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Journal of Chromatography A, 798 (1998) 1–8

JOURNAL OF
CHROMATOGRAPHY A

Automated amino acid determination by high-performance liquid chromatography with 2-(9-anthryl)ethyl chloroformate as precolumn reagent

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Abstract

A fully automated HPLC method for the determination of amino acids in protein hydrolysates has been developed, using 2-(9-anthryl)ethyl chloroformate and precolumn derivatization. This UV sensitive reagent reacts rapidly with both primary and secondary amines at room temperature to form stable carbamate derivatives. The reaction conditions have been thoroughly investigated. A complete separation of protein hydrolysates is accomplished within 40 min. The results are compared to those of the well known 9-fluorenylmethyl chloroformate method. Detection limits for UV and fluorescence detection were found to be 0.5 pmol and 0.06 pmol, respectively. © 1998 Elsevier Science B.V.

Keywords: Derivatization, LC; Anthrylethyl chloroformate; Amino acids

1. Introduction

Precolumn derivatization in combination with reversed-phase liquid chromatography is today the most used technique for determination of amino acids. The combination of high separation effectivity and accuracy of modern HPLC instruments and the availability of suitable precolumn reagents makes it possible to determine minute amounts of amino acids with high precision. Precolumn derivatization of amino acids is most often based on nucleophilic substitution reactions. The purpose is then to obtain derivatives with good detection properties. This can be achieved by a variety of reagents. For example fluorescein isothiocyanate (FITC) [1] and *o*-phthaldialdehyde/alkylthiols (OPA/R-SH) [2] which give highly fluorescent derivatives. Other reagents, like phenyl isothiocyanate (PITC) [3,4] and dimethylaminoazobenzenesulphonyl chloride (DABSCl) [5], are only used with UV detection. Dansyl chloride (Dns-Cl) [6], 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) [7,8] and 9-fluorenylmethyl chloroformate (FMOC) [9–12], can be used with either fluorescence or UV detection.

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PII S0021-9673(97)00987-4

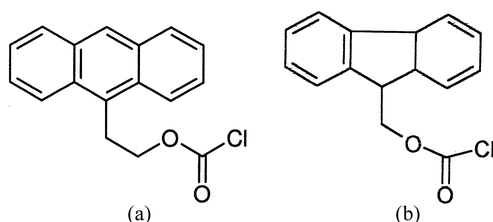

Fig. 1. Structures of (a) 2-(9-anthryl)ethyl chloroformate (AEOC), (b) 9-fluorenylmethyl chloroformate (FMOC).

The FMOC reagent was developed to act as a blocking group for the amine function in the peptide synthesis. The reagent is not optimised regarding chromophoric properties for quantitative spectrophotometric determination. The combination of a, for amines, very reactive function such as chloroformates and a strong absorption moiety in the molecule will result in an attractive reagent. Then the anthracene chromophore is an interesting compound with high molar absorptivity around 253 nm and fluorescence quantum yield of 0.36 in cyclohexane [13]. In the search for an alternative agent to FMOC to protect amino acids Kornblum and Scott [14] initially attempted to synthesize 9-anthrylmethyl chloroformate, however, the compound was not stable. Sörensen [15] found that the 2-(9-anthryl)ethyl chloroformate (AEOC) was stable in its crystalline form. This reagent was successfully used in fluorometric HPLC methods to determine polyamines [16] and phenols [17], and has also been used for amino acids in combination with capillary electrophoresis and microcolumn LC [18].

In this paper we describe a fully automated method for determination of protein amino acids by the use of AEOC. Furthermore, is a comparison to the FMOC method made for three protein hydrolysates. The structures of AEOC and FMOC are shown in Fig. 1.

2. Experimental

2.1. Equipment

The automated amino acid determination was made by using a ternary gradient delivery system (Varian 9012 Q, Varian, Sunnyvale, CA, USA) with Varian 9100 autosampler and a Varian 9050 UV detector with a Shimadzu RF-530 fluorescence spectromonitor (Kyoto, Japan) coupled to the outlet of the UV-detector. Integration of the peaks was made with Varian Star Chromatography data system. The method was also tested on an HP 1090 Chromatograph (Hewlett-Packard, Palo Alto, CA, USA) with autosampler and diode array detector. The detection limits were determined using a Waters 490 (Waters, Taunton, MA, USA) for UV detection and a Schoeffel FS 970 for fluorescence detection. The molar absorptivities for AEOC and FMOC were determined using a Cary 1E UV visible spectrophotometer (Varian). Amino acid standard (AA-S-18) was obtained from Sigma (St. Louis, MO, USA). The FMOC reagent was obtained from Fluka Chemie AG (Buchs, Switzerland). The AEOC reagent was prepared from 2-(9-anthryl)ethanol and phosgene as described earlier [15,16,19,20]. All solvents were of HPLC grade.

2.2. Derivatization procedure

The autosampler was programmed to mix 25 μl of the AEOC reagent (10 m*M* in acetonitrile) with 25 μl of the sample buffered with 250 m*M* $NaBO_4$ (prepared by mixing the sample with 1 *M* $NaBO_4$ pH 9.2 and H_2O to the desired concentration). After a waiting period of 3 min excess reagent was removed from the aqueous phase by addition of *n*-hexane to the mixture in five consecutive portions of 30 μl each, with mixing in between. 10 μl of the aqueous phase was injected after a 2 min wait to ensure phase separation. The derivatization was performed at room temperature.

2.3. Separation

A ternary gradient elution scheme was used for the separation of the amino acid derivatives. The scheme was developed to meet similar conditions as has been performed for the separation of amino acids derivatized with 9-fluorenylmethyl chloroformate [21]. The eluents were two buffer solutions consisting of sodium acetate (15 m*M*) and tetramethylammonium hydroxide (10 m*M*), with pH adjusted by addition of phosphoric acid to (A) 3.0, and (B) 5.5, respectively, and (C) acetonitrile with 10% methanol.

Elution scheme:

min	% A	% B	% C
0	66	0	34
15	12	48	40
37	25	5	70
40	20	0	80

The separations were carried out on a Varian Amino-Tag column (ODS, 5 μm, 15×4.6 cm) with a mobile phase flow of 1.8 ml/min. The column was equilibrated during the automated derivatization procedure, which took approximately 10 min. The upper pressure limit for the system was set to 250 atm and the separation was performed at room temperature (1 atm=101 325 Pa).

3. Results and discussion

3.1. Reaction conditions

Amino acids react rapidly with the AEOC reagent in aqueous alkaline mixtures (Schotten-Baumann conditions) at room temperature to form stable carbamates. The reaction medium is a mixture of buffered aqueous sample and acetonitrile, the latter to dissolve the reagent.

We found that *n*-hexane had to be added in five smaller portions rather than one large to ensure proper extraction of excess reagent. No difference could be found between the two different autosamplers used.

Reaction time (0.5–5 min), pH (7–10) and excess of reagent (2–10 times) were chosen as variables in a study of the reaction conditions. All investigated amino acids react totally in less than 1 min at a pH between 7 and 10 if the reagent excess is kept at least at three times the total sample concentration. The time passed before *n*-hexane is added does not affect the yield in the investigated interval, which shows that the reaction is very rapid. The pH of the reaction mixture does not have any effect on the peak areas with the exception of tyrosine (Fig. 2). Tyrosine forms double labelled derivatives by reaction with the phenolic group. The formation is slower at the lower pH and may not be complete if

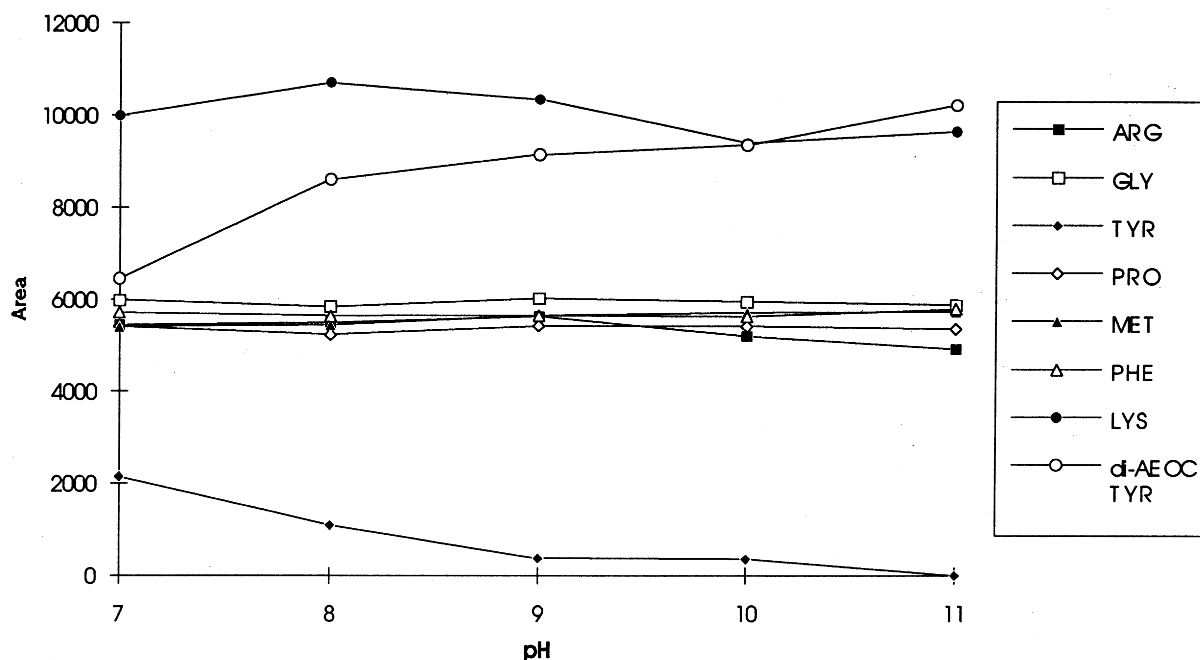

Fig. 2. Reaction yield for eight AEOC derivatized amino acids plotted against pH (reaction time=5 min).

the reagent is not in large excess. At conditions as a pH of 9.2 and a 10 times excess of reagent the quantitation of tyrosine is secured by the total formation of the double labelled derivative.

The amino acid derivatives were found to be stable over 1 week in darkness at room temperature except for histidine which broke down with concomitant formation of the mono-derivative. Assuming first order kinetics the half-life of the bis-histidine derivative is 26 h. Fig. 3 shows the plot of peak areas for bis-histidine and its breakdown product against the time passed from derivatization to injection. The plot is based on the derivatizations of four histidine standards. The formation of mono-labelled histidine seems to be quantitative. This suggests that the breakdown product of the bis-AEOC histidine may be included for quantitative determinations; however further investigations are needed.

The formed reaction by-product bis[2-(9-anthryl)ethyl] carbonate (BAEC) [16] is almost totally removed by the hexane extraction. The BAEC peak was found to be relatively unstable and after 5 h no BAEC peak could be seen in the chromatograms.

The linearity of response was controlled in the interval 0.5–400 μ*M*. All amino acids showed good linearity in this range (mean r^2=0.9987) (Table 1).

Table 1
Linearity and repeatability of AEOC derivatized amino acids for the described method

Amino acid	Linearity (0.5–400 μ*M*)	Repeatability (n=6) Peak area	Retention time (min)	
	r^2	%R.S.D.	t_r	S.D.
Arginine (R)	0.9980	4.5	8.7	0.14
Serine (S)	0.9993	3.0	13.4	0.24
Aspartic acid (D)	0.9994	2.6	14.1	0.24
Glutamic acid (E)	0.9995	2.4	15.0	0.28
Threonine (T)	0.9995	2.3	16.5	0.27
Glycine (G)	0.999	12.0	17.9	0.21
Alanine (A)	0.9992	2.9	20.8	0.14
Proline (P)	0.9993	3.6	23.0	0.11
Methionine (M)	0.9998	3.5	24.8	0.06
Valine (V)	0.9997	3.4	25.8	0.06
Phenylalanine (F)	0.9995	3.1	27.5	0.05
Isoleucine (I)	0.9993	3.1	28.1	0.06
Leucine (L)	0.9992	3.7	28.3	0.06
Cystine (C-C)	0.9996	3.6	30.5	0.03
Histidine (H)	0.9960	6.5	34.6	0.07
Lysine (K)	0.9920	4.7	35.7	0.10
Tyrosine (Y)	0.9995	6.6	37.8	0.09
Average	0.9987	3.6		0.13

3.2. Separation

The main variables for the optimization of the

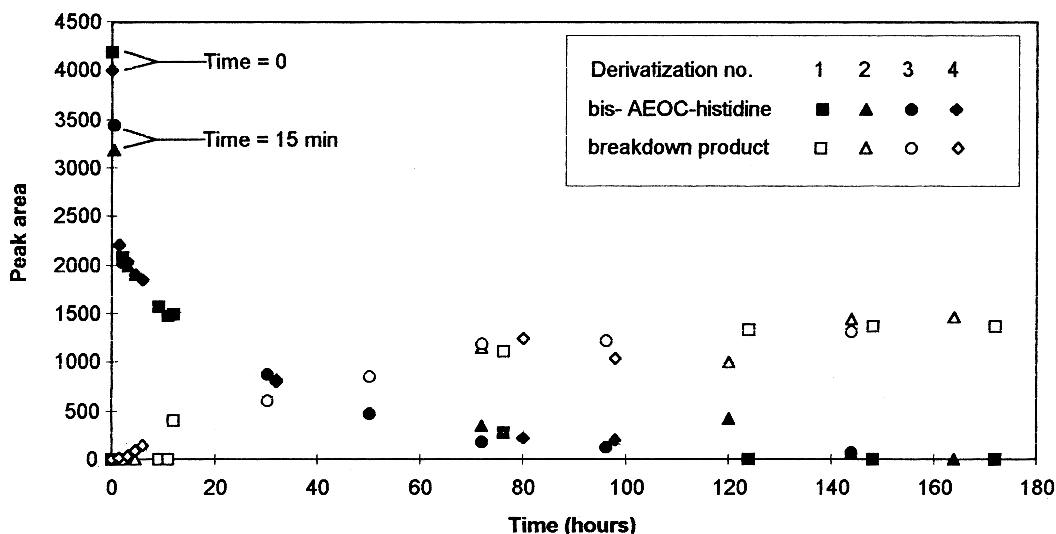

Fig. 3. Plot of peak areas for bis-AEOC-histidine and the mono labelled breakdown product against time passed from derivatization to injection. The plot is based on four derivatizations.

separation are the pH of the elution buffers and the composition of organic solvents. The retention of the derivatives generally decreases with increasing pH while the non-ionic 9-anthrylethanol (AEOH) by-product is unaffected. The effect of pH on the retention of the arginine derivative is relatively large, which is exploited to elute that derivative early in the chromatogram (Fig. 4). When the monoderivative of histidine is present this elutes first. Isoleucine and leucine are badly resolved, which is a problem if there is a large difference in concentration between these amino acids. Another drawback is the relatively long analysis time (40 min). It might be possible to overcome these problems by using other mobile phases or columns.

When compared to the FMOC method [21], the AEOC derivatives generally need a higher concentration of organic modifier to be eluted.

The precision of the procedure showed a mean relative standard deviation of the peak areas to be 3.6% and a mean standard deviation of the retention times of 0.13 min (Table 1).

3.3. Detection

The mean limits of detection for the AEOC derivatives of cysteine, histidine and alanine was determined to be 0.5 pmol for the UV detection and 0.06 pmol for the fluorescence detection. These values were obtained by dilution of a derivatized sample.

Detection limits with packed capillary LC has earlier been reported to be 0.4 pmol for UV detection and 0.3 fmol for monoderivatized amino acids and argon-ion laser-induced fluorescence detection [18].

When using UV absorbance detection the detectability of different substances under comparable conditions is dependent on the molar absorptivity of the substances at the specified wavelength. The molar absorptivities of the threonine derivative of AEOC and FMOC in water were found to be ε_{254}= 150 000 $l\,mol^{-1}\,cm^{-1}$ and ε_{264}=17 000 $l\,mol^{-1}\,cm^{-1}$, respectively, which would indicate about 9 times higher sensitivity for AEOC compared to FMOC. The calibration curves for AEOC and FMOC derivatives shows an even greater difference between these two reagents with about 17 times higher sensitivity for AEOC.

The importance of the absorptivity was further confirmed by comparing the lower detection limits of the AEOC reagent and the PITC reagent. Under comparable conditions using the same chromatographic equipment the mean detection limits for threonine, alanine and proline were 0.6 pmol (256 nm, S/N=3) for the AEOC derivatives and 7.1 pmol (254 nm, S/N=3) for the PTC derivatives. A comparison made by Engström et al. [18] gave a similar difference between the AEOC and the FMOC methods.

The anthryl moiety exhibits exceptionally high absorbance around 256 nm; however, other wavelengths may also prove useful. The UV absorption spectrum of AEOC-threonine (Fig. 5) shows that absorption also occurs at longer wavelengths (351, 366 and 388 nm). Those wavelengths can be used for confirmation. Furthermore, the characteristic spectrum can be used for identification with a diode array or fast scan UV detector.

High quantum yield is equally crucial for low detection limits in fluorescence spectroscopy as is the absorbance. Comparisons made between AEOC and FMOC [16,18] shows that although FMOC has a higher quantum yield, AEOC is the more sensitive, which is due to the pronounced higher absorbance.

The fluorescence response was similar for all amino acids except for the bifunctional amino acids cystine, histidine, lysine and tyrosine. The signals for these compounds were diminished with the most prominent effect for the cystine derivative. This can be seen when the chromatograms in Fig. 4a and b are compared. The separation is carried out with the two different detectors in series. The lower fluorescence quantum yield in the double derivatized amino acids presumably is due to intramolecular quenching.

3.4. Applications

In order to show the accuracy of the method, hydrolysates of bovine serum albumine (BSA), soybean and sunflower seed were derivatized and separated with the described method (Fig. 6a–c). The same hydrolysates were then analyzed by the FMOC method described by Cunico et al. [21]. The results obtained from the two methods are compared in Table 2. No significant difference is found between the two methods. The relatively large difference in

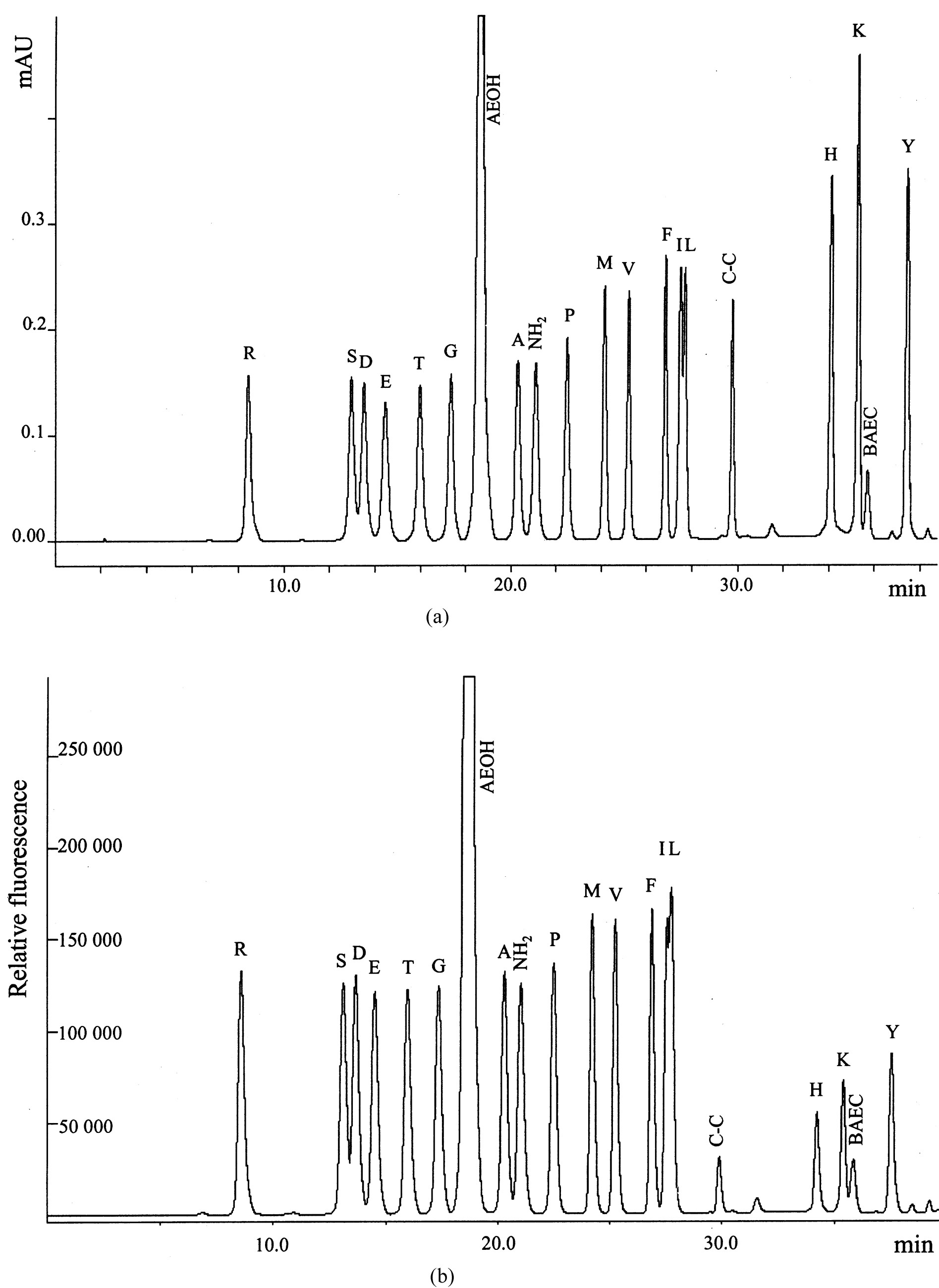

Fig. 4. Separation of an amino acid standard solution (250 μ*M*) each. (a) UV detection at 256 nm. (A=Arg, S=Ser, D=Asp, E=Glu, T=Thr, G=Gly, A=Ala, P=Pro, M=Met, V=Val, F=Phe, I=Ile, L=Leu, C-C=Cystine, H=His, K=Lys, Y=Tyr, AEOH=9-anthrylethanol, BAEC=bis (2-(9-anthryl)ethyl) carbonate). (b) Fluorescence detection with excitation wavelength=256 nm, and emission wavelength=420 nm.

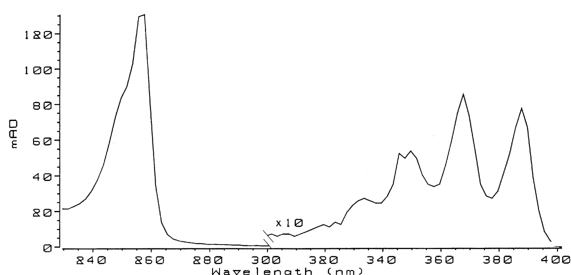

Fig. 5. UV-absorbance spectrum of AEOC-threonine. Absorbance maxima at 256, 351, 366 and 388 nm.

the ratio between the two methods for histidine in BSA and proline in soybean may origin from poor hydrolysis or sample contamination. Smaller peptides and other contaminants may coelute with the amino acids. The literature value for BSA hydrolysates [22] and determinations of both BSA and soy bean hydrolysates made by Woo et al. [23] indicates that the values obtained with the AEOC method are correct. The large deviations for the tyrosine peak in the sunflower seed and soy bean hydrolysates are probably due to incomplete formation of the double labelled derivative with the FMOC method. The derivatization of tyrosine with FMOC is, as well as for AEOC, very pH sensible [9].

The correlation between the two methods for the three samples was: $r=0.974$ (BSA) $r=0.874$ (soybean) and $r=0.994$ (sunflower seed).

4. Conclusions

The 2-(9-anthryl)ethyl chloroformate reagent is similar to 9-fluorenylmethyl chloroformate in aspects of derivatisation and separation. The difference lies in the spectral properties of the chromophore, resulting in one order of magnitude lower detection limit by UV absorbance for the anthracene derivatives of amino acids. The described method shows

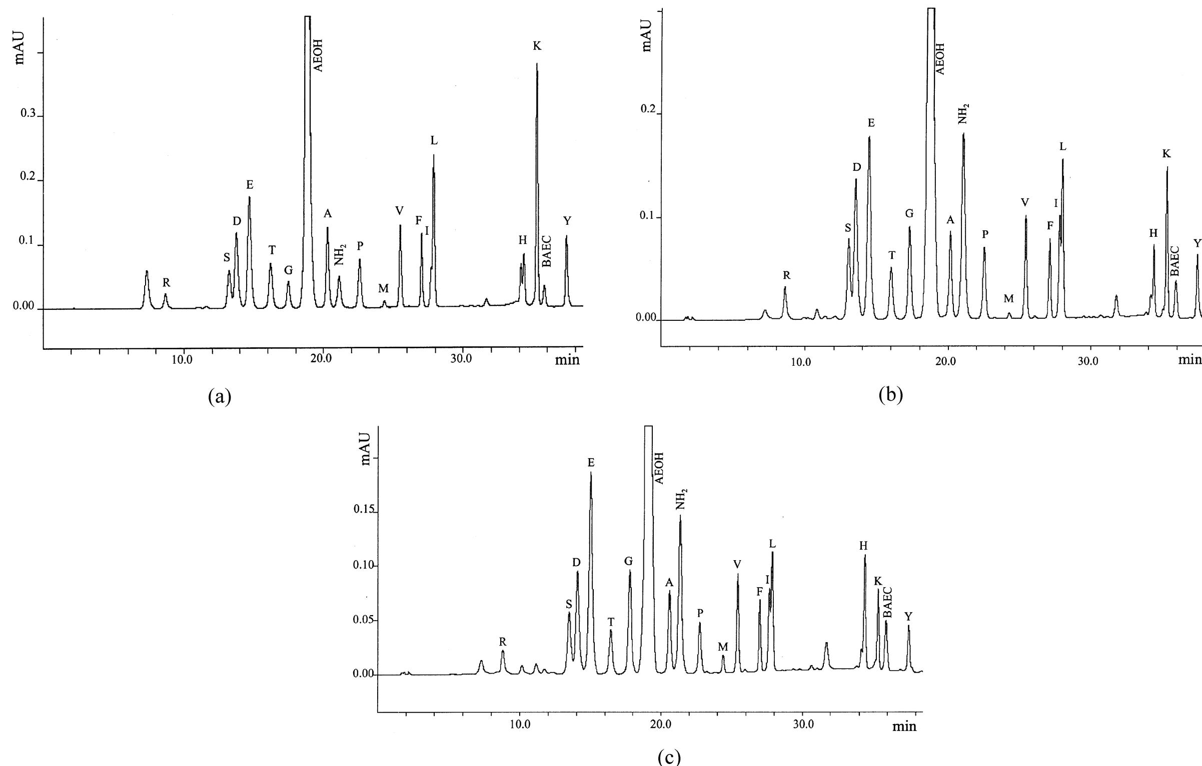

Fig. 6. Separation of AEOC derivatized hydrolysates. UV-detection 256 nm. (a) BSA hydrolysate. (b) Soybean hydrolysate. (c) Sunflower seed hydrolysate.

Table 2
Comparison between the AEOC and FMOC methods for hydrolysates of BSA, soybean and sunflower seed

Amino acid	BSA			Soybean			Sunflower seed		
	AEOC	FMOC	Ratio	AEOC	FMOC	Ratio	AEOC	FMOC	Ratio
Arg	84	65	1.3	102	71	1.4	88	81	1.1
Ser	219	190	1.2	275	243	1.1	230	219	1.0
Asx	473	439	1.1	517	475	1.1	372	393	0.9
Glx	695	687	1.0	733	704	1.0	779	889	0.9
Thr	277	258	1.1	192	168	1.1	157	158	1.0
Gly	148	130	1.1	307	282	1.1	346	394	0.9
Ala	385	[a]		258	[a]		213	[a]	
Pro	235	225	1.0	231	542	0.4	148	147	1.0
Met	28	[b]		14	[b]		37	[b]	
Val	299	279	1.1	252	232	1.1	211	225	0.9
Phe	235	223	1.1	177	146	1.2	142	147	1.0
Ile	125	97	1.3	208	177	1.2	170	172	1.0
Leu	642	523	1.2	364	319	1.1	247	283	0.9
His	124	200	0.6	103	119	0.9	149	204	0.7
Lys	446	502	0.9	168	194	0.9	97	111	0.9
Tyr	178	151	1.2	102	60	1.7	72	20	3.6

[a] Alanine coeluted with the NH_2 peak.
[b] Methionine was not detectable.

good correlation with the FMOC method when used for protein hydrolysates.

Acknowledgements

The authors would like to thank Varian Chromatography Systems, Sweden, for the loan of HPLC equipment. The Swedish Natural Science Research Council is acknowledged for financial support.

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