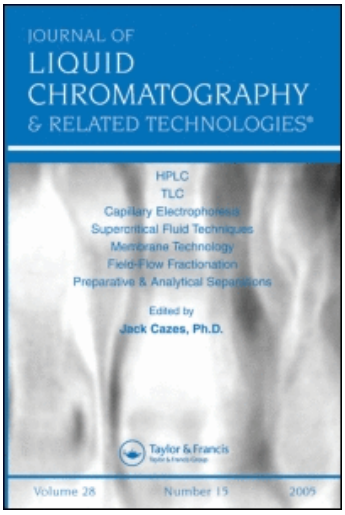

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Automated Optimised High Performance Liquid Chromatographic Analysis of Pre-Column O-Phthaldialdehyde-Amino Acid Derivatives

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AUTOMATED OPTIMISED HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF PRE-COLUMN O-PHTHALDIALDEHYDE-AMINO ACID DERIVATIVES

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ABSTRACT

The feasibility of injecting o-phthaldialdehyde/2-mercaptoethanol reagent and amino acid mixture simultaneously, from separate injector vials in order to allow automation of HPLC pre-column amino acid derivative analysis was investigated. The modified duel injection system described proved to be successful. Reaction of amino acids and reagent takes place during flushing of the injection loop. This is immediately followed by the introduction of the formed derivatives into the main HPLC eluent stream leading to the chromatographic column. The described procedure gave reproducible results with no loss in sensitivity. Further established was the optimum pH of reagent for derivative formation and of eluents for the separation of derivatives.

INTRODUCTION

Automated amino acid analysis systems in use today are based predominately on the ninhydrin procedure.[1] This procedure requires expensive specialized equipment, analysis times of generally 2 to 3 hours, and high running costs.

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Over the past several years numerous high performance liquid chromatographic (HPLC) amino acid analysis procedures have been described. The latter is rapid and simple and the instrument can be used for other types of analyses if necessary. The amino acid HPLC methods include both pre-column and post-column derivatising. However, various factors, primarily the instability of amino acid derivatives, have made the automation of these methods (particularly pre-column derivatization) extremely difficult. Jones, Paabo et al[2] have recently described a non-automated procedure in which o-phthaldialdehyde was used successfully as a pre-column derivatizing reagent.

A mixture containing 26 of these derivatives was efficiently resolved by these workers with an analysis time of less than 35 minutes. In this report the automation of Jones' method was investigated. The optimum reagent pH and solvent pH whereunder the amino acid derivatives are formed and subsequently separated and detected were also determined with this study. The applicability of the developed automated analysis to beerwort and beer is demonstrated.

MATERIALS AND METHODS

Apparatus

The HPLC system used for this study consisted of:

(a) Micromeritics Model 750 solvent delivery system;

(b) Micromeritics Model 731 column compartment with variable temperature control from ambient to 150°C;

(c) Micromeritics Model 725 auto-injector modified to inject the contents of two sample vials simultaneously through a 5 µl injection loop;

(d) Micromeritics Model 753 ternary solvent mixer;

(e) Micromeritics Model 740 Microcomputer based module with printer.

(f) Kratos FS Fluorimat detector equipped and set up as follows: 20 µl flow cell, a FSA 403 excitatation filter and FSA 111 lamp for excitation in the 330-375 nm range. The emission was measured with a FSA 426 (418 nm cut-off) filter, sensitivity dial setting at 6.6 units and the range 0.05;

(g) Hewlett Packard Model 3390A recorder/integrator;

(h) Altex Ultrasphere ODS column (250 x 4.6 mm); particle size 5µm

and

(i) Brownlee Guard Column with a 5 µm C18 cartridge fitted between the solvent delivery system and sample injector for filtering of the eluent only;

(j) Solvent A was tetrahydrofuran : methanol : 0.05 M sodium acetate (pH 7.5) 4:95:400 and solvent B was methanol : 0.05 M sodium acetate (pH 7.5) 8:2. Solvent C was methanol. Further details of the chromatographic procedure are given in the figure legends.

Reagents and Standards

Amino acid standards, internal standard β-alanine, sodium acetate, boric acid and 2-mercaptoethanol were purchased from Merck Chemicals. O-phthaldialdehyde was obtained from Sigma Chemical Co. HPLC grade methanol used as an eluent was purchased from Waters Associates and Merck Chemicals.

Preparation of o-phthaldialdehyde/2-Mercaptoethanol reagent

200 mg of o-phthaldialdehyde was dissolved in 5 cm^3 absolute methanol. To this was added 200 µl 2-mercaptoethanol and 90 cm^3 0,4 mol dm^{-3} boric acid (pH = 10.3). The mixture was flushed with argon and stored in a refrigerator.

Preparation of Amino Acid and Internal Standard Solutions

A mixture of 19 primary amino acids (excluding cystine) and 4-amino butyric acid was made so as to match approximately the

concentration of amino acids in a standard lager beerwort (e.g. Table I). The solution was transferred to smaller bottles, 3 drops of pentachlorophenol solution (330 mg PCP/50 cm^3 ethanol) added to each bottle as a preservative and stored at -20°C. The internal standard, βeta-alanine solution (50 mg/dm^3) was prepared separately and treated in the same manner as the amino acid standard mixture.

Beerwort and Beer

Beerwort and beer samples were centrifuged at 14 000 rpm, filtered through Kieselguhr to degas and finally Millipore filtered (0,45 μm).

Preparation of samples and reagent for Chromatography

200 μl of internal standard solution was added to 200 μl aliquots of amino acid standard solution, beerwort and beer respectively. Each sample was then made up to 800 μl with 0,6% Brij 35. The 800 μl aliquots of the prepared samples were then placed in every alternate injection vial of the auto-injector. 800 μl aliquots of the o-phthaldialdehyde/2-mercaptoethanol reagent solution were placed in the open positions left between the samples.

RESULTS AND DISCUSSION

A modified version of the conditions of chromatography described by Jones, Paabo et al[2] produced the amino acid standard mixture chromatogram shown in Figure I. The optimum column temperature for chromatography was found to be 38°C. This also reduced column backpressure and would ultimately lead to increased column life. Diluting standard mixture samples with methanol enhanced fluorescence of peaks generally and specifically lysine. However, the use of the surfactant Brij-35 was continued as methanol forms a precipitate when added to beerwort.

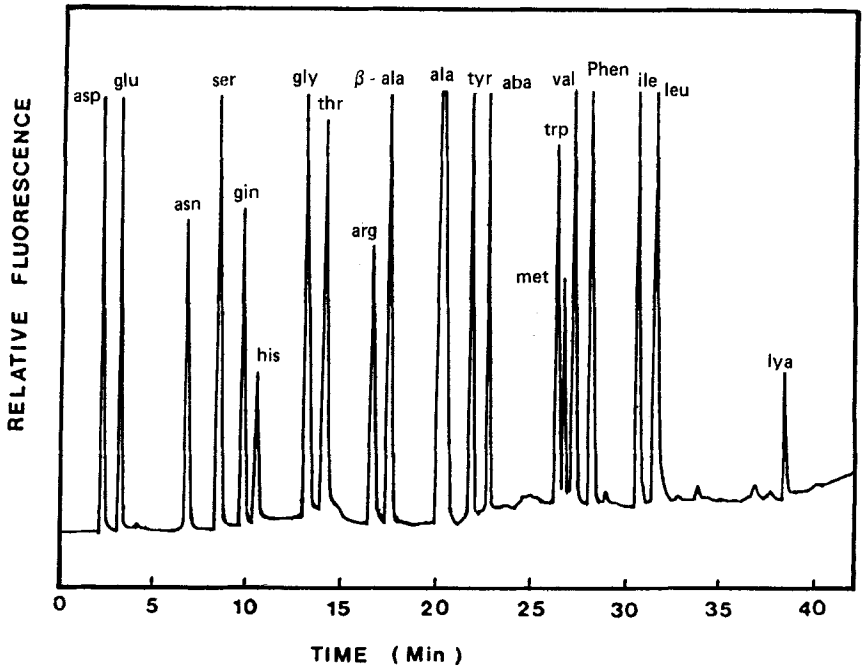

Figure I. Chromatogram of automated analysis of a standard aminoacid mixture. Conditions: Solvent A, methanol :0.05M potassium acetate (pH 7.5):tetrahydrofuran, 400:95:4; Solvent B, methanol : 0.05M potassiumacetate (pH 7.5), 8:2, Solvent C, methanol; program: 100% A isocratic step for 11 min, inject sample, 100% A to 80% A, 20% B linear step in 15 min,80% A, 20% B isocratic step for 3 min, immediate step to 41% A, 59% B, 41% A, 59% B isocratic step for 1 min., immediate step to 40% A, 60% B, 40% A, 60% B isocratic step for 3 min, linear step to 30% A, 70% B in 10 min, linear step to 100% C in 10 min, 100% C isocratic step for 3 min,return to initial conditions; flow rate of 1.7 ml min^{-1}.

Quantification of the amino acids using the method of manual pre-sample injection derivatization proved to be extremely tedious and intricate. Experimentation showed that reaction products are formed immediately after addition of o-phthaldialdehyde/ 2-mercaptoethanol reagent to the amino acid mixture. If the derivatized sample is allowed to stand for more than 2 minutes prior to analysis, a decrease in fluorescence on analysis is observed. A delay of ± 30 minutes on analysis of the derivatized sample would

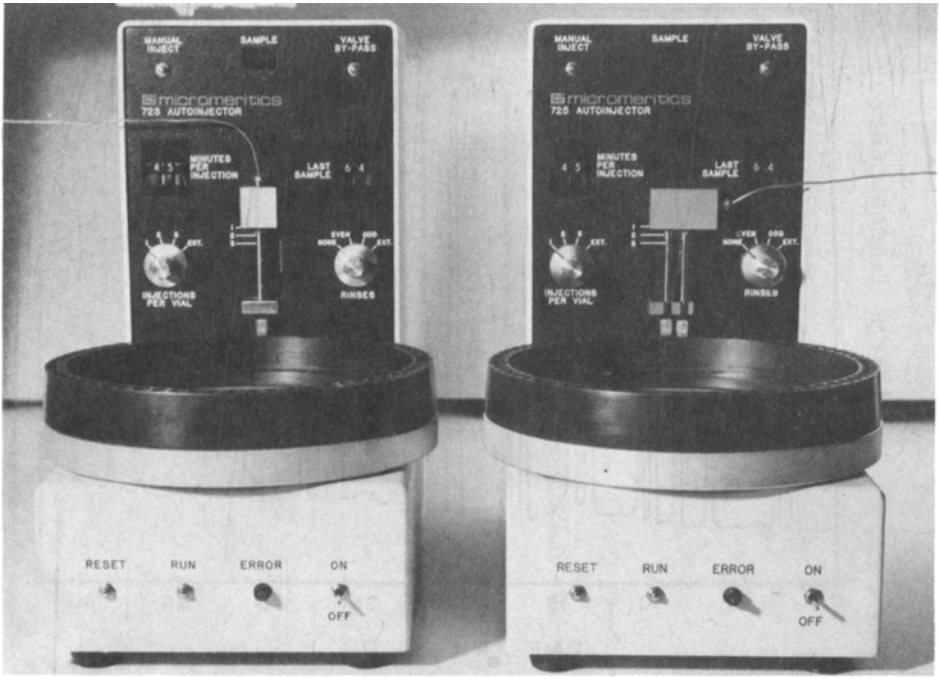

Figure IIa Figure IIb

Normal Auto-injector and the Modified Version which allows for Simultaneous Injection of Reagent and Sample.

give unreliable results. This demands that the derivatized samples must be prepared immediately prior to analysis.

The feasibility of automating derivative formation by the simultaneous injection of sample and reagent from separate vials was investigated. Results showed that the 5 µl injection loop allowed sufficient mixing and reaction time for complete derivatization. Increasing the reaction period by 90 seconds by delaying the actual injection of the derivatized sample on the column did not significantly affect derivative fluorescence.

As illustrated in Figure IIA the Micromeritics Model 725 auto-injector principle of operation is simply the manual

displacement of the sample from a vial, through an inverted type syringe action. The sample is displaced through the HPLC injection loop. Sample flushing the loop is run off into waste. Once the auto-injector has displaced the full contents of the vial, the injector loop containing a fixed volume of sample, is switched into the main HPLC eluent stream. Analysis of the sample can now take place.

To allow the simultaneous injection of reagent and sample, the auto-injector had to be modified (Figure IIB). The auto-injector syringe holder was replaced with one housing two injection needles. The needle housing which has only one outlet, has an internal Tee-junction which links the two injection needles. Mixing of reagent and sample can thus take place during displacement thereof from the vials to the injector loop.

A further modification was made to the auto-injector to allow the vial tray to advance two places instead of one. Reagent and sample are loaded alternatively into the tray. With the above modifications the instrument can be safely left running unattended.

A detailed description of the design and operation of the modified auto-injector was given by J.C. Hodgin at the 1983 Pittsburgh Conference.[3]

The reactant, o-phthaldialdehyde/2-mercaptoethanol solution, proved to be stable at room temperature. As a result, the auto-injector could be loaded with reagent and sample for overnight analyses. With the internal standard, βeta-alanine, the automated analysis of 11 each beerwort and beer samples produced the results shown in Table I.

The optimum solvent pH (7.5) was found by obtaining amino acid standard mixture chromatograms at eluent pH ranging from 5.5 to 11.5. The pH of solvents A and B were adjusted individually after mixing of buffer, methanol and THF. Table II reflects the results of this experiment. Using peak areas as the basis of calculations, pH

TABLE I

Results of Automated Analysis of Pre-column o-Phthaldialdehyde/ 2-Mercaptoethanol Amino Acid Derivatives of 11 Beerwort and 11 Beer samples

Amino Acid	Beerwort			Beer		
	$\bar{t}_r$ (Min)	$\bar{c}$ (mg/dm^3)	s (mg/dm^3)	$\bar{t}_r$ (Min)	$\bar{c}$ (mg/dm^3)	s (mg/dm^3)
Asp	2.25	62.4	0.4	2.25	1.8	0.2
Glu	3.18	56.0	0.4	3.21	2.1	0.2
Asn	6.77	111.8	0.6	6.85	Trace	-
Ser	8.53	71.8	0.4	8.67	0.8	0.1
Gln	9.80	81.1	0.2	10.00	1.0	0.1
His	10.50	61.0	0.6	10.72	8.6	0.1
Gly	13.37	42.7	0.4	13.55	9.9	0.2
Thr	14.31	61.4	0.2	14.51	1.1	0.1
Arg	16.79	170.8	1.3	17.03	15.7	0.2
β-Ala[a]	17.76	50.0	-	17.93	50.0	-
Ala	20.42	97.5	1.0	20.90	15.4	0.3
Tyr	21.63	114.2	0.5	21.71	68.9	0.5
Trp	25.10	44.5	0.5	25.13	12.5	0.3
Met	25.36	38.7	0.2	25.41	Trace	-
Val	25.79	92.0	0.6	25.85	4.3	0.3
Phen	26.51	140.9	0.8	26.60	5.8	0.8
Ile	28.46	83.2	0.4	28.60	0.7	0.3
Leu	29.38	139.5	0.8	29.54	5.9	0.1
Lys	36.53	171.2	0.6	36.66	Trace	-

a Internal Standard
s Standard deviation of mean concentrations
$\bar{c}$ Mean concentration of amino acids
$\bar{t}_r$ Mean retention time

O-phthaldialdehyde/2-mercaptoethanol reagent was prepared with the 0,4M boric acid pH ranging from 6 to 12. With the differing pH reagents amino acid standard mixture chromatograms were now obtained. Using reagent pH 10.4 and peak areas as the basis of calculations, Table III was compiled. From the table itis clear that pH10.3 is the optimum as fluorescence intensity of formed derivatives are the highest.

Example chromatograms of beerwort and beer are shown in figures III and IV respectively.

TABLE II

Percentage Fluorescence Difference[a] of Opa-Amino Acid Derivatives with Change in Solvent pH

Amino Acid	Norm 5.9	-al[a]	SOLVENT PH 6.0	6.5	7.0	7.5[c]	8.0	10.0	10.3	11.0	11.5
Asp	-12	-	-2	15	35	52	58	80	85	74	93
Glu	-20	-	1	42	42	55	61	80	86	84	94
Asn	0	-	2	38	52	55	58	67	63	63	61
Ser	-30	-	5	50	87	94	98	102	118	112	114
His	35	-	1	*	75	259	280	438	435	445	435
Gln	-7	-	0	*	31	44	47	54	53	57	58
Gly	-21	-	10	50	80	105	104	111	121	124	124
Thr	1	-	8	56	92	179	182	206	200	214	211
Arg	-1	-	5	32	50	72	72	81	212	*	*
β-Ala[b]	-8	-	6	42	60	82	98	102	29	*	*
Ala	-4	-	4	43	42	136	148	160	116	*	*
Tyr	-2	-	2	20	66	161	186	192	172	*	*
ABA	-8	-	5	14	-26	-22	-9	-12	8	*	*
NH_4^+	-3	-	21	55	29	55	41	*	*	*	*
Trp	-10	-	33	38	116	205	287	*	*	*	*
Met	10	-	8	22	37	68	99	115	130	111	103
Val	3	-	6	13	17	25	31	26	54	53	46
Phen	2	-	13	29	70	115	137	153	201	178	185
Ile	3	-	6	17	26	41	51	57	76	67	71
Leu	10	-	17	23	46	70	90	97	140	114	128
Lys	-22	-	-3	83	57	198	284	478	537	*	471

a Sodium acetate buffer pH 5.9 (2)
b Internal Standard
c pH 7.5 Optimum. pH > 7.5 harmful to reverse phase column.
ABA, 4-aminobutyric acid present in beer wort and beer.
* Merged peaks

TABLE III

Percentage Fluorescence Difference[a] of Opa-Amino Acid Derivatives with Change in Opa-Reagent pH

Amino Acid	OPA REAGENT PH										
	7.0	8.0	9.0	9.5	10.0	10.3	10.4[a]	10.5	11.2	11.5	12.0
Asp	*	-79	-73	-54	-25	8	-	-4	-23	-40	-50
Glu	*	-64	-46	-33	-18	5	-	-3	-15	-33	-54
Asn	*	-79	-73	-54	-25	8	-	4	-23	-40	-50
Ser	*	-53	-36	-20	-8	3	-	-2	-15	-28	-16
His	*	c	c	c	-12	0	-	-8	-40	-61	-79
Gln	*	-16	-12	-4	2	6	-	0	-41	-47	-73
Gly	*	-33	-7	-7	-7	5	-	-2	-5	-5	-5
Thr	*	-54	-44	-31	-12	3	-	2	-53	-63	-92
Arg	*	-27	-15	-10	-8	7	-	-5	-10	-33	-53
β-Ala[b]	*	-47	0	0	0	0	-	0	0	0	-22
Ala	*	-50	-37	-24	-15	3	-	-6	-7	-17	-24
Tyr	*	-61	-29	-21	-12	4	-	-5	-16	-39	-47
NH_4^+	*	-70	-24	-7	-1	0	-	-11	-40	-44	-40
Trp	*	-32	-27	-16	-11	-2	-	-6	-8	-16	-52
Met	*	-30	-23	-17	-10	0	-	-7	-7	-27	-63
Val	*	-38	-30	-20	-14	3	-	-9	-10	-15	-62
Phen	*	-50	-37	-24	-15	3	-	-6	-8	-18	-24
Ile	*	-53	-44	-31	-18	2	-	-7	-10	-11	-63
Leu	*	-42	-31	-17	-9	-1	-	-3	-9	-17	-28
Lys	*	-60	-23	-10	-4	0	-	-3	-33	-52	-91

a Boric acid pH 10.4 (2)
b Internal Standard
c Peak not integrated
* Fluorescence response poor

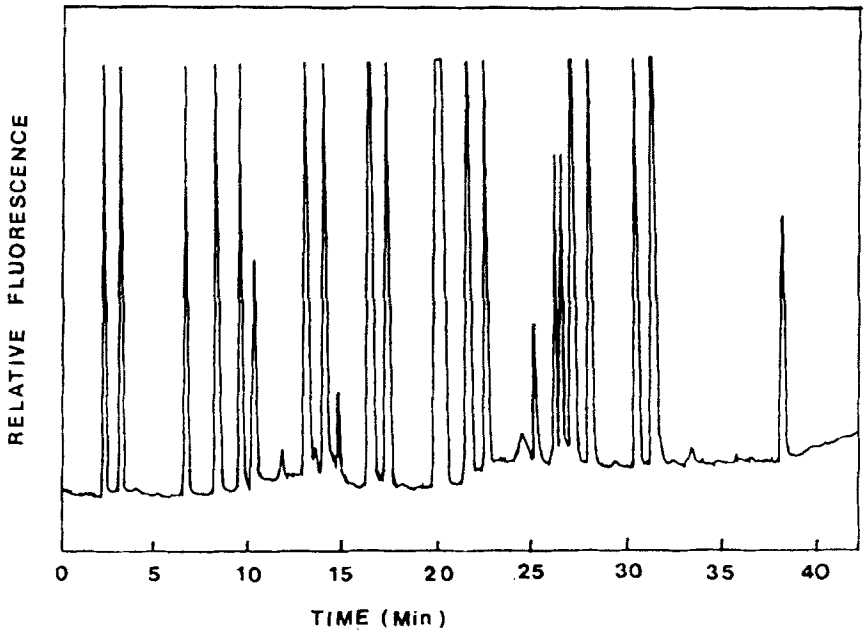

Figure III: Chromatogram of Automated Analysis of Beerwort.
Conditions: Identical to those in Figure I.

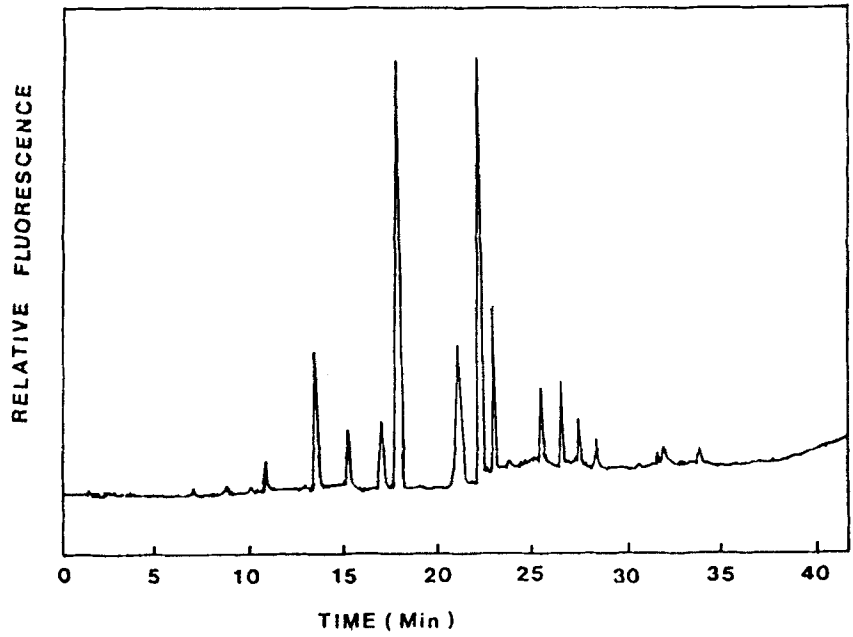

Figure IV: Chromatogram of Automated Analysis of Beer.
Conditions: Identical to those in Figure I.

7.5 was chosen as the optimum. Indications are that for pH > 8.0 fluorescent intensity generally tend to become constant followed by a decrease from pH 10.3 upwards. It is further known that solvent pH > 7.5 is harmful to the reverse phase column used.

CONCLUSIONS

The method described here has been used as a routine procedure in our laboratory. The accuracy described has been maintained over the life of the column which is generally more than 2 000 sample injections.

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