ELSEVIER

Journal of Chromatography A, 672 (1994) 109–115

JOURNAL OF CHROMATOGRAPHY A

Comparison of the recovery of amino acids in vapour-phase hydrolysates of proteins performed in a Pico Tag work station and in a microwave hydrolysis system ☆

Enikö Tatár, Mohamed Khalifa, Gyula Záray, Ibolya Molnár-Perl*

Institute of Inorganic and Analytical Chemistry, L. Eötvös University, P.O. Box 32, H-1518 Budapest 112, Hungary

(First received August 23rd, 1993; revised manuscript received February 22nd, 1994)

Abstract

An exhaustive study was made to determine the amino acid composition of lysozyme in hydroysates performed in a CEM microwave oven under various conditions [0.621–0.827 MPa (90–120 p.s.i.g.) for 5, 10, 20 and 40 min], determined by HPLC as their phenylthiocarbamyl derivatives. The results showed that the CEM microwave hydrolysis system works reproducibly up to 0.689 MPa (100 p.s.i.g.) pressure only. Uniformly optimum parameters for all components present in hydrolysates were not found. The recoveries of the components at 0.621 MPa (90 p.s.i.g.) (after 20 and 40 min) and at 0.689 MPa (100 p.s.i.g.) (after 10 20 and 40 min) varied from 63 to 111%. The reproducibility of the measurements was <8.0% (relative standard deviation).

1. Introduction

Pioneering microwave hydrolyses have been achieved in cooking apparatus [1–4] and proved to be very promising; *e.g.*, for native and oxidized ribonuclease A and insulin B [1], after 5 min of microwave hydrolysis the recoveries of amino acids were the same as or higher than those obtained by the conventional method (6 *M* HCl, 110°C, 24 h).

Outstandingly high recoveries have been reported for microwave hydrolysed N-*tert.*-butoxycarbonyl (N-t-Boc) amino acid resin esters [2], with the only exception being N-t-Boc-leucine (97.6%). All the other amino acids provided 99.7–113.8% recoveries, with the exception of the serine derivative, the recovery of which was repeatedly 343%. However, pressure and temperature conditions were not reported [1,2]. Subsequently [3,4], hydrolyses of lysozyme performed with methanesulphonic acid + tryptamine [3-(2-aminoethyl)indole] and with hydrochloric acid provided favourable results, in both instances applying optimum conditions, *i.e.*, 160°C for 45 min for methanesulphonic acid + tryptamine [3] and 178–180°C for 4 min for 6 *M* HCl (the optimum temperature was tentatively estimated by the "indirect method" [4], conducting preliminary calibration of the microwave oven by use of several organic compounds with known melting points).

A special "microwave hydrolysis system" supplied with a capping station, a manifold, a

* Corresponding author.

☆ Presented at the *9th Danube Symposium on Chromatography, Budapest, August 23–27, 1993.* The proceedings of the symposium were published in *J. Chromatogr. A*, Vol. 668, No. 2 (1994).

0021-9673/94/$07.00
SSDI 0021-9673(94)00184-B

turntable with vials and a fibre-optic thermometer (for details see Experimental) was introduced by CEM in 1989 [5]. Originally [5], the vapour-phase microwave hydrolysis of methionyl human growth hormone was performed in the CEM apparatus at 178°C and 0.910 MPa (132 p.s.i.g.) for 8, 10 and 12 min. The results obtained [6] were poorer than those published previously [1–4]. Identical optimized hydrolysis conditions for all sixteen amino acids tested were not found. The maximum recoveries of the single components varied between 93 and 145%, depending on the experimental conditions used, with the exceptions of threonine and serine (93% and 77%, respectively).

On the other hand, the microwave technique showed beneficial effects in the hydrolysis of hydrophobic contact-containing peptides [7], although the recoveries of amino acids not involved in the hydrophobic linkages of the same peptides were not presented.

Special applications of microwave irradiation [8–10], such as its utilization in protein detection [8], in proline determination [9] and in the determination of the components of tripeptides [10] have been reported.

An increased hydrolysis time (2 and 4 h at 125°C) for a bovine serum albumin sample provided nearly theoretical amino acid yields [11]. However, such a large increase in hydrolysis time makes the application of the very expensive microwave technique questionable. In Hewlett-Packard application note [6], the traditional gas-phase hydrolysis (24 h at 110°C) was compared with fast gas-phase hydrolysis (90 min at 150°C) and microwave heating gas-phase hydrolysis (20–25 min at 150°C). The data obtained by the three methods for eleven peptides and proteins were in an excellent agreement, but reproducibility data were not reported.

Recently, a review has been published [12] outlining the possibilities of the microwave technique in analytical chemistry. It was stated that because of the few references available, researchers are hesitating to purchase this expensive apparatus.

Continuing our earlier studies (performed in order to optimize the determination of amino acids in protein hydrolysates as their phenylthiocarbamyl [PTC] derivatives by HPLC [13–18]), in this work we surveyed the diverse literature data with respect to the recovery of amino acids from microwave-hydrolysed proteins [1–2] and examined the impact of microwave hydrolysis on the composition of lysozyme as an example in comparison with what was obtained from hydrolyses [13–18] carried out in a Pico Tag work station system.

2. Experimental

2.1. Materials

Triethylamine (TEA), phenyl isothiocyanate (PITC), amino acids and proteins were obtained from Sigma (St. Louis, MO, USA) and Serva (Heidelberg, Germany). HPLC-grade acetonitrile and methanol were purchased from Reanal (Budapest, Hungary). All other reagents were of the highest purity available.

2.2. Apparatus

Hydrolyses were performed both in a Pico-Tag work station (Waters, Milford, MA, USA) (henceforth, work station) and in a CEM (Matthews, NC, USA) MDS-2000 microwave hydrolysis system, consisting of three major components, the vessel capping station, the vapour- and liquid-phase hydrolysis vessels and the turntable and valve panel for vacuum evacuation and nitrogen purging (henceforth, CEM hydrolysis system). The same hydrolysis and derivatization tubes (Waters) were used for both types of hydrolysis apparatus.

2.3. Hydrolysis of proteins

Lysozyme (0.014–0.016 g, weighed with analytical precision) was dissolved in 10 ml of 0.1 *M* HCl. Standards of free amino acids in a mixture containing 5 μmol/ml of each were prepared in 0.1 *M* HCl. Tryptamine was dissolved in distilled water (2 μg/μl). Into a 50 × 6 mm I.D. tube, 5 μl of amino acid solution or 20 μl of lysozyme

solution were injected and 15 μl of tryptamine stock solution were added to each. Twelve tubes were placed in a Waters vacuum vial. The vial was then attached to the work station manifold and the solvent removed under vacuum. After drying, the vacuum was released and the samples were ready for hydrolyses.

Hydrolyses which serve as the basis of comparison were carried out in the work station at 145°C for 4 h, as described previously [16,17]. In the microwave hydrolyses, four tubes were hydrolysed simultaneously in three vessels, using the same procedure (at the same pressure and temperature). Of the four vessels available (in order to have four parallel hydrolysis tests), two tubes were placed in one vessel and 1-1 tube in the two others. The fourth vessel was reserved for the fibre-optic thermometer. Hydrolyses were performed in the presence of 5 and 10 ml of distilled HCl, poured into all four vessels. After sealing the vessels by means of the electronic capping station and connecting them with the turntable to the apparatus (both to the fibre-optic thermometer and to the valve panel), the vessels were evacuated and purged with nitrogen five times in succession.

Microwave hydrolyses were carried out at 0.621 MPa (90 p.s.i.g.) and 0.689 MPa (100 p.s.i.g.) for 5, 10, 20 and 40 min, and at 0.758 MPa (110 p.s.i.g.) and 0.827 MPa (120 p.s.i.g.) for various reaction times (0–20 min). The hydrolysed sample-containing tubes (after releasing the caps of the vessels with the help of the capping station) were wiped from the outside and were transferred into reaction vials of the work station. The vials were attached to the manifold and dried to a constant minimum reading [*ca.* 65 mTorr (8.65 Pa)]. The hydrolysed samples were then ready for derivatization.

2.4. Derivatization of amino acids with PITC

Free amino acids and hydrolysed proteins, previously dried under vacuum, were re-dried after adding 10 μl of ethanol–water–TEA (2:2:1) to each tube. Thereafter, to each re-dried sample, 20 μl of derivatization reagent [ethanol–TEA–H_2O–PITC (7:1:1:1)] were added and vortex mixed. The test-tubes containing derivatized samples were then transferred to the reaction vials, installed in the work station, evaporated to a minimum reading of *ca.* 65 mTorr and held at this constant reading for 20 min.

The derivatized standards were dissolved by stepped addition of 50 μl of acetonitrile, 50 μl of H_2O and 500 μl of 0.05 *M* sodium acetate solution (pH 7.2); 20-μl aliquots of standards contained *ca.* 1700 pmol of each amino acid.

2.5. Chromatography

The system used was a Liquochrom Model 2010 liquid chromatograph (Labor MIM, Budapest, Hungary) which consisted of a two-Liquopump 312/1 solvent-delivery system and a Type OE-308 UV detector with a wavelength range of 195–440 nm. Samples were injected in 20-μl volumes using an injector supplied by Labor MIM. The 150 mm × 4.0 mm I.D. columns (BST, Budapest, Hungary) contained Hypersil ODS bonded phase (5 μm) (Shandon, Runcorn, UK). Eluents we kept under a blanket of nitrogen. The solvent system consisted of two eluents: (A) 0.05 *M* sodium acetate (pH 7.2) and (B) 0.1 *M* sodium acetate–acetonitrile–methanol (46:44:10, v/v/v) titrated with glacial acetic acid or 50% sodium hydroxide to pH 7.2. The gradient, which was optimized for the separation, was from 0 to 100% B in 22 min, then 5 min with 100% B, followed by 2 min with 100% A. After an additional 3 min, elution with solvent A was performed. Thereafter the system was ready for the next injection.

3. Results and discussion

3.1. Microwave parameters

Advances in the HPLC determination of amino acids in protein hydrolysates now ensure rapid derivatization and elution procedures and the rate-limiting step in amino acid analysis remains the hydrolysis. Therefore, a need exists to speed up the hydrolysis step in protein analysis.

A shortened time (4 h at 160°C) for liquid-phase hydrolyses was introduced by Gehrke *et al.* [19], and a useful vapour-phase technique (4 h at 145°C) is offered by Waters (work station) [20]. Both of these possibilities provided the same values for amino acid components as the classical liquid-phase procedure (6 *M* HCl, 24 h, 110°C). Recently we demonstrated [17] that by means of the work station the same recovery can be obtained for all essential amino acids, also under milder conditions (145°C for 2 h and 125°C for 5 h).

From our detailed experience with the work station [13–17], we intended to start with probably the most advantageous microwave conditions proposed for the CEM hydrolysis system, *i.e.*, with the shortest hydrolysis time, which means also with the highest pressure [0.827 MPa (120 p.s.i.g.)], as advised in the manual [21]. The power applied was 650 W throughout.

At the beginning of our experiments with the CEM station, we attempted to work with the shortest possible time and at the highest pressure [0.827 MPa (120 p.s.i.g.)] However, of the four hydrolysis flasks supplied with the apparatus, only one was tight enough to withstand this pressure. The producer states [22] that it is not possible to operate the CEM station at maximum pressure, *i.e.*, above 0.621 MPa (91 p.s.i.g.). Therefore, remaining on the safe side, our investigations were performed at constant pressures, *i.e.*, at 0.621 and 0.689 MPa (90 and 100 p.s.i.g.) for different hydrolysis times (under the latter conditions the CEM hydrolysis system works excellently).

3.2. Microwave hydrolysis and recovery study

Lysozyme (see also refs. 15–17) was used as a standard protein. The results obtained were compared with the data provided by the work station applying vapour-phase hydrolysis for 4 h at 145°C (Table 1, values in braces). The following conclusions can be drawn.

(1) General optimum conditions applicable to all amino acids apparently cannot be established and the laboratory routine will always be some sort of compromise.

(2) In accordance also with literature data [4,11], the yields of threonine, cyst(e)ine, isoleucine, valine and tryptophan were lower than the values of the reference methods under all conditions tested.

(3) Nearly the theoretical recovery could be obtained for serine, in order of listing, at 0.621 and 0.689 MPa (90 and 100 p.s.i.g.) for 10 and 5 min, similar to that reported at 180°C for 4 min (pressure not given) [4]. After longer hydrolysis times (40, 40 and 20 min), even under mild conditions at 0.621, 0.689 and 0.379 MPa (90, 100 and 55 p.s.i.g.), considerable losess of serine were observed, 15, 27 and 10% [17], respectively.

(4) The recovery of histidine is usually high, being 175% at 180°C after 4 min [4]. According to our data, the histidine recovery increases with increasing pressure and hydrolysis time at 0.621 MPa (90 p.s.i.g.) (55, 86, 83 and 100%) and at 0.621 MPa (100 p.s.i.g.) (90, 96, 100 and 104%), respectively (see Table 1).

(5) Tyrosine proved to be very sensitive to the hydrolysis conditions: (i) at 180°C after 4 min only an 80% recovery was reported [10], whereas (ii) according to our results at 0.621 MPa (90 p.s.i.g.) the cleavage of its peptide bond needed at least 10 min.

(6) Regarding the expected amount of lysine, the cleavage of its peptide bond needs stronger conditions: at 0.621 MPa (90 p.s.i.g.) after only 40 min and at 0.689 MPa (100 p.s.i.g) after any hydrolysis time the amount of lysine proved to be satisfactory. The reported [10] 119% yield at 180°C after 4 min is unlikely, to our knowledge.

(7) The recoveries of the rest of amino acids, are within acceptable experimental error.

3.3. Reproducibility

The microwave parameters proved to be highly reproducible: working at constant pressures the chosen values were reached in 2.0–2.5 min, and during the period of the hydrolyses the temperature at 0.621 ± 0.02 MPa (90 ± 3 p.s.i.g.) varied between 152 and 157°C (Fig. 1a), and at 0.689 ± 0.02 MPa (100 ± 3 p.s.i.g.) between 154 and 157°C (Fig. 1b). In our system, at 0.379 MPa

Table 1
Recovery of amino acids from microwave hydrolysate of lysozyme obtained under different conditions

Conditions			Amino acid (%)[a]																	
Pressure (MPa) (p.s.i.g.)	Time (min)	Reproducibility	Aspartic acid	Glutamic acid	Serine	Glycine	Histidine	Threonine	Alanine	Proline	Arginine	Tyrosine	Valine	Methionine	Cyst(e)ine	Isoleucine	Leucine	Phenylalanine	Tryptophan	Lysine
			(20) {18.0}	(5) {5.0}	(18) {6.6}	(12) {10.1}	(1) {1.0}	(7) {5.2}	(12) {12.6}	(2) {2.0}	(11) {9.0}	(3) {2.9}	(6) {4.9}	(2) {1.9}	(8) {8.9}	(6) {4.7}	(8) {7.5}	(3) {2.5}	(6) {5.1}	(6) {6.6}
0.621	5	*A*	79	84	84	92	55	53	73	73	60	69	39	83	53	70	82	43	53	66
(90)		S.D.	1.2	6.3	1.5	2.7	3.2	2.0	3.6	6.1	4.3	5.0	3.6	4.0	4.5	2.7	1.3	4.9	2.5	3.9
		R.S.D. (%)	1.5	7.6	1.8	2.9	5.8	3.7	4.3	8.4	7.2	7.2	9.2	4.8	8.5	3.9	1.6	11.4	4.7	5.9
0.621	10	*A*	95	82	103	105	86	88	104	105	93	95	70	98	66	86	97	68	83	87
(90)		S.D.	0.73	5.5	1.8	3.0	5.7	2.1	1.0	7.8	1.7	1.1	3.5	10.3	10.8	2.6	1.4	9.8	10.7	13.9
		R.S.D. (%)	0.8	6.7	1.7	2.9	6.6	2.3	1.0	7.4	1.8	1.2	5.0	10.5	16.4	3.0	1.4	14.4	12.9	16
0.621	20	*A*	108	75	96	110	83	93	99	98	98	100	68	102	90	92	94	63	92	86
(90)		S.D.	0.5	4.7	1.6	1.9	5.8	2.1	1.9	0.3	1.3	6.0	6.1	2.6	6.3	1.8	2.4	12.6	3.0	1.0
		R.S.D. (%)	0.5	6.7	1.7	1.7	7.0	2.3	1.9	3.3	1.3	6.0	8.9	2.5	7.0	2.0	2.5	2.0	3.3	1.2
0.621	40	*A*	80	105	85	105	100	78	97	96	101	94	82	96	81	97	100	76	79	100
(90)		S.D.	2.6	4.7	2.4	1.4	1.1	8.6	0.4	1.1	0.6	4.1	6.2	6.0	5.8	1.5	0.3	4.6	4.8	3.4
		R.S.D. (%)	3.2	4.7	2.8	1.3	1.1	1.1	0.4	1.1	0.6	4.4	7.5	7.3	7.2	1.5	0.3	6.0	6.1	3.4
0.689	5	*A*	84	85	106	87	94	86	85	85	86	115	60	105	71	64	82	95	85	100
(100)		S.D.	13.9	6.5	14.8	4.0	1.4	12.2	2.0	5.9	5.1	7.9	0.5	5.7	15.2	1.3	1.2	5.5	12.2	4.8
		R.S.D. (%)	16.6	7.7	14.0	4.6	1.5	14.2	2.4	6.9	5.9	6.9	0.9	5.4	21.4	0.2	1.5	5.8	14.4	4.8
0.689	10	*A*	86	90	95	98	96	77	106	100	85	97	75	92	73	78	89	111	80	101
(100)		S.D.	0.39	2.0	2.1	0.7	4.0	1.2	0.8	2.0	1.5	3.9	3.2	5.3	2.9	2.3	1.3	5.9	5.8	2.2
		R.S.D. (%)	0.45	2.2	2.2	0.7	4.2	1.5	0.8	2.0	1.8	3.9	4.2	5.8	4.0	3.0	1.5	5.3	7.3	2.2
0.689	20	*A*	92	95	84	98	100	77	108	100	89	93	88	93	95	85	105	110	67	99
(100)		S.D.	2.6	5.6	3.4	1.4	2.0	4.2	5.2	3.7	1.2	5.9	4.8	6.7	5.5	4.8	2.5	4.6	5.4	3.2
		R.S.D. (%)	2.8	5.9	4.0	1.4	2.0	0.54	4.8	3.7	1.4	6.3	5.4	7.2	5.8	5.7	2.4	4.2	8.0	3.2
0.689	40	*A*	95	101	73	101	104	76	108	100	96	95	98	92	88	94	104	112	63	102
(100)		S.D.	4.7	3.6	2.3	4.2	4.4	1.6	4.3	7.7	2.8	3.8	4.5	6.9	4.6	4.5	3.3	3.6	4.7	4.7
		R.S.D. (%)	4.9	3.6	3.1	4.2	4.2	2.2	4.0	7.7	2.9	4.0	4.6	7.5	5.2	4.8	3.2	3.2	7.4	4.6
Literature values		Ref.																		
n.m.	4	10	92	88	102	94	175	100	100	n.m.	91	80	98	105	92	104	89	104	n.m.	119
0.379(55)	20	17	100	102	90	93	100	90	100	91	93	110	87	95	93	93	99	97	n.m.	87

[a] Expressed as percentages of data obtained from hydrolyses performed in the work station values, in parentheses, literature values given in ref. 19; values in braces, values of lysozyme components expressed in pmol; these serve as the basis of comparison. *A* = Averages of four separate hydrolyses performed in three vials; S.D. = standard deviation; R.S.D. = relative standard deviation; n.m. = not measured.

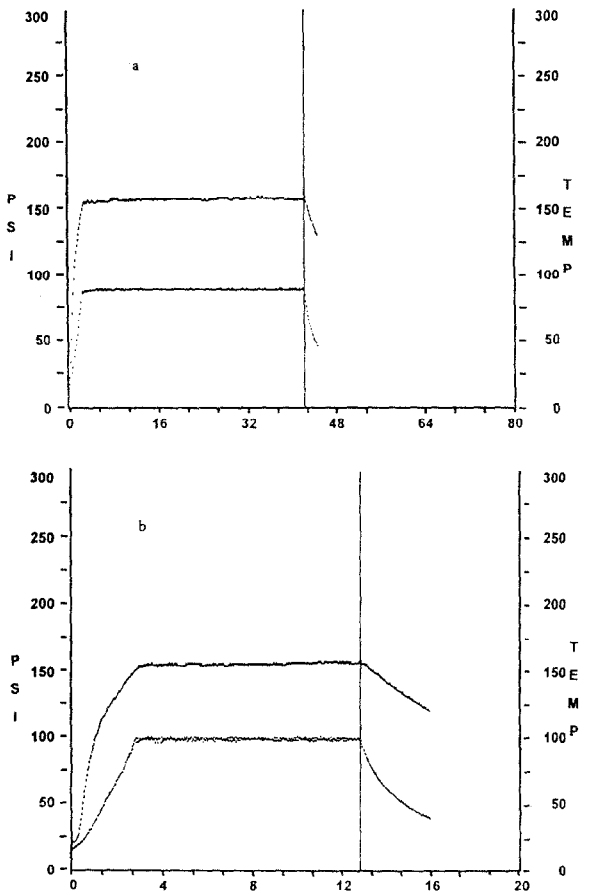

Fig. 1. Variation of temperature (upper curve) and pressure (lower curve) for a microwave hydrolysis system operating at (a) 0.621 MPa (90 p.s.i.g.) and (b) 0.689 MPa (100 p.s.i.g.). The traces are the original curves given by the CEM apparatus, *i.e.*, values on the abscissa are the hydrolysis time in minutes, and on the ordinate both the temperature (in °C) and the pressure (in p.s.i.) are presented.

(55 p.s.i.g.) the temperature did not exceed 125°C (Fig. 1a and b), in spite of the fact that strictly the same parameters as reported in ref. 11 were followed.

Generally, the reproducibility, characterized by the relative standard deviation (R.S.D.) (Table 1), is higher than in previous experiments [13–17]. In the case of microwave-hydrolysed lysozyme, the highest error can be attributed mainly to incomplete hydrolysis [tests performed at 0.621 MPa (90 p.s.i.g.) for 5 and 10 min and at 0.689 MPa (100 p.s.i.g.) for 5 min, and also for all values for phenylalanine]. Regarding the other data, the reproducibility of these measurements proved to be <8.0% (R.S.D.).

4. Conclusions

The microwave irradiation procedure shortened the hydrolysis time of lysozyme considerably. As optimum hydrolysis conditions could not be defined uniformly for all essential amino acids, we suggest its use in the following cases: (1) the sample to be hydrolysed contains a limited and known number of peptide bonds (mainly synthetic short-chain peptides); the optimization of the conditions seems to be easier than for natural proteins and/or protein matrices; and (2) the microwave apparatus is needed also for other purposes, or the samples to be hydrolysed require the entire capacity of the apparatus.

Acknowledgement

This work was supported by the Hungarian Academy of Sciences (Project Numbers OTKA I/3 2284 and I/4 T5053).

References

[1] S.T. Chen, S.H. Chiou, Y.H. Chu and K.T. Wang, *Int. J. Pept. Protein Res.*, 30 (1987) 572.
[2] H.M. Yu, S.T. Chen, S.H. Chiou and K.T. Wang, *J. Chromatogr.*, 456 (1988) 357.
[3] S.H. Chiou and K.T. Wang, *J. Chromatogr.*, 448 (1988) 404.
[4] S.H. Chiou and K.T. Wang, *J. Chromatogr.*, 491 (1989) 424.
[5] C. Woodward, L.B. Gilman and W.G. Englehart, *Int. Lab.*, 20 (1990) 40.
[6] R. Grimm, *Hewlett-Packard Publication Number 1-5091-4585E*, Hewlett-Packard, Avondale, PA, 1992.
[7] L.B. Gilman and C. Woodward, in *Current Research in Protein Chemistry*, Vol. 23, Academic Press, New York, 1990, p. 13.
[8] H. Engelhardt, M. Kramer and H. Waldhoff, *Chromatographia*, 30 (1990) 523.

[9] K. Kolar and H. Berg, in *Meat and Meat Products*; *Publication 1990-04-17 KK Ba K 0924a*, Swedish Meat Research Institute, 1990.
[10] A. Pecavar, M. Prosek, D.F. Temeljotov and J. Marsel, *Chromatographia*, 30 (1990) 159.
[11] S.A. Margolis, L. Jassie and H.M. Kingston, *J. Autom. Chem.*, 13 (1991) 93.
[12] A. Sinquin, T. Görner and E. Dellacherie, *Analusis*, 21 (1993) 1.
[13] M. Morvai, V. Fábián and I. Molnár-Perl, *J. Chromatogr.*, 600 (1992) 87.
[14] I. Molnár-Perl and M. Morvai, *Chromatographia*, 34 (1992) 132.
[15] I. Molnár-Perl, M. Pintér-Szakács, M. Morvai and V. Fábián, in I. Ishiguro, R. Kido, T. Nagatsu, Y. Nagamura and Y. Ohta (Editors), *Advances in Tryptophan Research 1992*, Fujita Health University Press, Toyoake, 1993, pp. 195–198.
[16] I. Molnár-Perl, M. Pintér-Szakács and M. Khalifa, *J. Chromatogr.*, 632 (1993) 57.
[17] I. Molnár-Perl and M. Khalifa, *Chromatographia*, 36 (1993) 43.
[18] I. Molnár-Perl, *J. Chromatogr.*, 661 (1994) 43.
[19] C.W. Gehrke, L.L. Wall, J.S. Absheer, F.E. Kaiser and R.W. Zumwalt, *J. Assoc. Off. Anal. Chem.*, 68 (1985) 811.
[20] *Pico Tag Work Station Operator's Manual*, Manual Number 86746, Revision C, Waters, Milford, MA, 1988.
[21] W.G. Engelhart, personal communication.
[22] *Installation and Operation Manual: Microwave Sample Preparation System MD-S 2100*, CEM, Matthews, NC, 1991, p. IV-14-24.