Journal of Chromatography, 270 (1983) 378–382
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,150

Note

Automated pre-column amino acid analyses by reversed-phase high-performance liquid chromatography

MICHAEL J. WINSPEAR and ANN OAKS*
Biology Department, McMaster University, Hamilton, Ontario L8S 4K1 (Canada)
(Received June 14th, 1983)

Previous reports have shown that derivatives of amino acids with *o*-phthaldialdehyde (OPA) can be separated by reversed-phase high-performance liquid chromatography (HPLC)[1–2]. The derivatization is quick, reproducible and sensitive. The major problem with the method is that the fluorescent derivatives are unstable and thus derivatized samples should not be left for automatic analysis. We have developed an automated derivatization procedure, using a microprocessor-controlled three-pump HPLC system to avoid this problem. The method has been used to analyze the amino acid composition of cereal seed proteins.

EXPERIMENTAL

Apparatus

The HPLC system was a Beckman Model 334 ternary solvent gradient liquid chromatograph. Sample injections were performed with a Beckman Model 500 automatic sampler equipped with a 20-μl sample loop. Separation of components was on an Altex Ultrasphere-ODS column (250 × 4.6 mm, particle size 5 μm), protected by a guard column (25.0 × 4.6 mm) packed with Ultrapack-ODS (Altex, particle size 10 μm). Detection was with a Gilson Spectra/GLo fluorometer, 15 μl flow-cell, with excitation at 360 nm and emission at 455 nm. Peak areas were integrted using a Shimadzu Chromatopac C-RIA data processor.

Reagents

The water used was distilled from glass after deionization and filtration to remove organics. Methanol (HPLC grade) and sodium acetate were obtained from BDH. Tetrahydrofuran (analytical grade) was obtained from Sargent-Welch. The mixed solvents were passed through Durapore filters (Millipore, pore size 0.45 μm) and degassed under vacuum before use.

Methods

Automated derivatization of amino acids with OPA was accomplished by employing a microprocessor-controlled pump to pump OPA. Amino acid samples were pumped with the automatic sampler. The OPA and amino acids were combined in a mixing tee just prior to injection. The samples were reacted in the injector loop

0021-9673/83/$03.00

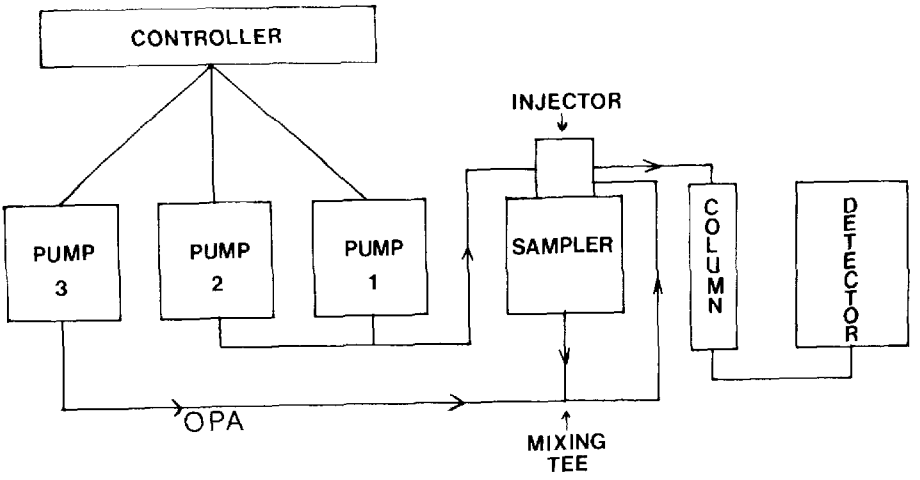

Fig. 1. Schematic arrangement of components for automated amino acid analysis by reversed-phase HPLC.

with the solvent flow halted, for 2 min before loading onto the column (See Fig. 1). The flow of OPA solution [40 m*M* in 0.4 *M* sodium borate buffer, pH 9.4–methanol (90:10)] is 2.0 ml/min. The flow of sample is approximately 4 ml/min.

Chromatography

The conditions were modified from the method of Jones *et al.*[2]. The column is equilibrated with solvent A (tetrahydrofuran–methanol–0.5 *M* acetate buffer, pH 5.9 (1:15:80). The amino acid derivatives were eluted by a gradient of solvent A to 100% solvent B (methanol–0.05 *M* acetate buffer, pH 5.9 (80:20) over a period of 85 min (see Fig. 2 for gradient). Flow-rate was 0.8 ml/min. Column pressure was approx. 2500 p.s.i. ($\approx$17.2 MPa).

RESULTS AND DISCUSSION

Separation and quantitation of OPA-amino acid derivatives can be accomplished efficiently by reversed-phase HPLC with fluorescent detection. The reaction of OPA and amino acids occurs rapidly and quantitatively in the presence of mercaptoethanol at ambient temperatures[1]. However, the derivatives are unstable at normal temperatures. Thus, leaving derivatized amino acid samples in vials on an autosampler results in rapid loss of fluorescence (Table I). This can be overcome by reacting amino acids with OPA immediately before chromatography, as outlined under *Methods*. Fluorescence of some amino acid decrease within a 30-min reaction time. However, there is little effect on fluorescence for reaction times up to 10 min (Table I). As a result of these tests a standard time of 2 min was chosen for the reaction.

Linearity of the detector response with amino acid concentration was tested for several amino acids. It appears that the reaction is linear to an amino acid concentration of at least 1 m*M*. Routinely, total amino nitrogen concentration in samples was kept at less than 1 m*M*. This results in at least a 20-fold excess of OPA.

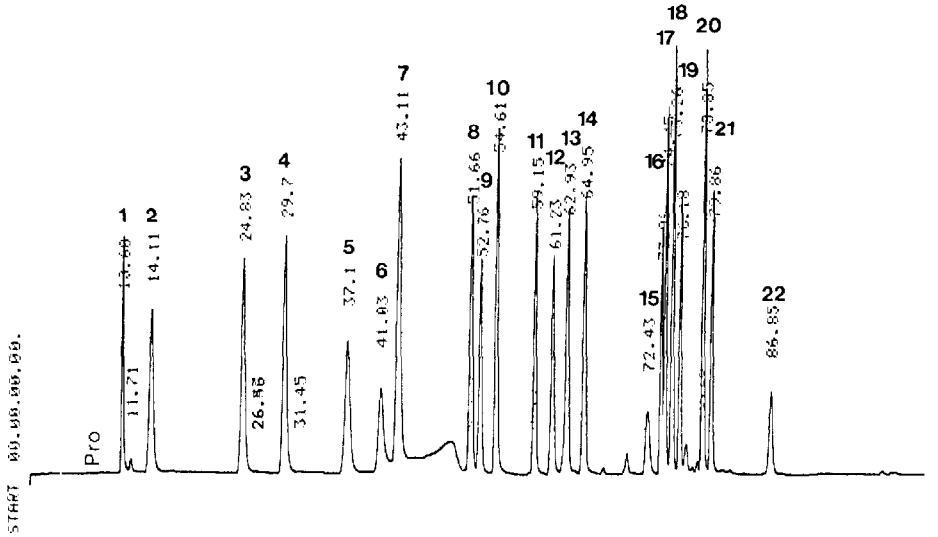

Fig. 2. Standard separation of OPA-amino acid derivatives. Peaks: 1 = cysteic acid; 2 = Asp; 3 = Glu; 4 = Asn; 5 = Ser; 6 = His; 7 = Gln; 8 = Gly; 9 = Thr; 10 = Arg; 11 = β-Ala; 12 = Ala; 13 = Tyr; 14 = GABA; 15 = NH_3; 16 = Trp; 17 = Met; 18 = Val; 19 = Phe; 20 = Ile; 21 = Leu; 22 = Lys.

TABLE I

STABILITY OF OPA-AMINO ACID DERIVATIVES

Amino acid	*Peak area (× 10⁶)*					
	Derivatization time					
	1 min	*2 min*	*4 min*	*10 min*	*30 min*	*2 h in sample vial*
Asp	0.964	1.484	1.884	1.510	2.081	0.174
Glu	1.516	1.627	1.947	1.606	1.653	0.229
Asn	1.589	1.690	2.020	1.733	1.670	0.318
Ser	1.334	1.486	1.597	1.447	0.693	N.D.
His	0.899	0.964	1.069	0.981	0.686	N.D.
Gln	2.449	2.566	2.810	2.829	2.163	0.398
Gly	1.776	1.939	1.832	1.880	0.254	N.D.
Thr	1.202	1.307	1.583	1.446	1.035	0.126
Arg	1.960	2.011	2.146	2.033	1.311	0.190
Ala	1.567	1.595	1.516	1.492	0.143	N.D.
Tyr	1.660	1.701	1.954	1.949	1.711	0.761
γ-Aminobutyric acid	3.486	3.579	3.497	3.160	0.593	N.D.
NH_3	0.278	0.372	0.418	0.402	N.D.	N.D.
Trp	0.829	0.895	0.968	0.899	0.942	0.267
Met	1.806	1.949	2.125	1.959	1.627	0.361
Val	1.864	1.949	2.544	2.248	2.149	0.661
Phe	1.202	1.328	1.179	1.177	1.198	0.574
Ile	1.855	1.914	2.019	1.806	0.551	0.681
Leu	1.179	1.246	1.288	1.216	0.889	0.224
Lys	0.957	0.841	0.718	0.774	0.163	0.192

Reproducibility of ten automatic injections was tested (Table II). Variability of peak areas increased with a larger number of injections indicating a drift in the reaction with time. Use of an internal standard corrected this drift, although it did not significantly decrease variability for smaller numbers of samples. Routinely now, standards are injected after five test samples and a suitable internal standard is included in all samples.

TABLE II

REPRODUCIBILITY OF AUTOMATED HPLC ANALYSIS OF AMINO ACIDS

(A) Calculated from S.D. of samples 1–5 and 6–10, (B) calculated from S.D. of samples 1–10.

Amino acid	*Coefficient of variation*					
	Peak area		*Internal standard (γ-aminobutyric acid)*		*Internal standard (β-Ala)*	
	A	*B*	*A*	*B*	*A*	*B*
Glu	4.8	6.1	3.7	3.9	4.5	4.9
Asn	3.9	3.8	4.0	4.2	5.1	6.9
Ser	6.6	9.0	6.5	7.2	4.3	4.1
His	4.5	4.2	6.0	3.6	6.7	8.4
Gln	5.0	6.7	2.5	3.2	6.1	8.0
Gly	6.6	8.4	5.9	6.4	4.1	3.9
Thr	8.5	10.7	8.0	8.8	5.6	5.3
Arg	3.4	4.0	2.6	2.7	3.9	5.5
β-Ala	5.8	9.1	5.7	6.6	–	–
Ala	8.6	10.4	3.1	4.8	1.2	1.9
Tyr	4.6	7.1	2.9	4.3	4.4	4.5
γ-Aminobutyric acid	5.1	5.3	–	–	5.7	6.6
NH_3	6.9	8.5	9.8	9.4	7.1	7.2
Trp	6.0	9.0	6.0	7.0	3.7	3.7
Met	6.0	9.0	6.0	7.1	4.8	3.7
Val	3.9	3.7	3.8	3.6	5.8	8.0
Phe	7.7	10.8	7.8	9.5	6.2	6.2
Ile	2.8	2.9	4.9	5.7	4.9	8.5
Leu	6.6	9.6	4.3	6.8	5.5	5.4
Lys	7.7	8.5	8.4	8.3	8.9	8.8
Mean	5.7	7.2	5.3	5.9	5.2	5.9

The major causes of variability in the automated pre-column OPA derivatization sytem would come from errors in the mixing ratios of sample and reagent, degradation of amino acids over time, changes in reactivity of the OPA reagent over time and fluctuations in temperature of reaction or chromatography. Time of reaction can be precisely controlled and time of chromatography does not vary significantly. The first error consideration is likely the most important. Variation in the mixing ratio can occur due to buildup of residue in the injector lines, causing back-pressure changes, variation in the flow-rate of sample due to changes in the pumping pressure or variation in the amount of reagent pumped at different times of the pump stroke cycle.

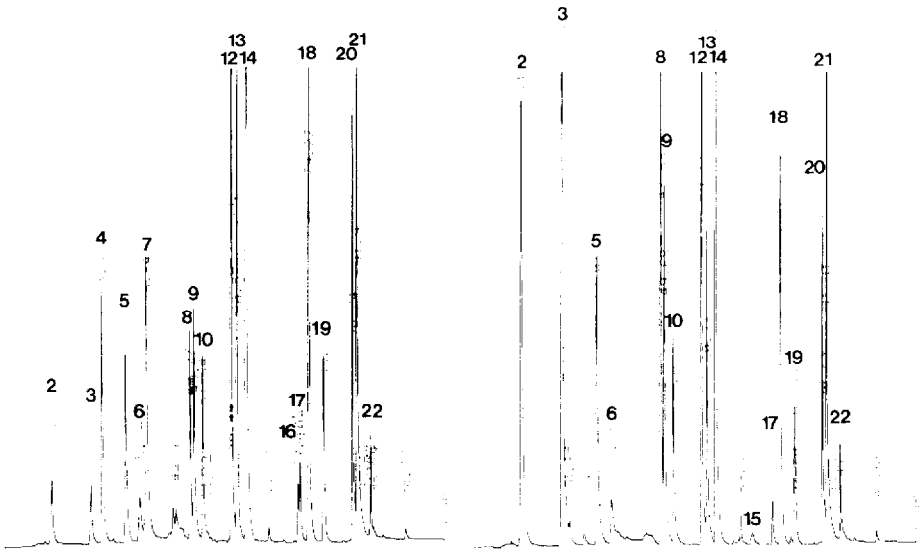

Fig. 3. Amino acids released from maize endosperm powder by enzymatic digestion (left) and acid hydrolysis (right). Peaks as in Fig. 2.

The rapid and efficient separation of the amides and acidic amino acids by reversed-phase HPLC is valuable in the examination of enzyme rections involving these amino acids. Separations involving only Asp, Glu, Asn and Gln can be accomplished isocratically in less than 20 min. The buffer used for this separation is 0.02 *M* sodium phosphate pH 6.8–methanol (70:30). A complete amino acid analysis using gradient elution can be run in 120 min, including reaction time and reequilibration of the column. The method is sensitive to picomole quantities of amino acid injected. Proline, which does not react with OPA, elutes in the void volume.

Proline, hydroxyproline and cysteine can be estimated in picomole quantities by derivatization with 4-chloro-7-nitrobenzofurazan and separations by reversed-phase chromatography[3]. If interfering amino acids (ornithine, lysine or hydroxylysine) are not present in significant quantities, proline can be estimated with ninhydrin[4] directly on extracts, although sensitivity is low (approx. 10 nmoles). This test is satisfactory for the analysis of proline in cereal endosperm proteins.

Enzymatic digestion of proteins by the method of Winkler and Schön[5] preserves the amides, methionine, tryptophan and cysteine. The amino acids released by enzymatic digestion or by acid hydrolysis of maize endosperm proteins have been compared using the HPLC method of analysis (see Fig. 3). The combination of enzyme digestion of proteins and HPLC analysis of the amino acids is an accurate efficient method of estimating nutritional quality of cereal seed proteins.

REFERENCES

1 P. Lindroth and K. Mopper, *Anal. Chem.*, 51 (1979) 1667–1674.
2 B. N. Jones, S. Paabo and S. Stein, *J. Liquid Chromatogr.*, 4 (1981) 565–586.
3 H. Umagat, P. Kucera and L.-F. Wen, *J. Chromatogr.*, 239 (1982) 463–474.
4 F. P. Chinaro, *J. Biol. Chenm.*, 199 (952) 91–95.
5 U. Winkler and W. J. Schön, *STI/PUB/496*, International Atomic Energy Agency, Vienna, 1979, pp. 343–351.