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## Note

# Sensitive and rapid amino acid analysis of peptide hydrolysates by high-performance liquid chromatography of *o*-phthaldialdehyde derivatives

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The amino acid composition of peptides isolated from biological sources provides essential information for their chemical characterization. Since such peptides are often obtained in only very small amounts a sensitive and accurate determination of the amino acid composition is essential. Recent reports indicate that amino acids derivatized with *o*-phthaldialdehyde (OPT) can be separated by reversed-phase high-performance liquid chromatography (HPLC) and detected with high sensitivity[1–6]. We found that some of the previously described solvent systems resulted in insufficient resolution when applied to a Nucleosil 5 $C_{18}$ column. In this paper a solvent system optimized for the Nucleosil 5 $C_{18}$ column is described. This system separates OPT derivatives of all amino acids common to peptide hydrolysates with good resolution. An evaluation of chromatographic conditions and quantitative properties is presented, resulting in a reproducible and sensitive method for the amino acid analysis of small amounts of biologically active peptides.

## EXPERIMENTAL

### *Apparatus*

HPLC was performed with a Waters Assoc. assembly consisting of two Model 6000A pumps, a Model 660 solvent programmer and a U6K injector; a Nucleosil 5 $C_{18}$ column (250 × 4.6 mm I.D., particle size 5 μm, Chrompack, Middelburg, The Netherlands) was used. The column effluent was monitored with an Aminco Fluoro-Monitor equipped with a Corning 7-51 primary filter (330 nm cut-off) and a Wratten 2A secondary filter (420 nm cut-off). The flow cell volume was 18 μl.

### *Chromatography*

In the standard elution program the mobile phase was formed by a gradient of sodium citrate buffer ($A_I$ or $A_{II}$) and methanol (B) according to the following schemes:

$$\text{I: } 0\% \xrightarrow{\text{linear (5 min)}} 30\% \xrightarrow{\text{isocratic (5 min)}} 30\% \xrightarrow{\text{linear (20 min)}} 65\%\ \text{B}$$

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[solvent $A_I$ was 0.1 *M* sodium citrate (pH 6.5) premixed with methanol (4:1, v/v); solvent B was methanol] or

$$\text{II: } 20\% \xrightarrow{\text{linear (5 min)}} 45\% \xrightarrow{\text{isocratic (5 min)}} 45\% \xrightarrow{\text{linear (20 min)}} 75\%\ \text{B}$$

[solvent $A_{II}$ was 0.1 *M* sodium citrate (pH 6.5); solvent B was methanol]

Chromatography was performed at a flow-rate of 1.0 ml/min at ambient temperature.

*o-Phthaldialdehyde–2-mercaptoethanol (OPT–ME) reagent*

*o*-Phthaldialdehyde (Sigma, St. Louis, MO, U.S.A.) was dissolved in methanol to a concentration of 56 mg/ml. One volume of this solution was mixed with nine volumes of 0.4 *M* sodium borate buffer, pH 9.5, and 4 μl of 2-mercaptoethanol per ml of reagent mixture were added. Addition of 1 μl of 2-mercaptoethanol per ml reagent each week was sufficient to maintain a constant reagent strength during two months.

*Derivatization*

The derivatization procedure was standardized in the following way. Samples containing 50–200 pmole of individual amino acid residues were made up in 25 μl of quartz-distilled water, and 50 μl of the OPT–ME reagent was added ($t = 0$ sec). After thorough mixing at ambient temperature, 50 μl of the reaction mixture were taken up in the HPLC syringe at $t = 60$ sec. The syringe was placed in the sample loading port of the injector unit and at $t = 90$ sec the total volume was injected into the sample loading loop. At $t = 120$ sec the sample was loaded onto the column.

*Sample preparation*

For HPLC analysis, standard mixtures containing 1–50 pmole of each amino acid per μl were prepared. Peptides were hydrolysed in 50–100 μl 6 *M* hydrochloric acid containing 0.1 % thioglycolic acid in evacuated sealed glass tubes at 110°C for 16 h[7]. Performic acid oxidation of cyst(e)ine-containing peptides to the corresponding cysteic acid peptides was carried out prior to hydrolysis essentially according to a modified procedure of Hirs[8,9].

## RESULTS AND DISCUSSION

*Chromatography*

After attempts with several solvent systems to separate the OPT–amino acid derivatives on a Nucleosil 5 $C_{18}$ column, optimal conditions were found in the use of a gradient of 0.1 *M* sodium citrate buffer, pH 6.5, and methanol. Fig. 1 shows the separation of all amino acid derivatives common to peptide and protein hydrolysates and the change of effective methanol concentration in a typical run.

During evaluation of the present system the influence of buffer composition, buffer concentration and pH has been examined. Unsatisfactory resolution was obtained when the sodium citrate concentration was lowered from 0.1 to 0.05 *M*. Replacement of sodium citrate by sodium acetate or the use of sodium citrate mixed with sodium acetate buffers in various ratios resulted in insufficient separation between

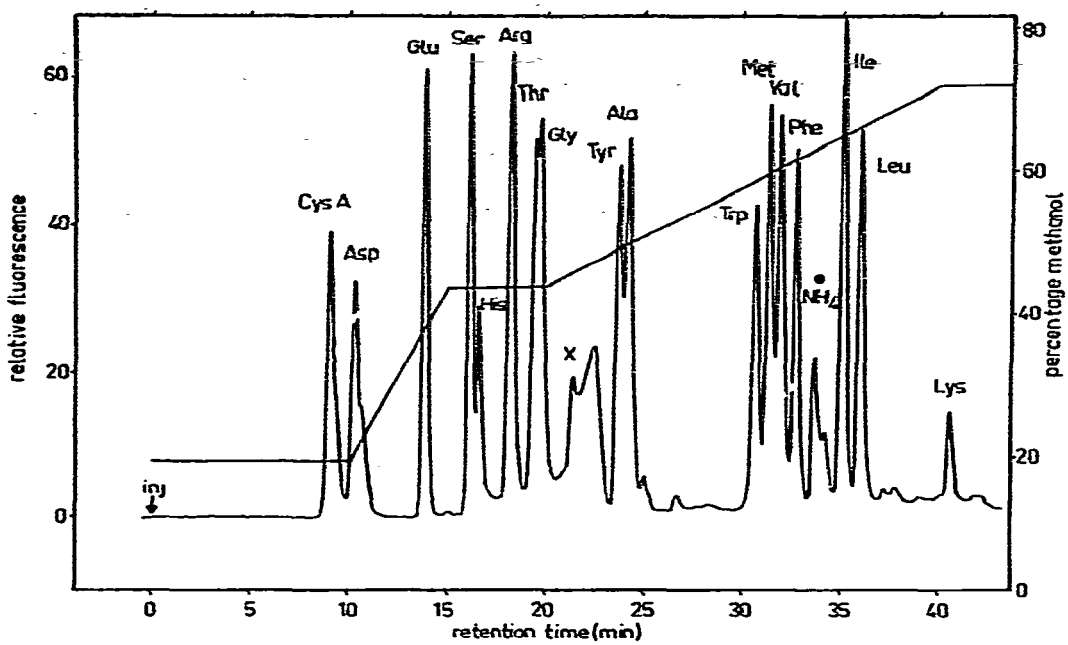


Fig. 1. Elution profile of OPT derivatives of amino acid standards (80 pmole each) on a Nucleosil 5 $C_{18}$ column. The gradient was formed between 0.1 *M* sodium citrate, pH 6.5, and methanol (system II, see Experimental). The calculated actual methanol concentration in the effluent expressed as volume percentage is shown. Chromatography was performed at ambient temperature (22°C). The flow-rate was 1.0 ml/min. Component X originates from the OPT–ME reagent. Amino acids are indicated by conventional abbreviations except cysteic acid (CysA).

OPT–Thr and OPT–Gly. The effect of pH of the 0.1 *M* sodium citrate buffer was examined over the range pH 5.5–6.5. The overall elution profile and resolution of the OPT–amino acids was found optimal at pH 6.5.

In some HPLC systems OPT–Thr and OPT–Gly are not separated or are separated only with low resolution[1,6]. Although in the present system they were sufficiently separated for quantitative determination we tried to improve the resolution by including 1 % tetrahydrofuran in the mobile phase, following the suggestions of earlier reports[3,5]. The addition of 1 % tetrahydrofuran to the sodium citrate solution led to loss of separation between the OPT pairs Ser/His, Thr/Gly, and Tyr/Ala. In our set-up, use of tetrahydrofuran in combination with 0.05 *M* sodium acetate, pH 5.9, resulted in co-elution of OPT–Thr and OPT–Gly, of OPT–Tyr and OPT–Ala, and of OPT–Trp and OPT–Met, while the elution order of OPT–Thr/Gly and OPT–Arg was reversed.

In the present system, OPT–amino acid derivatives were separated with good resolution as single components except for OPT–His. Secondary peaks of OPT–His were present: a relatively small, sharp peak eluted with a retention time of approximately 17 min and a broad asymmetric component eluted between 20 and 23 min (Fig. 1). The first peak was used for quantitation. This phenomenon appeared to be dependent on the pH of the aqueous elution buffer.

*Quantitative properties*

The reaction time of the OPT-derivatization reaction and the stability of OPT–amino acid derivatives are known to influence the fluorescence intensity of the products after HPLC[2,3,5]. Therefore, a stringently standardized procedure of derivatization and chromatography was employed. For evaluation of the reproducibility of the procedure the retention times and fluorescence responses of consecutive analyses of amino acid standard mixtures were measured. These experiments allowed us to evaluate the deviation in retention times, the linearity of and deviation in the fluorescence responses, and to calculate the fluorescence factor of each OPT–amino acid (Table I). The average deviation in the retention times was *ca.* 0.1 min. A linear relationship between fluorescence response, measured as peak height, and amount of amino acid was found in the tested range of 20–200 pmole. The calculated linear regression of the standard curves had excellent correlation coefficients (Table I). From the slopes of the standard curves the fluorescence factors were calculated and normalized to OPT–Glu (Table I). The average deviation in fluorescence response measured as peak height was 3.3%.

TABLE I

CHARACTERISTICS OF AMINO ACID ANALYSIS BY REVERSED-PHASE HPLC OF OPT DERIVATIVES: RETENTION TIMES, LINEARITY OF FLUORESCENCE, FLUORESCENCE FACTORS AND PRECISION

| *OPT–amino acid derivative* | *Retention time* ± S.D. (min)* | *Correlation** coefficient of linearity* | *Fluorescence*** factor* | *Deviation in[§] fluorescence response (%)* |
|---|---|---|---|---|
| CysA | 8.7 ± 0.1 | 0.9998 | 0.96 | 2.0 |
| Asp | 10.0 ± 0.2 | 0.9993 | 0.59 | 4.4 |
| Glu | 13.7 ± 0.1 | 0.9987 | 1.00 | 1.8 |
| Ser | 16.1 ± 0.1 | 0.9994 | 1.09 | 1.1 |
| His | 16.7 ± 0.1 | 0.9974 | 0.37 | 6.2 |
| Arg | 17.8 ± 0.1 | 0.9999 | 1.49 | 1.5 |
| Thr | 19.3 ± 0.2 | 0.9997 | 1.23 | 1.8 |
| Gly | 19.7 ± 0.1 | 0.9997 | 1.00 | 3.6 |
| Tyr | 23.2 ± 0.2 | 0.9990 | 1.06 | 2.7 |
| Ala | 23.9 ± 0.2 | 0.9994 | 0.87 | 2.5 |
| Trp | 30.2 ± 0.1 | 0.9985 | 0.81 | 4.6 |
| Met | 30.9 ± 0.1 | 0.9993 | 1.35 | 3.5 |
| Val | 31.6 ± 0.1 | 0.9990 | 1.23 | 3.7 |
| Phe | 32.4 ± 0.1 | 0.9990 | 1.03 | 3.3 |
| Ile | 34.9 ± 0.1 | 0.9998 | 1.56 | 3.4 |
| Leu | 35.8 ± 0.1 | 0.9997 | 1.08 | 3.9 |
| Lys | 40.1 ± 0.1 | 0.9804 | 0.26 | 6.2 |

* Measured from the chromatograms of seven consecutive runs of 20, 20, 40, 80, 120, 160 and 200 pmole of each amino acid residue.

** The correlation coefficient of linearity of the fluorescence response (measured as peak height) of the individual OPT–amino acid derivatives of the same consecutive runs mentioned under footnote*.

*** The fluorescence factor is defined as the normalized slope of the fluorescence response (measured as peak height) versus the amount of OPT–amino acid (Glu = 1.00). Data were obtained as under footnote*.

[§] Calculated from the peak heights of 120 pmole of each amino acid determined from three consecutive runs.

The signal-to-noise ratio obtained from 10 pmole of amino acid was 10, resulting in a detection limit in the order of 3 pmole.

Three synthetic naturally occurring peptides, human β-endorphin, vasopressin, and oxytocin, were subjected to acid hydrolysis and their amino acid compositions were determined using the system described in this paper. The data obtained from a single determination are presented in Table II.

TABLE II

AMINO ACID COMPOSITION OF PEPTIDES

Data were obtained in a single analysis of 187 (a), 164 (b), and 191 pmole (c), respectively. Oxytocin and arginine–vasopressin were oxidized by performic acid treatment prior to acid hydrolysis, resulting in some loss of tyrosine and the simultaneous generation of a component with a retention time similar to that of OPT-Ser. n.d. = Not detectable.

| *Amino acid residue* | *Human β-endorphin*[a] | *Oxytocin*[b] | *Arginine–vasopressin*[c] |
|---|---|---|---|
| CysA | n.d. (0) | 1.99 (2) | 1.91 (2) |
| Asp | 2.04 (2) | 1.00 (1) | 1.00 (1) |
| Glu | 3.04 (3) | 1.09 (1) | 1.07 (1) |
| Ser | 1.62 (2) | 0.25 (0) | 0.35 (0) |
| His | n.d. (0) | n.d. (0) | n.d. (0) |
| Arg | 0.03 (0) | 0.04 (0) | 0.98 (1) |
| Thr | 2.96 (3) | n.d. (0) | n.d. (0) |
| Gly | 2.81 (3) | 1.00 (1) | 1.11 (1) |
| Tyr | 2.00 (2) | 0.54 (1) | 0.74 (1) |
| Ala | 1.82 (2) | 0.03 (0) | 0.06 (0) |
| Met | 1.01 (1) | n.d. (0) | n.d. (0) |
| Val | 1.29 (1) | 0.05 (0) | 0.06 (0) |
| Phe | 1.98 (2) | 0.02 (0) | 0.93 (1) |
| Ile | 1.74 (2) | 0.91 (1) | 0.04 (0) |
| Leu | 1.99 (2) | 0.96 (1) | 0.04 (0) |
| Lys | 4.87 (5) | n.d. (0) | n.d. (0) |

## CONCLUSION

The HPLC method described here provides a technique which allows quantitative analysis of amino acids in the low pmol range with good precision and reproducibility. The method is rapid and easy to perform and does not require specialized equipment. Applied to peptide hydrolysates it provides an excellent tool for the chemical characterization of peptides obtained in very small amounts.

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