promote complete mixing before passing to either the L-AAO reactor or the D-AAO reactor. This flow was then mixed with the reagent solution propulsed by a Gilson Minipuls 2 peristaltic pump. A guard column (silanol 3 ml, Analytichem, USA) was inserted into the reagent line to adsorb impurities. The combined flows were thoroughly mixed in a second knotted reactor (75 cm, 0.51 mm i.d.) before reaching the HRP reactor. The absorbance was monitored in a 11-μl flow-through cell (LKB, Sweden, Model 2141).

The activity of the different IMERs was tested in the flow injection analysis (FIA) mode using the same set-up as shown in Fig. 1, the sole difference being that the analytical column and the LC pump were removed.

Results and Discussion

Enzymatic detection reactions

L- and D-amino acids are deaminated by L-AAO and D-AAO, respectively, in the presence of molecular oxygen and water to give equimolecular amounts of the oxo-acid, ammonia and hydrogen peroxide:

$$\text{L-amino acid} + O_2 + H_2O \xrightarrow{\text{L-AAO}} \text{2-oxo acid} + NH_3 + H_2O_2, \quad (1)$$

$$\text{D-amino acid} + O_2 + H_2O \xrightarrow{\text{D-AAO}} \text{2-oxo acid} + NH_3 + H_2O_2. \quad (2)$$

Different techniques have been applied to monitor these reactions, e.g. amperometric [18], conductometric [27], chemiluminiscence [28, 29], and fluorescence methods [30]. Hydrogen peroxide can also be enzymatically converted in a successive indicator reaction by the action of HRP to form a red-coloured complex (a quinoneimine) with an absorbance maximum at 514 nm [31]:

$$H_2O_2 + \text{4-AP} + \text{DCPS} \xrightarrow[\text{DCP}]{\text{HRP}} \text{quinoneimine} + H_2O. \quad (3)$$

Stereoselective detection system

In the design of analytical IMERs used in the post-column chromatographic mode, the conversion efficiency should be optimized owing to its relation to the mean residence time which, in turn, is related to the dispersion [32, 33]. This should be taken into consideration to prevent band broadening of the chromatographic separation [34].

The selectivity and apparent activities of the immobilized L-AAO and D-AAO preparations were measured in the FIA mode by 50-μl injections of 0.5 mM amino acid solutions. A 0.1 m phosphate buffer, pH 7.0, was pumped (0.3 ml min^{-1}) through the injector to either the L-AAO or the D-AAO reactor. Chromogens were pumped (0.6 ml min^{-1}) in a second channel to a confluence point where the stream mixed with the sample stream. The volumes of the L-AAO reactors were 50 and 85 μl for the azo and the glutaraldehyde coupling, respectively. The volumes of the D-AAO reactors were 122 μl for the azo and 64 μl for the glutaraldehyde coupling, respectively. The results obtained with the L-AAO and D-AAO reactors are shown in Figs 2 and 3, respectively, and estimated as relative conversion values which are calculated in relation to that for phenylalanine [9, 18, 35]. The amino acids are considered according to the chemistry of their side chains. Both enzymes present a broad selectivity, but neither L-AAO nor D-AAO in either preparation showed any activity for non-chiral (glycine), or for aspartic, glutamic acids, or threonine.

The conversion efficiencies for L-phenylalanine were 90 and 82% for the azo and glutaraldehyde coupling, respectively. The conversion efficiencies for D-phenylalanine were 46 and 24% using the azo and glutaraldehyde coupling, respectively. L-AAO, azo coupling, showed conversion efficiencies higher than 55% for seven amino acids: leucine (92%), isoleucine (56%), phenylalanine (90%), tyrosine (90%), tryptophan (86%), cysteine (61%) and methionine (90%). Histidine, arginine, glutamine, alanine and

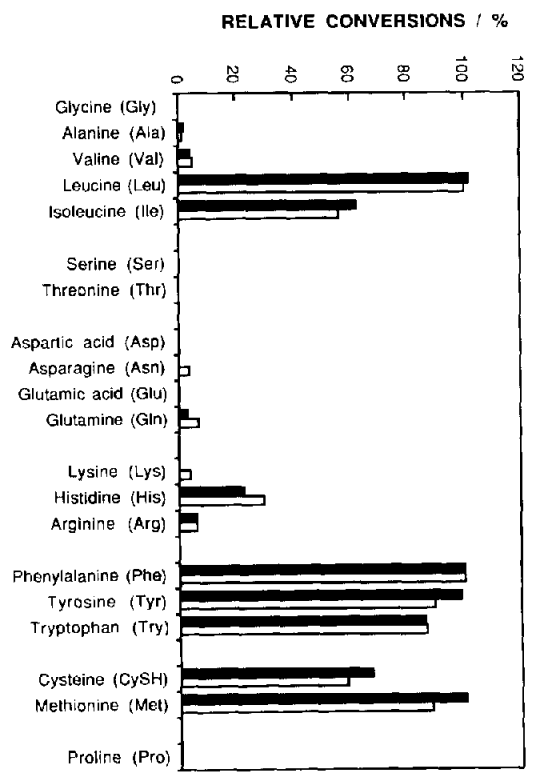

Figure 2
Relative conversion efficiencies of immobilized L-AAO for different L-amino acids using glutaraldehyde (□) and azo (■) coupling. For details, see text.

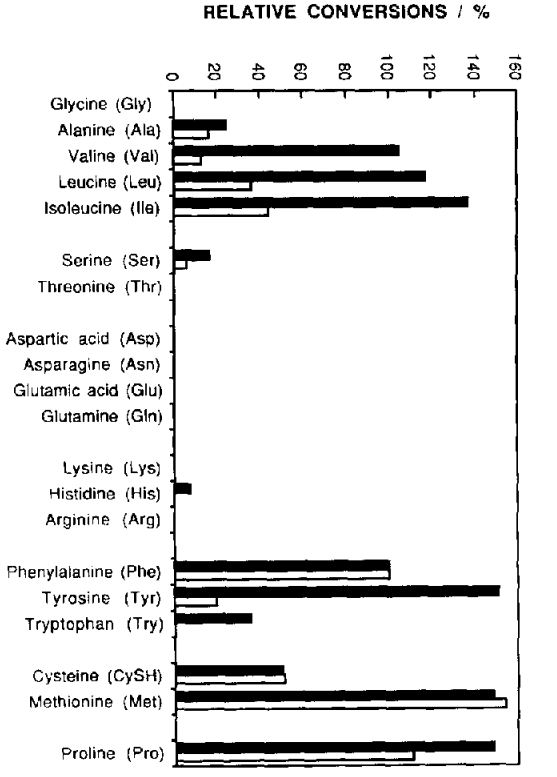

Figure 3
Relative conversion efficiencies of immobilized D-AAO for different D-amino acids using glutaraldehyde (□) and azo (■) coupling. For details, see text.

valine were oxidized with a lower efficiency due to the lower activity of the free enzyme for these amino acids [35]. D-AAO, with azo coupling, showed conversion efficiencies higher than 45% for: valine (48%), leucine (54%), isoleucine (63%), phenylalanine (46%), tyrosine (69%), methionine (68%) and proline (68%). Conversion efficiencies between 10 and 25% were found for alanine (11%), tryptophan (16%) and cysteine (23%). These conversions are evaluated in relation to the response for injections of stoichiometric amounts of hydrogen peroxide.

The azo coupling yields higher relative conversions mainly for D-AAO, indicating that different active enzyme loadings ($\alpha = k_2$ [E]/ $D_s K_M^{app}$) [36] were achieved in the azo and in the glutaraldehyde coupling. The loading factor depends on the inherent dissociation constant of the product from the enzyme (k_2), the concentration of enzyme in the support ([E]), the diffusion coefficient of the substrate (D_s), and the apparent Michaelis constant (K_M^{app}). Whether the differences found are due to different concentrations of active enzymes on the supports, or on a change in the kinetics of the immobilized enzymes, or both, after the different immobilization procedures, cannot be deducted. Similar results have been found for immobilized HRP [24]. The different chemistry involved in the two coupling procedures may modify the activity of the immobilized enzyme and contribute significantly to the conversion efficiency and ultimately to the band broadening observed [34].

Applications

The method was applied for the stereoselective determination of L- and D-amino acids in spiked serum samples with the manifold shown in Fig. 1. The mobile phase (0.4 ml min^{-1}) was a 75 mM sodium citrate buffer at pH 4.5. The make-up flow (0.3 ml min^{-1}) consisted of a 0.1 M pyrophosphate buffer at pH 10.4, making the enzymatic reaction proceed at pH 7.0 in the IMERs. The reagent

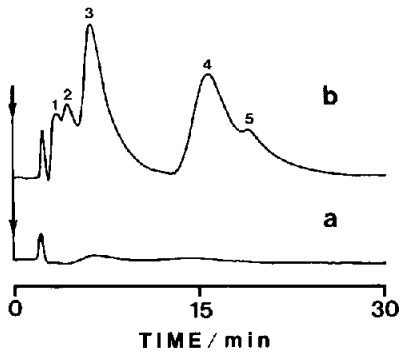

Figure 4
Chromatographic separation and post-column catalytic detection of: (a) deproteinized, filtered and 10× diluted human serum; and (b) spiked serum with (2) L-CySH 500 μM, (3) L-Leu 100 μM, (4) L-Phe 100 μM, and (5) L-His 500 μM using L-AAO and HRP reactors. (1) represents an unknown peak. For details, see text.

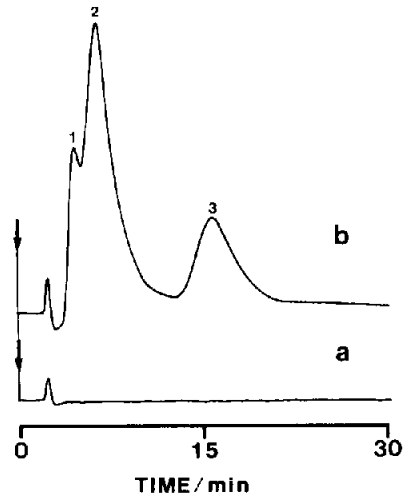

Figure 5
Chromatographic separation and post-column catalytic detection of: (a) deproteinized, filtered and 10× diluted human serum; and (b) spiked serum with (1) D-CySH 500 μM, (2) D-Leu 250 μM and (3) D-Phe 500 μM using D-AAO and HRP reactors. For details, see text.

solution (0.3 ml min^{-1}) consisted of: 1 mM 4-AP, 4 mM DCPS and 1 mM DCP [31]. The volumes of the reactors used in Figs 4 and 5 were 85 μl (1.5 mm i.d., length 48 mm) and 122 μl (2.4 mm i.d., length 27 mm) for the L-AAO glutaraldehyde coupling and the D-AAO azo coupling reactor, respectively. The peroxidase reactor was 177 μl (1.1 mm i.d., length 52.6 mm in volume).

Figure 4 shows the chromatograms obtained with a 100-μl injection of: (a) deproteinized, and 10 times diluted human serum; and (b) the same diluted serum after addition of L-phenylalanine (100 μM), L-leucine (100 μM), L-cysteine (500 μM) and L-histidine (500 μM). Figure 5 shows the chromatograms obtained when the D-AAO reactor was used with 100 μl of deproteinized, and with 10 times diluted human serum injected (a). The upper chromatogram (b) corresponds to spiked serum (10×) with D-phenylalanine (500 μM), D-leucine (250 μM) and D-cysteine (500 μM). Samples were filtered through sterile Millipore 0.22-μm membranes before being injected into the LC system.

Conclusion

The present work shows the usefulness of post-column enzymatic reactions for enantioselective detection in LC using achiral stationary and mobile phases. Further work is in progress to extend and improve the separation step. Wider applications of the method include the use of other enzymes, such as aminopeptidases and carboxypeptidases in the pre-column mode, enabling separation and detection of the released amino acids using the stereoselective amino acid oxidases, extending the application of LC–IMER (pre- and post-column mode) into new and promising areas.

Acknowledgements — The authors wish to thank the Waters Division of Millipore in Sweden, Carnegie Medicine AB and LKB AB for financial support or for the loan of instrumentation. Financial support from the National Energy Administration (STEV) and the Swedish Board for Technical Development (STU) is gratefully acknowledged.

References

[1] T.E. Kelly, in *Clinical Chemistry* (L.A. Kaplan and A.J. Pesce, Eds), pp. 832–850. C.V. Mosby, St Louis, MO (1984).
[2] I.M. Moodie, G.S. Shephard and D. Labadorios, *J. High Resolut. Chromatogr.* **12**, 509–516 (1989).
[3] M. Radjai and R.T. Hatch, *J. Chromatogr.* **196**, 319–322 (1980).
[4] N. Kiba and M. Kaneko, *J. Chromatogr.* **303**, 396–403 (1984).
[5] N. Seiler and B. Knodgen, *J. Chromatogr.* **341**, 11–21 (1985).
[6] C. de Jong, G.J. Hughes, E. van Wieringen and K.J. Wilson, *J. Chromatogr.* **241**, 345–359 (1982).
[7] L.A. Allison, G.S. Mayer and R.E. Shoup, *Anal. Chem.* **56**, 1089–1096 (1984).
[8] T. Kawasaki, M. Maeda and A. Tsuji, *J. Chromatogr.* **328**, 121–126 (1985).
[9] K. Miyaguchi, K. Honda and K. Imai, *J. Chromatogr.* **303**, 173–176 (1984).
[10] H. Brückner and M. Mausch, *Chromatographia* **28**, 487–492 (1989).
[11] K.H. Schleifer and O. Kandler, *Bact. Rev.* **36**, 407–412 (1987).

[12] B.W. Bycroft (Ed.), *Dictionary of Antibiotics and Related Substances*. Chapman and Hall, New York (1988).
[13] J.J. Corrigan, *Science* **164**, 142–149 (1969).
[14] A. Fox, S.L. Morgan, in *Instrumental Methods for Rapid Microbiological Analysis* (W.H. Nelson, Ed.), p. 135. VCH, Deerfield Beach, FL (1985).
[15] W.L. Hinze, T.E. Riehl, D.W. Armstrong, W. DeMond, A. Alak and T. Ward, *Anal. Chem.* **57**, 237–242 (1985).
[16] P. Jadaud, S. Theloan, G.R. Schonbaum and I.W. Wainer, *Chirality* **1**, 38–44 (1989).
[17] R. Kuropka, B. Miller, H. Höcker and H. Berndt, *J. Chromatogr.* **481**, 380–386 (1989).
[18] T. Yao and T. Wasa, *Anal. Chim. Acta* **209**, 259–264 (1988).
[19] G. Marko-Varga, *Anal. Chem.* **61**, 831–838 (1989).
[20] H. Jansen and R.W. Frei, in *Selective Sample Handling and Detection in High-Performance Liquid Chromatography*, Part B (R.W. Frei and K. Zech, Eds), pp. 208–233. Elsevier, Amsterdam (1989).
[21] G. Marko-Varga, E. Domínguez, B. Hahn-Hägerdal and L. Gorton, *J. Pharm. Biomed. Anal.* **8**, 817–823 (1990).
[22] G. Gübitz, in *Selective Sample Handling and Detection in High-Performance Liquid Chromatography*, Part A (R.W. Frei and K. Zech, Eds), pp. 154–207. Elsevier, Amsterdam (1988).
[23] G. Marko-Varga and L. Gorton, *Anal. Chim. Acta* **234**, 13–29 (1990).
[24] B. Olsson and L. Ogren, *Anal. Chim. Acta* **145**, 87–99 (1983).
[25] R.A. Messing and H.H. Weetall, U.S. Pat., 3 515 538 (1970).
[26] E. Domínguez, G. Marko-Varga, B. Hahn-Hägerdal and L. Gorton, *Anal. Chim. Acta* **213**, 139–150 (1988).
[27] D.W. Taylor and T.A. Nieman, *J. Chromatogr.* **368**, 95–102 (1986).
[28] H. Jansen, U.A.Th. Brinkman and R.W. Frei, *J. Chromatogr.* **440**, 217–223 (1988).
[29] S. Igarashi and W.L. Hinze, *Anal. Chim. Acta* **225**, 147–157 (1989).
[30] N. Kiba and M. Kaneko, *J. Chromatogr.* **303**, 396–403 (1984).
[31] B. Olsson, *Mikrochim. Acta* **2**, 211–221 (1985).
[32] G. Johansson, L. Ögren and B. Olsson, *Anal. Chim. Acta* **145**, 71–85 (1983).
[33] Y.T. Shih and P.W. Carr, *Anal. Chim. Acta* **167**, 137–144 (1985).
[34] R.W. Frei, H. Jansen and U.A.Th. Brinkman, *Anal. Chem.* **57**, 1529A–1539A (1985).
[35] L.A. Lichtenberg and D. Wellner, *Anal. Biochem.* **26**, 313–319 (1968).
[36] L.D. Bowers, *Anal. Chem.* **58**, 513A–530A (1986).

[*Received for review 25 April 1990*]