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Automated HPLC-Amino Acid Determination of Protein Hydrolysates by Precolumn Derivatization with OPA and FMOC and Comparison with Classical Ion Exchange Chromatography

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Key Words

Column liquid chromatography
Ion exchange chromatography
Quantitative amino acid analysis
OPA/FMOC precolumn derivatization
Cheese

Summary

An improved HPLC method for the quantitative determination of the amino acids from hydrolysed cheese proteins and peptides is described. Some important improvements to the existing method are suggested. The addition of piperidine-4-carboxylic acid (PICA) as a hydrolysis-resistant internal standard enabled the quantification of secondary amino acids. The following analytical parameters have been determined: repeatability, detection limit and linearity in the measuring range. A statistical comparison with the classical ion chromatographic method gave an excellent correlation for all determined amino acids. Both methods are free of artifacts and systematic errors. Compared with ion chromatography, HPLC shows the following advantages: faster equilibration of the column, shorter retention times, more stable baseline, narrower peaks and more sensitive fluorescence detection. A drawback is the slightly lower repeatability for some amino acids.

Introduction

The shift in amino acid analysis away from classical ion chromatographic amino acid analysers (IEC) [1] to more universal reversed phase HPLC systems has been delayed by the lack of reproducible methods and by the time consuming sample preparation [2–8]. A recently proposed new method [9] using o-phthalaldehyde/3-mercapto propionic acid (OPA/MPA) and 9-fluorenyl-methylchloroformate (FMOC) for precolumn derivatisation can be fully automated and since it requires only a small sample (1 µl) is suited both to liquid as well as gas phase hydrolysis techniques. Quantitative determination of all amino acids except cystine, cysteine and tryptophan, and to some extent methionine, can be achieved by using ODS columns of different dimensions, different fluorescent parameters and by the addition of a new internal standard for secondary amino acids.

Experimental

Sampling

Selected cheese samples were prepared from internal cheese experiments or obtained from the retail trade. If possible a 1–2 cm thick rind was cut off and discarded. The hard cheese samples were ground at room temperature, whilst semi-hard cheese were cooled to 4 °C and soft cheese were frozen before grinding.

Reagents and Ancillary Apparatus

The reagents used were: Hexane purum (Fluka) for degreasing; 370 g l^{-1} hydrochloric acid pro analysi, phenol pro analysi, tri-sodium citrate dihydrate from Merck for hydrolysis; amino acid standard 1 µmol ml^{-1}, OPA/MPA-Reagent, FMOC-Reagent conc. in acetonitrile, borate buffer 0.4 mol l^{-1} pH 10.4 from H.-P., sodium acetate trihydrate pro analysi, titriplex III pro analysi, sodium hydroxide pro analysi from Merck, tetrahydrofuran puriss. p.a., acetonitrile and methanol for HPLC, sarcosine (SAR), piperidine-4-carboxylic acid (PICA) from Fluka, deionised water from a Milli-Q installation (Millipore), and L-norvaline (NVA) (Sigma) for the HPLC determinations; amino acid standard (Sarasin), tri-lithium citrate tetrahydrate for amino acid determination, citric acid monohydrate pro analysi, lithium chloride, Brij solution 300 g l^{-1} for amino acid determination, lithium hydroxide pro analysi, dimethylsulfoxide for spectroscopy, ninhydrin pro analysi, 2,2′-thiodiethanol, octanoic acid, hydrindantine dihydrate for amino acid determination, acetic acid pro analysi from Merck for additional IEC determinations.

The following ancillary apparatus was used: Sorvall mixer (Sorvall), analytical balance (Mettler), Pulverisette labora-

tory grinder (Fritsch), lyophilizer for degreasing; vacuum reaction tube (Pierce), Edwards vacuum pump (Zivy), Evapo-Mix evaporator (Kontron), oil bath (Lauda) for hydrolysis; column Hypersil ODS 250×4 mm, 5 µm (H.-P.), precolumn Hypersil ODS 20 × 4.0 mm (H.-P.), analytical balance (Mettler), disposable filters ACRO LC13 0.45 µm (Scan), polyethylene tubes 5 ml with stoppers (Milian), Millipore filter type RAWP 1.2 µm (Millipore), Millipore filter type FHUP 0.5 µm (Millipore), several volumetric flasks 10–1000 ml, volumetric pipettes 1–25 ml, measuring cylinders 200 ml/1000 ml, 1 ml transfer pipette with variable volume (Eppendorf), test tubes large 8 × 180 mm, Pasteur capillary pipettes 230 mm and 280 mm, beakers 25 ml, disposable syringes 5 ml for HPLC determinations; BTC resin 2710 (Sarasin), BTC-F resin for precolumn (Sarasin) for additional IEC determinations.

Instrumentation

HPLC Determination: Hewlett-Packard (HP) 1090M HPLC equipment with DR 5 binary solvent delivery system, variable volume injector with 25 µl syringe, temperature controlled autosampler (10 °C) and column compartment with a solvent preheating device was used in conjunction with a HP 1046A programmable fluorescence detector fitted with a 280 nm cut-off filter.

IEC Determination: A Biotronik LC 7000 amino acid analyser with ninhydrin post column derivatisation and UV detection with a Biotronik Photometer 7025 at 570 nm and 440 nm for primary and secondary amino acids served for the chromatographic amino acid determination of hydrolysed proteins and peptides. The separation column (140 × 3.2 mm) was filled with BTC 2710 resin, the precolumn (30 × 6 mm) with BTC F resin. The separation took place with five lithium citrate buffers (see Table I) at a buffer flow of 0.317 ml min^{-1} and a ninhydrin reagent flow of 0.156 ml min^{-1} (column temperature 56 °C). The chromatographic separation took 110 min.

Procedures

Degreasing of cheese samples: Degreasing of semi-hard and soft cheese should be executed under cool conditions (4 °C) whilst hard cheese may be degreased at room temperature. About 15 g of the grated cheese was weighed into a Sorvall beaker, 50 ml of n-hexane were added and the mixture homogenized for 30 s with the Sorvall mixer at a speed setting of 6. The cheese was left to precipitate for 1 min and the hexane was carefully decanted off. The degreasing procedure was repeated twice under the same conditions. The degreased residue was transferred to a 250 ml pear-shaped flask in a plastic housing (implosion protection) and the residual hexane removed under water pump vacuum for 1 h. The cheese powder was then completely desiccated by lyophilization for 48 h. Finally, the dried sample was ground for 2 min in a laboratory grinder (Pulverisette). The cheese extract so obtained could be stored in the dark for several months in glass containers under an inert atmosphere.

Hydrolysis: Approximately 200 mg of the cheese extract were weighed to a precision of 0.1 mg in a 25 ml glass beaker and treated with 8 ml of a 100 g l^{-1} tri-sodium citrate dihydrate solution for 2 min at room temperature. After addition of 6 ml of deionised water the mixture was kept at 40 °C and completely suspended by occasional stirring. This suspension was then transferred to a 100 ml volumetric flask and the internal standards added, before making up to the mark with deionised water.

- Internal standards for HPLC determination: 8.00 ml of norvaline solution (10 µmol ml^{-1}), 8.00 ml of sarcosine solution (10 µmol ml^{-1}) and 8.00 ml of piperidine-4-carboxylic acid (10 µmol ml^{-1}).
- Internal standard for IEC determination: 20.0 ml of norvaline solution (2.5 µmol ml^{-1}).

500 µl of this solution and 750 µl of 300 g l^{-1} hydrochloric acid (containing 2 g l^{-1} phenol) were transferred with an Eppendorf pipette through a long Pasteur capillary pipette to a Pierce tube. The Pierce tubes were cooled with liquid nitrogen, evacuated and sealed. The samples were hydrolysed for exactly 22 h in an oil bath at 110 °C – in order to guarantee complete hydrolysis the entire tube had to be immersed in the oil bath. After cooling the tubes were carefully cleaned, opened and the contents transferred with an extra-long Pasteur capillary pipette to large test tubes. The solution was evaporated to dryness (Evapo-Mix evaporator) at 40 °C at a vacuum of 1 mbar.

HPLC Determination

Test solution: The hydrolysis residue was dissolved in 2.0 ml of a 0.1 mol l^{-1} solution of hydrochloric acid and then filtered through a ACRO LC 13 (0.45 µm) disposable filter.

Standard solution: 5.00 ml of the HP amino acid standard solution (1 µmol ml^{-1}) were transferred to a 25 ml volumetric flask. 1.00 ml of the norvaline solution (5 µmol ml^{-1}), 1.00 ml sarcosine solution (5 µmol ml^{-1}) and 1.00 ml piperidine-4-carboxylic acid solution (5 µmol ml^{-1}) were added and the mixture made up to 25 ml with 0.1 mol l^{-1} hydrochloric acid.

Preparation of the mobile phase: Eluent A: 4.10 g of sodium acetate trihydrate and 40 mg Titriplex III were weighed into a 1 l volumetric flask, deionised water was added to a volume of about 950 ml, the pH was adjusted with 0.1 mol l^{-1} NaOH to 7.20 and the solution was made up to the mark with deionised water. 2.5 ml of tetrahydrofuran were added and the solution was well mixed and filtered through a 1.2 µm RAWP Millipore filter.

Eluent B: 2.72 g of sodium acetate trihydrate and 40 mg of Titriplex III were dissolved in 200 ml of deionised water in a 1 l flask and the pH adjusted to pH 7.20 with 0.1 mol l^{-1} NaOH. 800 ml of acetonitrile were added with good stirring and the solution was filtered through a 0.5 µm FHUP Millipore filter.

Injector parameters:

Slowdown Draw & Eject 5 – Mix 2 – Hold after Draw & Eject 0

1 Draw	5.0 µl from Vial#:	2 Borate buffer
2 Draw	1.0 µl from Vial#:	0 OPA/MPA reagent
3 Draw	0.0 µl from Vial#:	100 Needle wash
4 Draw	1.0 µl from Vial#:	Sample Sample

5	Draw 0.0 µl from Vial#:	100	Needle wash
6	Mix 7.0 µl cycles :	6	Reaction with OPA
7	Draw 1.0 µl from Vial#:	1	FMOC reagent
8	Draw 0.0 µl from Vial#:	100	Needle wash
9	Mix 8.0 µl cycles :	4	Reaction with FMOC
10	Inject		

IEC-Determination

Test solution: The evaporated hydrolysed residue was dissolved in 2.0 ml of lithium dilution buffer pH 2.20 (see below) and then filtered through a ACRO LC13 filter (0.45 µm).

Lithium dilution buffer pH 2.20: 141.0 g of tri-lithium citrate tetrahydrate were weighed into a 5 l volumetric flask, dissolved in about 4 l of deionised water and 12.5 ml of 2,2'-thiodiethanol and 0.5 ml of octanoic acid added. 115 ml of 370 g l^{-1} hydrochloric acid were carefully added. After cooling the pH was adjusted to 2.3 by dropwise addition of 370 g l^{-1} hydrochloric acid and the solution was made up to the mark with deionised water. The buffer was left for at least 12 hours before adjusting the pH to 2.20 by dropwise addition of 370 g l^{-1} hydrochloric acid.

Lithium acetate buffer pH 5.20: 964.8 g of lithium hydroxide were dissolved with constant stirring in 2 l of deionised water in a 10 l volumetric flask. 2.500 l of acetic acid were carefully added within 5 min (*very exothermic reaction*). After dissolution of all the lithium hydroxide and cooling to room temperature deionised water was added up to ca. 9 l. The pH was corrected to 5.2 by dropwise addition of acetic acid and deionised water was added up to about 9.9 l. Finally the pH was adjusted to 5.20 with acetic acid or concentrated lithium hydroxide solution and made up to the mark with deionised water.

Ninhydrin reagent: 825 g of dimethylsulfoxide were poured into a 1 l brown bottle. After stirring and N_2 flushing for 20 min, 20.0 g ninhydrin were dissolved. Again 20 min later, 688 mg of hydrindantine dihydrate and 250 ml of the lithium acetate buffer pH 5.2 were added, dissolved and flushed with N_2 for 20 min. The reagent has to be stored for at least 12 hours at 4 °C.

Standard solution: 5.00 ml of Benson Type H (2.5 µmol ml^{-1}) standard amino acid solution and 5.00 ml of the norvaline solution (2.5 µmol ml^{-1} in lithium dilution buffer pH 2.20) were added to a 100 ml graduated flask and made up to the mark with lithium dilution buffer pH 2.20.

300 g l^{-1} Brij Solution: 30.0 g of Brij were weighed into a 250 ml beaker and liquified in a hot water bath. Hot deionised water was added, with permanent stirring, to a volume of 100 ml.

Preparation of the mobile phase: The quantities of tri-lithium citrate tetrahydrate, citric acid monohydrate and lithium chloride listed in Table I were weighed into a 2 l graduated flask and dissolved in about 1.5 l of deionised water. The tabulated quantities of hydrochloric acid, methanol and Brij solution were then added. The flask was filled with deionised water and the pH adjusted to 0.1 above the required value with 370 g l^{-1} hydrochloric acid before filling to the mark with deionised water. The buffer was left for about 12 hours and the pH adjusted to the required value by dropwise addition of 370 g l^{-1} hydrochloric acid.

The separation program was run with the following buffer solutions: 18 min A, 26 min B, 19 min C, 8 min D, 45 min E, 15 min F and 35 min A. Temperature was programmed as follows: 34 °C during 30 min and 56 °C during 80 min.

Results and Discussion

Sample Degreasing

The nitrogen content was determined by the Kjeldahl method both before and after degreasing. The content of amino acids of the original cheese sample was calculated by using the total nitrogen content of the cheese sample.

Hydrolysis

During the described acid hydrolysis tryptophan and the sulfur containing amino acids cystine, cysteine and methionine were partially or entirely destroyed. These amino acids cannot be determined by this method. Tyrosine, threonine and serine decompose partially during hydrolysis. As the decompositions follow first order kinetics the initial concentration can be calculated. 92 % of serine and 96 % of threonine survived the above described conditions, the correction for tyrosine can be neglected. The duration and the temperature of hydrolysis have to be accurately controlled in order to allow precise correction.

Table I Buffer composition for IEC determination

Buffer	A	B	C	D	E	F	
pH value	2.70	3.06	3.57	4.00	3.53		
tri-Lithium citrate * $4H_2O$	18.8	22.6	33.8	37.6	10.0		g
Citric acid monohydrate	14.8	12.0	3.6				g
Lithium chloride				17.0	114.0		g
Methanol	120.0	100.0					ml
370 g l^{-1} Hydrochloric acid	11.0	12.0	16.0	17.0	4.0		ml
Brij solution 300 g l^{-1}	2.0	2.0	2.0	2.0	2.0	2.0	ml
Titriplex III						0.8	g
Lithium hydroxide						25.2	g

The hydrolysis tubes must be entirely submerged in the heating liquid, otherwise hydrolysis is incomplete and can range from 60 to 100 %, depending on the amino acid.

Application of the HPLC Determination

Even though the optimal excitation wavelength of OPA/MPA derivatives is situated at 230 nm, this wavelength was fixed at 340 nm in order to improve reproducibility for aspartic and glutamic acid. As only secondary amino acids were derivatised with FMOC, no interference by FMOC-OH which eluted after proline was observed (Figure 1). As lysine forms two derivatives with OPA/MPA, quantification was made by using the sum of both peaks.

Linearity of the HPLC determination: The correlation coefficient r of the linear regression curve (fluorescence

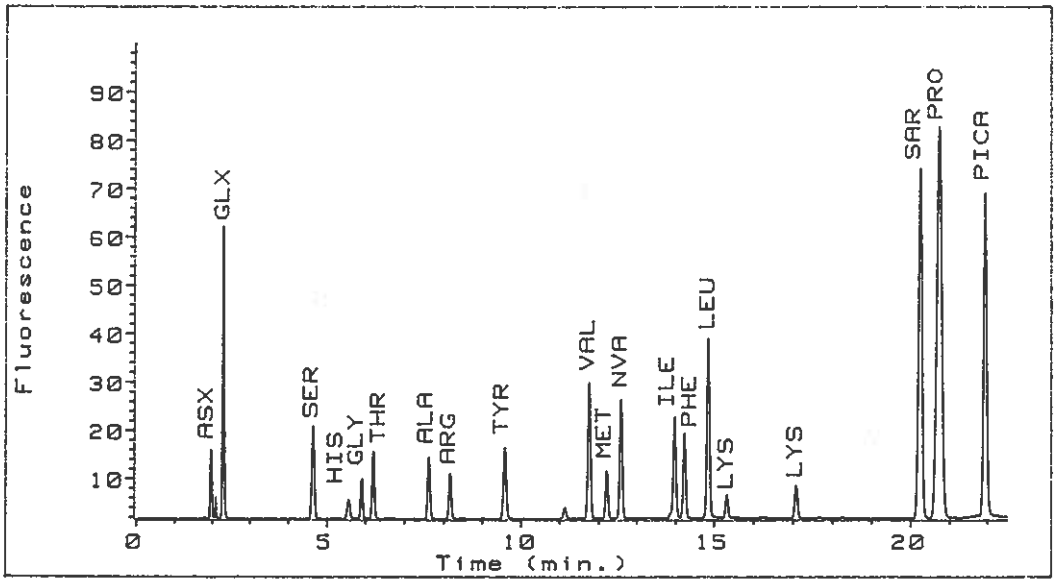

Figure 1

Amino acid chromatogram of cheese hydrolysate by Hewlett-Packard Type 1090 M HPLC equipment with fluorescence detection. Chromatographic conditions were as follows: solvent A, 30 mmol l^{-1} NaOAc pH 7.20 + 0.25 % tetrahydrofurane + 0.1 mol l^{-1} titriplex III; solvent B, 100 mmol l^{-1} NaOAc pH 7.20 + 80 % acetonitrile + 0.1 mol l^{-1} titriplex III; flow rate, 1.000 ml min^{-1}; column temperature, 42 °C. The derivatized amino acids were separated by a 24-min stepwise linear gradient from 3.3 to 40 % B over 20 min and 40 to 100 % B over 4 min. Column cleanup with 100 % B and re-equilibration required 7 min. The detector parameters were set to detect the OPA derivatives at the start of the program at Ex: 340 nm and Em: 455 nm, switched to Ex: 230 nm at 3 min and then changed at 18 min to detect the FMOC derivates Ex: 266 nm and Em: 313 nm.

Table II Detection limit for HPLC and IEC determination

Amino acid	Name	Detection limit HPLC [pmol μl^{-1}] (Signal/Noise = 3)	Detection limit IEC [pmol μl^{-1}] (Signal/Noise = 3)
ASX	Aspartic acid + Asparagine	1.0	6.3
GLX	Glutamic acid + Glutamine	0.8	8.2
SER	Serine	1.1	6.7
HIS	Histidine	1.8	5.9
GLY	Glycine	1.1	5.2
THR	Threonine	1.0	6.9
ALA	Alanine	1.1	5.9
ARG	Arginine	1.0	10.4
TYR	Tyrosine	0.8	4.8
VAL	Valine	0.8	4.6
MET	Methionine	0.8	5.1
ILE	Isoleucine	0.8	6.9
PHE	Phenylalanine	0.8	6.0
LEU	Leucine	0.8	7.8
LYS	Lysine	4.1	4.9
PRO	Proline	0.8	17.0

versus concentration) of all amino acids was better than 0.999 in the range tested (50–250 pmol μl^{-1}).

Detection limits and repeatability for HPLC and IEC determinations: Table II shows the detection limits of all the amino acids standards studied. Lysine was the only amino acid having a detection limit higher than 4 pmol μl^{-1}.

Table III shows the repeatability of a double preparation and determination. The variation coefficient had a maximum value of 3.2 percent.

Application of IEC Determination

The amino acid determination of hydrolysed proteins and peptides by IEC using the Biotronik amino acid analyser gave a good separation for all the amino acids studied (Figure 2). Quantitative determination of cystine, cysteine, methionine and tryptophan was not possible due to partial loss during acid hydrolysis.

Comparison of HPLC and IEC Determinations

Table IV shows the comparison between HPLC and IEC values (as a ratio in % of IEC).

Average deviations between HPLC and IEC determinations are given in Figure 3.

Table III Repeatability of hydrolysis procedure and determination with HPLC and IEC of two samples. Variation coefficient VC is given in percent (n = 2)

Amino acid	Average VC HPLC [%]	Average VC IEC [%]
ASX	1.5	0.4
GLX	1.1	0.2
SER	1.2	0.3
HIS	1.5	1.1
GLY	2.3	0.4
THR	0.9	0.4
ALA	1.1	1.0
ARG	1.2	2.2
TYR	1.3	1.6
VAL	0.8	0.4
MET	0.9	1.0
ILE	0.9	0.4
PHE	0.7	2.0
LEU	0.6	1.2
LYS	3.2	0.2
PRO	0.6	1.0

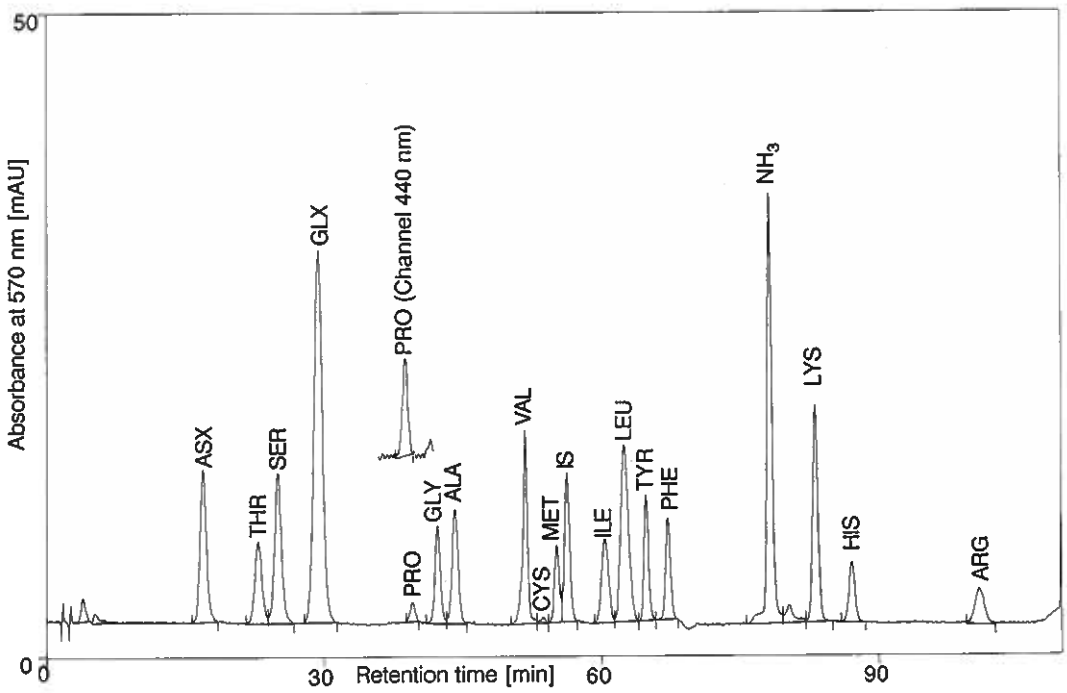

Figure 2

Amino acid chromatogram of cheese hydrolysate by Biotronik LC 7000 amino acid analyser with UV-detection. Chromatographic conditions were as follows: Solvent A to E were all lithium citrate buffers with different methanol content, ionic strength and pH values (Table I); buffer flow rate, 0.317 ml min^{-1}, ninhydrin reagent, 0.156 ml min^{-1} (at 56 °C); column temperature were set at 34 °C at the start of the chromatogram and changed to 56 °C at 30 min. Column cleanup with solvent F and re-equilibration required 41 min. The detection wavelengths were 570 nm for primary and 440 nm for secondary amino acids.

Table IV Comparison of IEC and HPLC determinations (given as a ratio: IEC = 100 %)

	Sample 1	Sample 2	Sample 3	Sample 4	Mean Value (n = 4)	Variation coefficient
	HPLC/IEC [%]	HPLC/IEC [%]	HPLC/IEC [%]	HPLC/IEC [%]	HPLC/IEC [%]	[%]
ASX	102	109	104	104	105	2
GLX	96	97	91	84	92	6
SER	99	101	97	98	99	1
HIS	99	96	100	95	97	2
GLY	97	112	102	98	102	6
THR	105	103	102	104	104	1
ALA	99	99	101	99	99	1
ARG	107	107	104	101	105	2
TYR	103	103	99	97	100	2
VAL	101	104	98	96	100	3
MET	103	102	95	102	101	3
ILE	108	105	104	103	105	2
PHE	102	110	103	96	103	5
LEU	105	106	102	99	103	3
LYS	99	90	95	100	96	4
PRO	89	96	86	94	91	4

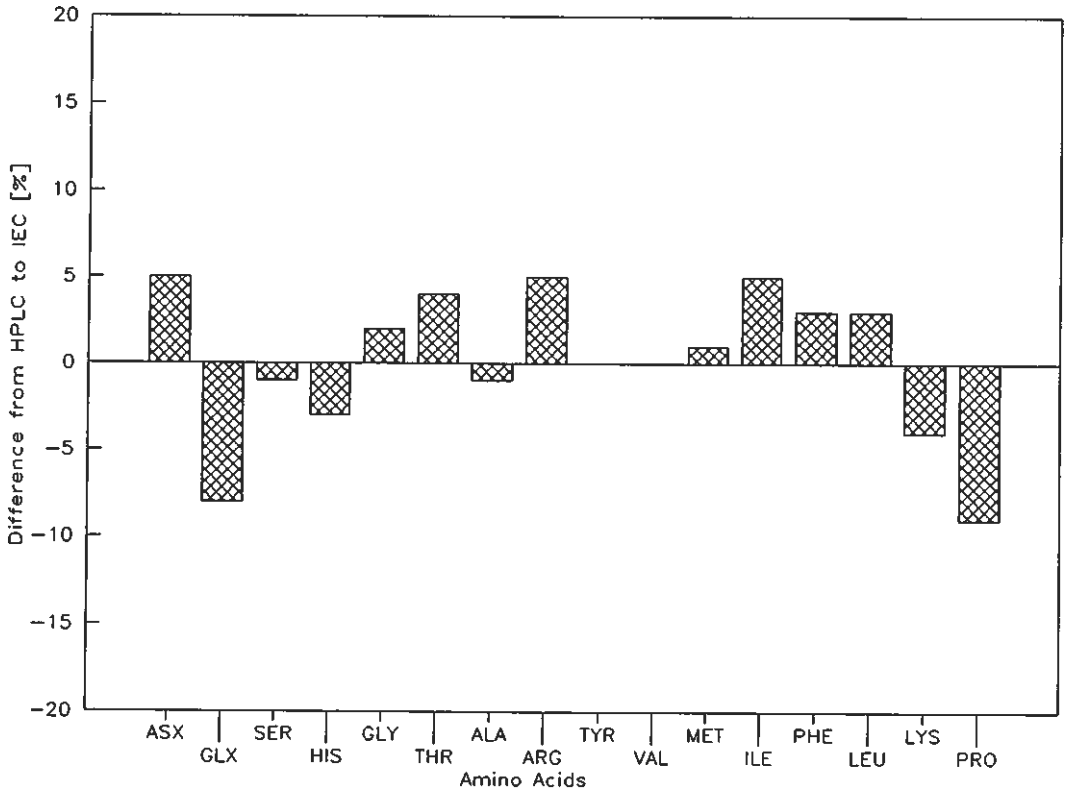

Figure 3
Mean value of difference between HPLC and IEC determination in percent.

Conclusion

The RP-HPLC method suggested in this work enables rapid, simple and very sensitive primary and secondary amino acid determination of protein hydrolysates. An automated precolumn derivatisation of the amino acids present was accomplished by using OPA/MPA for the primary, followed by FMOC for the secondary amino acids. Norvaline served as internal standard for primary and the newly introduced piperidine-4-carboxylic acid (sarcosine as a control) for secondary amino acids. As lysine forms two derivatives with OPA/MPA, quantification was made by using the sum of both peaks. The variation coefficient for the peak height for all determined amino acids was between 0.5–3.2 %.

Compared with classical IEC methods, the described method showed the following advantages: shorter retention times, narrower and better resolved peaks, more stable baseline and a lower detection limit, but a slightly lower repeatability for some amino acids. This method requires no specialised equipment other than standard HPLC equipment. The easy manual derivatisation under time control can be fully automated with a programmable autosampler. The reproducibility of the HPLC method and the good correlation with IEC speak in favour of its introduction for routine amino acid determination.

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