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Review

Derivatization and chromatographic behavior of the *o*-phthaldialdehyde amino acid derivatives obtained with various SH-group-containing additives

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Abstract

An overview is presented of HPLC methods currently in use to determine amino acids as their *o*-phthaldialdyde derivatives in the presence of various SH-group-containing additives. Crucial points that proved to influence the stability of the amino acid OPA derivatives have been discussed in detail: (i) the mol ratios of the OPA–SH-group-containing additive amino acid; (ii) the preparation and storage conditions of the OPA reagents; (iii) the optimum pH conditions for the interactions and elutions; (iv) the behavior of the, believed to be, less stable amino acids, such as glycine, β-alanine, γ-aminobutyric acid, histidine, ornithine and lysine.

Keywords: Reviews; Derivatization, LC; Amino acids; Amino acids, OPA derivatives

Contents

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1. Introduction

All of those analytical chemists who have worked and/or are working in the field of amino acid analysis and tried to use various derivatization techniques in their chromatographic determinations, know, that for simplicity and for minimizing work and time HPLC of the pre-column derivatized *o*-phthaldialdehyde (OPA) amino acids is the method of choice. The distribution of derivatization methods, proposed in order to quantitate amino acids by HPLC in two, very recent monographs [1,2] proves this statement (Tables 1 and 2). Since the very welcomed introduction [3] of the OPA method its popularity is continuously increasing; in the period from 1982 to 1998 its application represented 30.4% of all derivatizations performed in HPLC (Table 1), while 1 decade later, in the period 1992–1998, on the basis of the same selection, this figure increased to 72.5% (Table 2, 31.2·100/43.0), covering the 31.4% of all chromatographic techniques applied to the analysis of amino acids. Thus, it seems to be useful to compile all of those advantages/disadvantages, difficulties, chromatographic advancements and application areas that characterize this very common procedure.

Table 1
Distribution of quantitation methods used in HPLC of amino acids

Derivative	No.[a]	%[b]
Activated halide	42	8.4
Acyl halide	61	12.1
OPA	3	0.6
OPA–2-mercaptoethanol	92	18.3
OPA–ethanethiol	12	2.4
OPA–3-mercaptopropionic acid	17	3.4
OPA–NAC (chiral cysteines)	29	5.8
OPA in total	153	30.4
Dialdehyde (not OPA)	8	1.6
Phenylthiocarbamyl	95	18.8
Subst. phenylthiocarbamyl	32	6.3
Succinimidyl ester	16	3.2
Dabsyl	36	7.1
Dansyl	31	6.2
Lactone/ketoaldehyde	6	1.2
Cobalt and hydroxylamine	7	1.4
Solid-phase reagent	3	0.6
Sulfonic acid/halide	5	1.0
Miscellaneous	9	1.8
In total	504	

[a] Number of papers referred.
[b] Distribution %, (expressed in the total) taken from Ref. [1], appeared in the period 1982–1998.

Table 2
Distribution of the quantitation methods used in chromatography of amino acids

Method	Derivative	No.[a]	%[b]
HPLC	Underivatized	8	4.5
	Dabsyl	3	1.7
	Succinimidyl ester	9	5.0
	Dansyl	8	4.5
	Phenylthiocarbamyl	17	9.5
	Subst.phenylthiocarbamyl	5	2.8
	OPA–2-mercaptoethanol	8	4.5
	OPA–3-mercaptopropionic acid	3	1.7
	OPA–NAC (chiral cysteines)	7	3.9
	Substituted OPA	3	1.7
	FMOC+OPA–MPA	3	1.7
	OPA in total	24	31.2
	Activated halide	11	6.1
	In total	77	43.0
IEC		4	2.2
CE, CEC		37	20.7
GC		30	16.8
TLC		20	11.2
Others	Less common	11	6.1
In total		179	

[a] Number of papers referred.
[b] Distribution % (expressed in the total), taken from Ref. [2], appeared in the period 1992–1998.

2. Blank value, composition, lifetime and optimum storage condition of reagents

2.1. Blank value of the reagent

Recently [4–6], an exhaustive study was carried out in order to examine the characteristics of the OPA–3-mercaptopropionic acid (MPA) and OPA–*N*-acetyl-L-cysteine (NAC) reagents. These studies [4–6], were performed with UV photodiode array (DAD) and fluorescence (FL) detection, simultaneously. Results proved that the OPA–MPA and OPA–NAC reagents do have blank values both in the UV and in the FL regions. The quality and quantity of peaks originated from the reagents are depending on the age and certainly on their concentrations. It is to be emphasized, even when using the same preparation and storage conditions, that the amounts of the single impurities are different from reagent to reagent (in spite of the fact that the tendency concerning the increase and decrease of the single impurities, as a function of reagent's age and their quality are very similar). Consequently, to take into consideration the reagent peaks in order to subtract them from the coeluting amino acids is obligatory. In particular, in those cases when a relatively higher reagent concentration is needed, because amino acids are measured in extremely different concentrations, in the presence of their matrix [6]. Since the quality and quantity of reagent impurity peaks are changing, (the aged reagents contain less than the fresh ones), the blank value of the reagent should be tested at least every day.

As to the expected amount of impurity peaks using the OPA–MPA or OPA–NAC reagents, measured with the same reagent, between 50 min and 22 days, are changing: in the elution range of amino acid derivatives to be determined the amount of the coeluting impurities can vary between 50 and 300 p*M*, injected in total. Certainly, that which of the amino acids will be coeluting with which reagent peaks depends on the column and the gradient applied. Thus, the blank value depends upon the system and conditions.

As to the case of blank value, in general [3,7–16], OPA reagents with SH-group-containing additives are regarded as '' . . . nonfluorescent itself, and when present in excess does not break down or react to form fluorescent byproducts''. [7]. Although, selected observations [8–16], including the pioneer work [3] are in contradiction with the above general accepted impression and call attention to the fact, that in particular with the use of its large excess, the chromatographic elution of the reagent can result in numerous spurious peaks [8–15]. In the pioneer work [3] the author's warning is unambiguous: '' . . . the blank fluorescence is the main factor limiting the sensitivity of the method''. On the basis of this study the self-fluorescence of impurities was defined as the half of 1 μl 10^{-3} *M* alanine solutions applying the OPA–3-mercaptoethanol (MCE) reagent in 10 molar excess. From others [8,9] the fluorescence response of a matured 48-h-old OPA–MCE reagent '' . . . was found to be approximately 10 times less intense than the response seen from 100 p*M* of each OPA–MCE amino acid derivative . . . '' [8]. According to another experience [9] '' . . . when working with amounts of material less than about 100 p*M*, appropriate control must be run to compensate for the minute amounts of amino acids found in even the best of commercially available reagents.'' Further experiences [10–15] were mainly qualitative, advising one to discard the OPA–MCE reagent solution if contaminant peaks appear, earlier, if necessary, also at 2 months [10], or 1 week [11]: due to the extremely different, believed life time of the same reagent (note of the present paper's author). The impurity peaks proved to be extremely disturbing by using electrochemical detection (ED) [12–15]. In the case of the OPA–MPA reagent substantially more extraneous peaks were detected applying ED compared to the FL detection [12]. In order to improve sensitivity either special chromatographic condition [13] or the elimination of the excess reagent was proposed [14,15]. Scavenging the excess of the OPA–MCE reagent by adding iodoacetamide or *N*-ethylmaleimide resulted in limited success only [14]. Scavenging the excess of the OPA–*tert*.-butylthiol (BT) reagent with the excess of glycine and iodoacetamide [15] resulted in the quantitative elimination of interfering reagent peaks and in a detection limit of 50 a*M* amino acid. The stabilizing effect of nitrilotriacetic acid acting as a metal chelating agent was found to increase the OPA–MCE reagent stability at least fourfold in the sample vial of an automated system [16].

2.2. Composition of the reagent; derivatization conditions

Diversity of the reagent composition, the mol ratios of OPA to the SH-group-containing additive and to the amino acids to be derivatized, the pH, the buffer concentration and time of interactions suggested as optimum ones, in the case of the OPA–MPA and OPA–NAC derivatives have been summarized, in detail: they proved to be extremely different [5]. Thus, in this compilation the same correlations are shown for the reagent couples OPA–MCE [17–22] and the OPA–BT [15], seeing as these reagents were selected in recent papers [15,17–19]. As seen (Table 3), even in the last decade, few attention has been paid to any of most important parameters, such as the absolute concentration both of reactants and the buffer, the pH and time of derivatization reaction, storage condition and life time of the reagent, etc. All these uncertainties result in basically incomparable analytical data and consequences drown from them.

2.3. Life time and optimum storage conditions of reagents: dependence on the SH-group-containing additive

The behavior of the OPA–MPA and OPA–NAC reagents – stored in the refrigerator at 4°C – has been followed with DAD and FL detection, simultaneously, with storage from 50 min to 22 days [5]. It is worthy of mention that the older the reagents the smaller the amount of impurities found. Thus, the best solution of the problem might have been the use of aged reagents. Unfortunately, both reagents can be applied until 9 days, only, remaining on the safe side, in order to obtain quantitative derivatization [5].

The OPA–MCE(BT) reagent was reported to be appropriate for derivatization (Table 3) up to 1 day [18], 1 week [22,23], 2 weeks [20] or 1 month [19], usually saved in the refrigerator at 4°C [17–20,23], or at room temperature [15,22]. Others [15,17,21] do not attach substantial importance to these phenomena: as a basis of quantitative and reproducible derivatizations [5,6]. Concerning other conditions, the most desirable pH (pH 8–11.5) and/or the applied mol ratios of the reagent to the analyte ([OPA]/[amino acids]=5.5–$8\cdot10^3$) proved to be extremely different.

3. Reaction mechanism studies

The formation and composition of the fluorescent adduct – consisting of OPA, a primary amine or amino acid of primary amino group, formed in the presence of various SH-group-containing agents, incorporated also in the derivative – have been investigated in detail [24–37]. On the basis of spectral and NMR [24–28], kinetic [29,30], as well as on those of mass spectrometric (MS) evidence [31–33] the ''main'' reaction products proved to be 1-alkylthio-2-alkyl-substituted isoindoles [24–33]: obtained from 1–1–1 mol of OPA, NH_2 and SH-group-containing compounds with the elimination of 2 mol of water. Although, beside the ''main''

Table 3
Conditions in the derivatization of amino acids with the OPA–MCE and OPA–*tert*.-butylthiol (BT) reagents: literature data[a]

Related to the final volume						Storage of the reagent		Reaction time (min)	Detection			Ref.
OPA (*M*)	MCE, BT, (*M*)	Buffer		Mol ratios OPA					UV (nm)	Fluorescence		
		M	pH	MCE	Amino acid	a, r	Time			Excitation (nm)	Emission (nm)	
0.0364	0.0122	0.075	8.0	2.98	–	r	–	2	338	338	458	[17]
0.04	0.26	0.5	9.5	0.15	$2\cdot10^7$	r	1 d	1	–	–	–	[18]
0.037	0.105	0.4	8–9.7	0.35	$8\cdot10^3$	r	1 m	1–9.3	–	335	425	[19]
0.017	0.029	0.2	10.4	0.58	170	r	2 w	2.5	–	337	452	[20]
0.0738	0.130	–	9.5	0.57	5.5	–	–	3.5	268	330	450	[21]
0.0088	0.258	0.5	9.5	0.034	20–400	a	1 w	3.5–15	–	330	455	[22]
0.0027	0.0048	0.5	10.4	0.56	$1.8\cdot10^4$	r	1 w	2	–	330	408	[23]
0.030	0.040 BT	–	11.5	0.75	–	a	–	2		Electrochemical		[15]

[a] Abbreviations/indications: M=mol; a=ambient temperature; r=refrigerator; d=day; w=week; m=months.

product, very likely as a result of its decomposition (disproportionation/autooxidation), the presence of the 1,3-dithio-substituted-2-alkylisoindoles were also determined, based on NMR evidence [27], supported also by kinetic data [29,30]. Further products such as the dimer adduct of ethanedithiol, OPA and propylamine were also reported: formed nearly quantitatively from 0.5 *M* ethanedithiol and 1–1 *M* of OPA and propylamine [27]. Regarding the reaction between the two primary amino groups containing lysine and ornithine, providing multiple OPA derivatives, assumptions are available, only. MS proof of the composition of the OPA–MCE(ethanethiol)-C1–C5 aliphatic amines [31] and the OPA–β-aminothiol [32] adducts were performed off-line, by gas chromatography–mass spectrometry (GC–MS) as silyl derivatives [31,32]: subsequent to their HPLC separation, followed extraction into organic solvents and derivatization with bis(trimethylsilyl)trifluoroacetamide (BSTFA). The composition of the OPA–MCE–biogenic amine adducts have been identified one by one, also as silyl derivatives, by GC–MS [33], on the basis of their highly informative $[M]^{+}$ ions (extracting their OPA–ET adducts from aqueous solutions with diethyl ether, drying and silylating them with BSTFA). Recently, on-line HPLC–MS determinations [17,35,36] have been reported. HPLC–thermospray (TSP)-MS was applied in order to determine *S*-nitrosoglutathione in the presence of an enormous excess of reduced glutathione as their OPA–MCE derivatives [17].

The diastereomeric isoindoles of the OPA–NAC derivatives of alanine, valine, leucine phenylalanine, phenylglycine and their corresponding amides have been separated and identified also by HPLC–TSP-MS [35], all on the basis of their protonated molecular ions ($[M+1]^{+}$). The isotope enrichment of citrulline, arginine, tyrosine, valine and leucine has been measured by HPLC–electrospray (ESP)-MS as their OPA–MPA derivatives [36] working in the negative ion mode.

4. Stability of the amino acid derivatives

The stability dilemma and the use of the OPA method are inseparable. Special studies devoted exclusively to examine the stability of the amino acid OPA derivatives off-line from the chromatographic system [4,37] were performed. In addition several papers, dealing primarily with the HPLC of the OPA amino acids, somehow, became also involved in the stability phenomena: providing new SH-group-containing alternatives, improved derivatization/chromatographic conditions and/or new application areas. Before going into details it is worthy of mention that in the literature as model compounds of stability studies (from the beginning and thereafter continuously), glycine, γ-aminobutryic acid (GABA), β-alanine, histidine, ornithine and lysine have been selected as the less stable ones: in shortage of knowledge of their intrinsic characteristics, i.e., (i) these six amino acids provide more than one OPA derivative, (ii) resulting in products of similar high stability as the single OPA derivative furnishing amino acids [5,6,34]): so long as, the total of their FL or UV responses, i.e., the sum of derivatives obtained from the given amino acid were taken into account in their quantitation.

4.1. Comparative studies applying different SH-group-containing additives

4.1.1. Optimum conditions in the use of the o-phthaldialdehyde–3-mercaptoethanol reagent: comparisons of the stability of the o-phthaldialdehyde–3-mercaptoethanol and o-phthaldialdehyde–thioalcohol derivatives

In the 1980s exhaustive investigations were performed to clarify factors affecting the stability of the primary amino group with the OPA–MCE [38], OPA–3-mercapto-1-propanol (MCP) [38] and OPA–ethanethiol (ET) [39] reagents. The kinetic stability of isoindole products was evaluated under conditions similar to those for analytical application. As model compounds *n*-propylamine, GABA, β-alanine, glycine, β-aminobutyric acid and alanine have been tested [38,39]. Stability conditions of their derivatives obtained at 40.5°C and at pH 8.95, applying the mol ratio of OPA–MCE–amino acid=150:1430:1 ($1=1\cdot10^{-6}$ *M*) were characterized with the 50% decomposition of the product, expressed as the half time of their decomposition ($t/2$) which proved to be in order of listing 18.2, 21.1, 24.9, 30.8, 57.8 and 98.9 min. Note: the decomposition of β-alanine was four-times faster than that of alanine (98.9/24.9=

3.97). Decreasing the OPA concentration in OPA–MCE–β-alanine to $74.8 \cdot 10^{-6}$ *M* and $37.7 \cdot 10^{-6}$ *M*, the $t/2$ values became increased for 35.7 and 43.3 min. Increasing the OPA concentration from $149 \cdot 10^{-6}$ *M* to $298.9 \cdot 10^{-6}$ *M* and to $447.9 \cdot 10^{-6}$ *M* the rate of decomposition decreased to 16.5 and 12.0 min, respectively: proving the accelerating effect of the OPA excess. The superior stability of the OPA–MCP derivatives was compared by the $t/2$ of its β-alanine derivative which proved to be 346.6 min with the $149 \cdot 10^{-6}$ *M* OPA concentration (346.6/24.9=13.9). Consequently, these investigations resulted in two ways to alter the OPA derivatization reactions: changing of reactant ratios, taking into account that the high excess of OPA accelerates decomposition, and altering the SH-group component. In order to compare the stability of the OPA–MCE derivatives to the OPA–ET ones, investigations were carried out in the same laboratory [39], under the same analytical conditions, as the OPA–MCE and OPA–MCP ones [38]: ensuring the comparability of the stability of all derivatives under investigation. These results based on comparison the reaction times of 10% degradation of the OPA–MCE to the OPA–ET products proved stability enhancements of (ET/MCE) 5.6, 7.1, 9.4, 33.0, and 19.3 for GABA, β-alanine, glycine, β-amino-*n*-butyric acid and alanine, respectively. A direct comparison of the fluorescence intensities of the OPA–MCE and OPA–ET derivatives, was reported [9]: the fluorescence intensities of OPA–ET derivatives averaging seven times that of their MCE counterparts. Enhanced

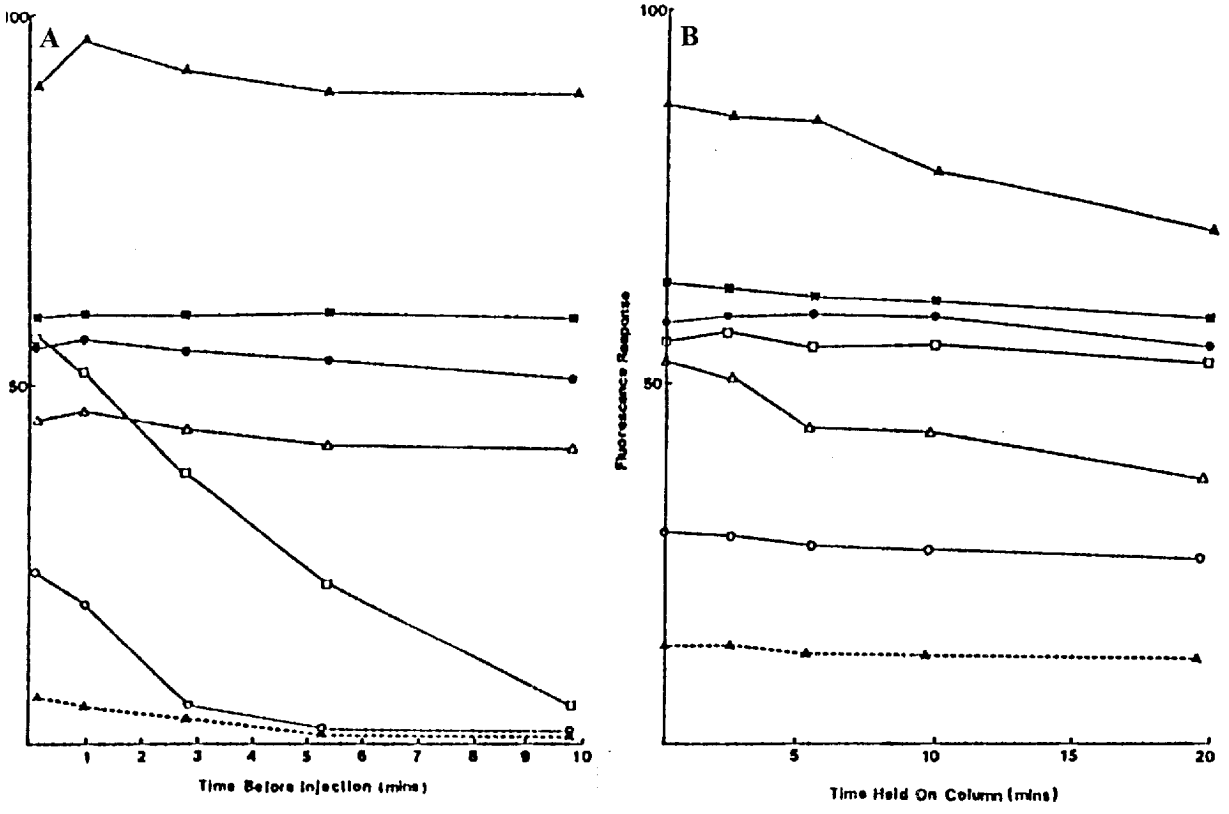

Fig. 1. Stabilities of the OPA-MCE amino acids tested with different reaction times before injections. (A) In the presence, (B) in the absence of the reagent's excess, the time held on-column when the solvent flow was stopped. Symbols: —▲—, glutamic acid; △, aspartic acid; □, glycine; ●, serine; ■, valine; ◦, lysine; --▲--, ornithine; (note: the pH of solutions were pH 6.5, the temperature was not given). Reproduced with permission from Ref. [8].

stability and fluorescence intensity has been described for glycine, GABA and β-alanine by increasing the reaction temperature to 50–60°C and substituting the MCE with ET or methanethiol [40]. A convincing approach regarding the destructive effect of the reagent excess was presented [8], by holding the OPA–MCE amino acids for various time intervals in the injection loop before injecting them onto the HPLC column (Fig. 1A) and compared them to those when 2 min after injection of the derivatives onto the HPLC column, the solvent flow-rate was stopped for different time interval to examine the stability of the OPA-MCE amino acid derivatives on the column (Fig. 1B). This experiment with glycine, lysine and ornithine explains the fact that the excess of OPA reagents are involved in the transformation of the first forming monothioisoindoles to the transformed isoindol derivatives [29,30,6,34].

Special suggestions were made to enhance the stability of the OPA-MCE reagent: by using nitrilotriacetic acid (NTAA), in the mass ratio of OPA-NTAA=5:1 resulted in its fourfold stability [16]. The increased methylalcohol concentration of the reagent improved the stability of the OPA-MCE [41] and OPA-ET [42] amino acids.

4.1.2. Comparisons of the stability of the o-phthaldialdehyde-3-mercaptopropionic acid and o-phthaldialdehyde-N-acetyl-L-cysteine amino acids to the o-phthaldialdehyde-3-mercaptoethanol(ethanethiol) amino acid derivatives and to each other

The stability of the OPA-MPA amino acids were compared with the corresponding OPA-MCE and the OPA-ET derivatives [43,44]. Evaluating the stabilities and responses of the three derivatives of alanine (Fig. 2, [43]) based on FL intensities and those of the three model solutions, based on the UV responses (334 nm) of the total of 17 amino acids (Fig. 3, [44]) proved the constant stability of the OPA–MPA derivatives.

The advantage of the OPA–NAC reagents over the OPA–MCE and OPA–ET ones, due to their chiral recognition characteristics, attracted only few scientists [7,45,46]. The introduction of NAC as SH-group-containing alternative was explained mainly in qualitative manner [45], i.e.: ‘‘Even the fluorescence of the glycine derivative, which is the most susceptible to autooxidation, was stable for 20 min. This indicates that the AcCys (NAC) derivatives of amino acids are much stable than their MCE or ET deriva-

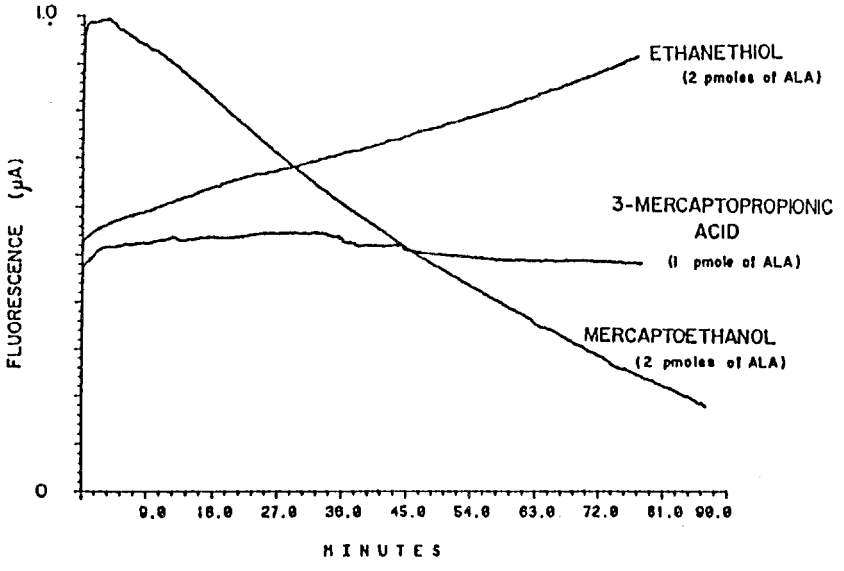

Fig. 2. The effect of various mercapto reagents on the stability of OPA–alanine. Reproduced with permission from Ref. [43].

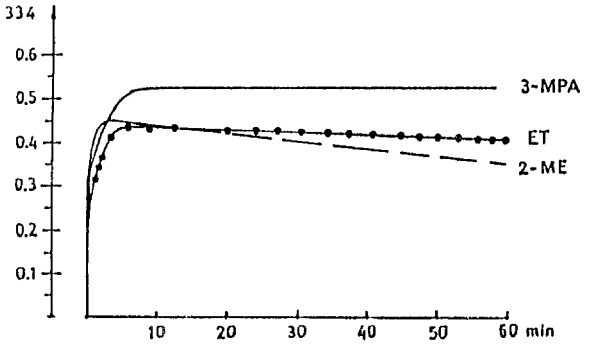

Fig. 3. The stability of the 17 OPA amino acid adducts illustrated as the UV response (334 nm) versus reaction time for the three mercapto reagents: UV absorbances of the total of 17 amino acids (2.5 n*M*/ml of each) obtained as the OPA–MCE, OPA–ET and OPA–MPA derivatives. Reproduced with permission from Ref. [44].

tives''. The comparison of the relative FL intensities of the OPA–NAC amino acids with the OPA–MCE amino acids [46] revealed that their intensities are in the same order of magnitude with the exception of proline previously oxidized by hypochlorite, which provided 20 times higher FL intensity than its corresponding OPA–MCE derivative. Kinetic–spectrophotometric studies on the formation and degradation of the OPA–NAC amino acid derivatives [7] have been performed under the same conditions as those of the OPA–MCE ones [37], in order to compare them in a reliable manner. (i) The formation both of the MCE and the NAC isoindoles are instantaneous but the formation rate constants of the NAC isoindoles, with three exceptions (aspartic acid, glycine, lysine), proved to be more accelerated. The formation rate seems to be affected by the amino

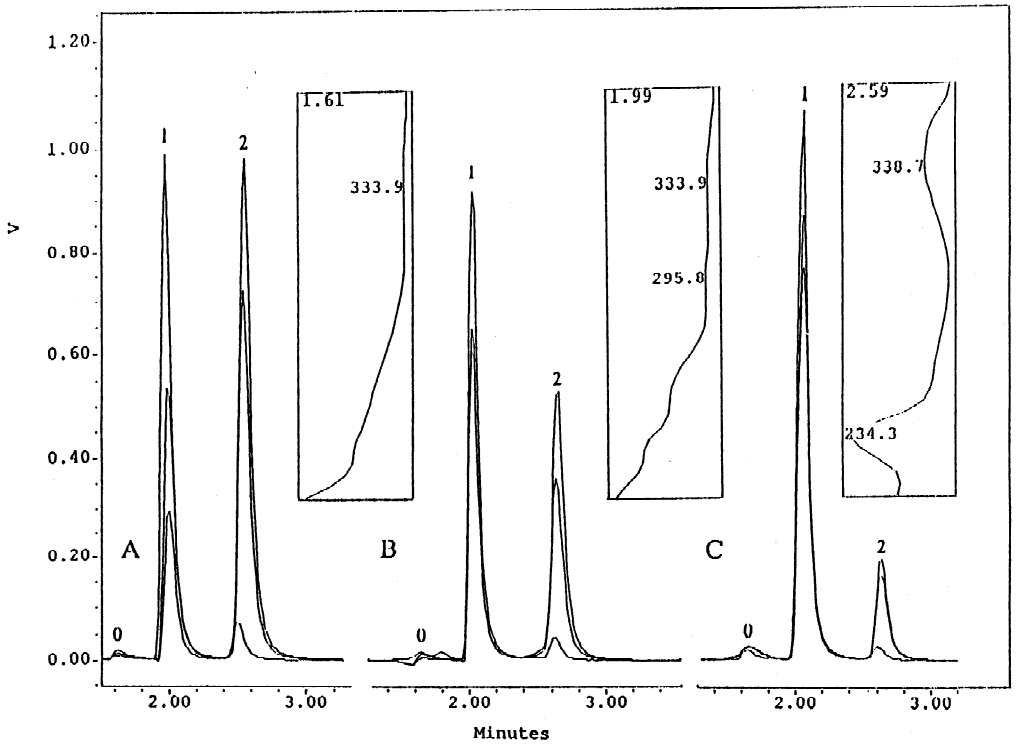

Fig. 4. Chromatograms (A, B, C) obtained by FL detection and DAD, applying the mol ratios of OPA–MPA–glycine=20:10:1 (A), OPA–MPA–glycine=20:60:1 (B), OPA–MPA–glycine=20:200:1 (C) ($1=1\cdot10^{-9}$ *M*) as a function of the reaction time (7 min, 3 h, 6 h). Peaks: 1, gly0; 2, gly1; 3, gly2. Chromatographic conditions: column, A and B eluents as in Refs. [5,6]. Isocratic elution, rate: 0.8 ml/min, 25% eluent B. Reproduced with permission from Ref. [34].

acid's structure, in particular by the substituent of the C atom, adjacent to the NH_2 group. (ii) Comparing the stability of the OPA–NAC amino acids to the corresponding OPA–MCE amino acids, selecting the less stable ones, such as GABA, β-alanine and glycine, they proved to be more stable by the factors of 37, 18 and 24, respectively, expressed in min, required for a 10% absorbance drop. The comparison of the stability of 24 OPA–MPA and OPA–NAC amino acid derivatives performed recently [4–6,34], (still under investigation), proved that all of them are significantly more stable than they are generally believed to be: (i) the single derivatives providing amino acids exhibited less than 4% degradation, even after 6 h reaction time. (ii) The amino acids providing more than one derivative (glycine, GABA, β-alanine, histidine, ornithine and lysine), evaluating the total of their peaks, are as stable as the single OPA-derivative furnishing amino acids [5].

4.2. Study on the multiple derivatives providing NH_2-group-containing compounds

The principle of the multiple derivatives providing amino acids proved to be the crucial point of their stability issue [5,6,34]. Earlier observations [18,47–53] did not attach great importance to this experiment. The first reference to the double peaks was related to the OPA–ET derivatives of lysine and ornithine [47]. The lower responses of the OPA–MCE derivatives of lysine and ornithine were assumed (not proved: author's note) [48] due "... to the presence of two fluorescent isoindole structures". Isotope detection study [49] of the OPA–MCE derivatives of glutamic acid, arginine and ornithine resulted in an additional five, in total six ornithine derivatives. The double peaks of the OPA–MPA–lysine was reported as the basis of its quantification [50], while in an other work [51] the presence of the

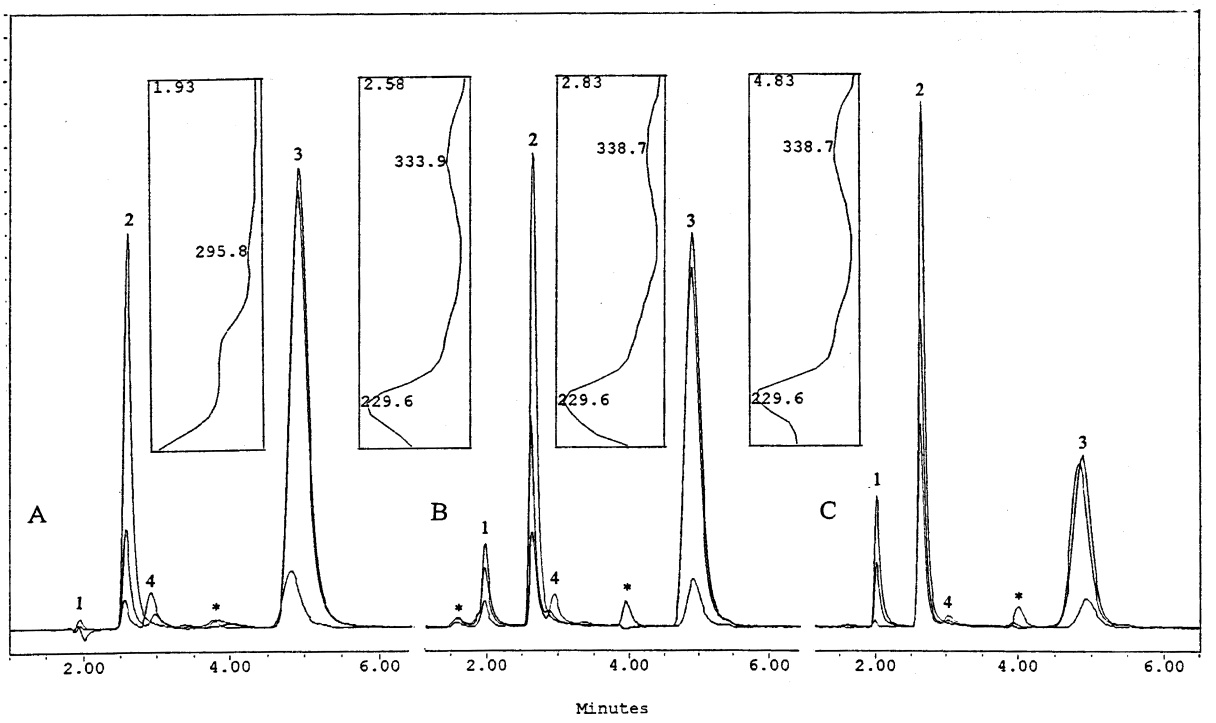

Fig. 5. Chromatograms (A, B, C), obtained by FL detection and DAD, applying the mol ratios of OPA–MPA–lysine=20:10:1 (A), OPA–MPA–lysine=20:60:1 (B), OPA–MPA–lysine=20:200:1 (C) ($1=1\cdot10^{-9}$ *M*) as a function of the reaction time (7 min, 3 h, 6 h). Peaks: lys1; 2, lys2; 3, lys3; 4, lys4; * impurity; (detailed data in Table 4). Chromatographic conditions: column, A and B eluents as in Refs. [5,6]. Isocratic elution, rate: 0.8 ml/min, 33% eluent B. Reproduced with permission from Ref. [34].

Table 4
Stability and characteristics of the OPA–MPA and OPA–NAC amino acid derivatives as a function of the mol ratios of the reactants and the reaction time[a]

Amino acid	OPA–MPA amino acids				OPA–NAC amino acids			
	Response (%)[b]			RR	Response (%)			RR
	7 min	3h	6 h		7 min	3 h	6 h	
OPA–MPA–amino acid=20:10:1 ($1=1\cdot10^{-9}$)								
Glycine0	2.5	1.5	1.1	11.4	5.6	4.2	3.5	11.3
Glycine1	89.6	39.2	20.8	13.6	91.3	46.3	25.9	12.4
Glycine2	7.9	59.3	78.1	18.4	3.1	29.2	45.1	14.4
In total	76	100	100		80	100	100	
OPA–MPA–amino acid=20:60:1 ($1=1\cdot10^{-9}$)								
Glycine0	4.2	2.4	2.1	10.5	4.7	4.7	4.0	12.6
Glycine1	90.2	60.1	46.9	13.5	62.2	67.9	54.7	12.8
Glycine2	5.6	37.5	51.0	18.9	3.0	27.4	41.3	14.7
In total	89	100	100		94	100	100	
OPA–MPA–amino acid=20:200:1 ($1=1\cdot10^{-9}$)								
Glycine0	2.4	4.1	5.2	10.9	3.2	3.8	3.9	10.7
Glycine1	94.7	79.4	73.7	13.5	95.5	86.4	81.8	12.9
Glycine2	2.9	16.5	21.1	17.0	1.4	9.8	14.3	12.9
In total	98	100	93		98	100	100	
OPA–MPA–amino acid=20:10:1 ($1=1\cdot10^{-9}$)								
Lysine1	–	0.2	0.5	–	–	–	–	
Lysine2	76.2	9.3	2.4	2.0	92.9	44.3	26.4	1.9
Lysine3	23.8	88.2	92.4	5.1	7.1	55.7	73.6	3.9
Lysine4	–	2.3	4.2	–	–	–	–	
In total	50	100	100		60	88	100	
OPA–MPA–amino acid=20:60:1 ($1=1\cdot10^{-9}$)								
Lysine1	4.9	6.9	9.0	–	–	0.5	1.7	
Lysine2	78.5	18.5	8.9	2.0	92.2	42.8	26.0	1.8
Lysine3	16.6	72.3	77.9	5.0	7.8	56.7	72.3	3.8
Lysine4	–	2.3	4.2	–	–	–	–	
In total	53	100	100		61	88	100	
OPA–MPA–amino acid=20:200:1 ($1=1\cdot10^{-9}$)								
Lysine1	0.8	7.3	15.1	–	–	1.8	4.2	
Lysine2	87.3	40.5	27.8	2.0	95.9	70.0	56.8	1.9
Lysine3	11.9	52.3	55.8	4.6	4.1	28.2	39.0	4.0
Lysine4	–	0.5	1.3	–	–	–	–	
In total	75	100	100		81	93	100	

[a] m, Min; h, hours; RR=FL–UV represent the response ratios of the fluorescence intensities versus the UV ones; indications as given in Figs. 4 and 5, values are corresponding to the peaks in Figs. 4 and 5; #=maximum Fl was measured after extended reaction time, mainly after 15–28 min.
[b] Expressed in the total.

OPA–MPA derivatives of lysine and ornithine was expected but could not be affirmed. The two GABA derivatives obtained with the OPA–isobutyryl-L/D-cysteine reagents were shown without comment [52]. The formation of the double OPA–MPA derivatives of hydroxyllysine was shown [53], instead of the expected two OPA–MPA–lysine and ornithine derivatives [47,49,50], which have not been found [53].

The double derivatives of reduced glutathione (GSH) and γ-glutamylcysteine (γ-Glcys) were detected as their OPA–MCE derivatives [18], after a 15-min reaction time under extremely high concentrations: the relative response ratios of GSH1/GSH2=50:21 and that of γ-Glcys1/γ-Glcys2=54:19, can be attributed to the extremely low mol ratios of OPA–MCE= 0.15:1 in the reagent.

Recently [5,34], exhaustive study was made in order to clarify the features of the most common amino acids, as their OPA–MPA and/or OPA–NAC derivatives providing multiple OPA products (before our results, the amino acids, such as glycine, β-alanine, GABA, histidine, ornithine, lysine, were believed to providing the less stable derivatives). From an analytical point of view, (based on the principle of their multiple OPA derivatives), problems with them have been solved: (i) their similar high stability compared to the single derivative forming amino acids has been unambiguously proved. (ii) The possibility of their reliable quantitation taking into account the total of their peaks both in model solutions [5] and also in natural matrices [6] was presented in detail. The structure of the successively formed derivatives was only assumed on the basis of their different spectral characteristics [5].

Without on-line LC–MS study, we aimed to clarify affecting factors accelerating the formation/transformation of multiple derivatives [34]. (With these investigations we expect to inspire researchers interested in the OPA amino acid topic to carry out MS investigations.)

The two selected factors affecting the transformation of the primarily formed products to their transformed ones are (i) the mol ratios of OPA to the SH-group additive (changes in the amounts of the glycine (Fig. 4A–C: glycine0–2), and lysine (Fig. 5A–C: lysine1–4) derivatives, detailed data in Table 4), and (ii) the pH of solution containing the OPA amino acids (Fig. 6, Table 5). Decreasing the mol ratios of OPA–MPA in the reagent from 1:0.5 (Figs. 4A and 5A) through 1:3 (Figs. 4B and 5B) to 1:10 (Figs. 4C and 5C), led to a spectacular rate reduction regarding the transformation of the first products to the forthcoming ones. For example, in all six cases, for both derivatives (OPA–MPA, and OPA–NAC), the peak ratios of glycine (Fig. 4A–C) and lysine

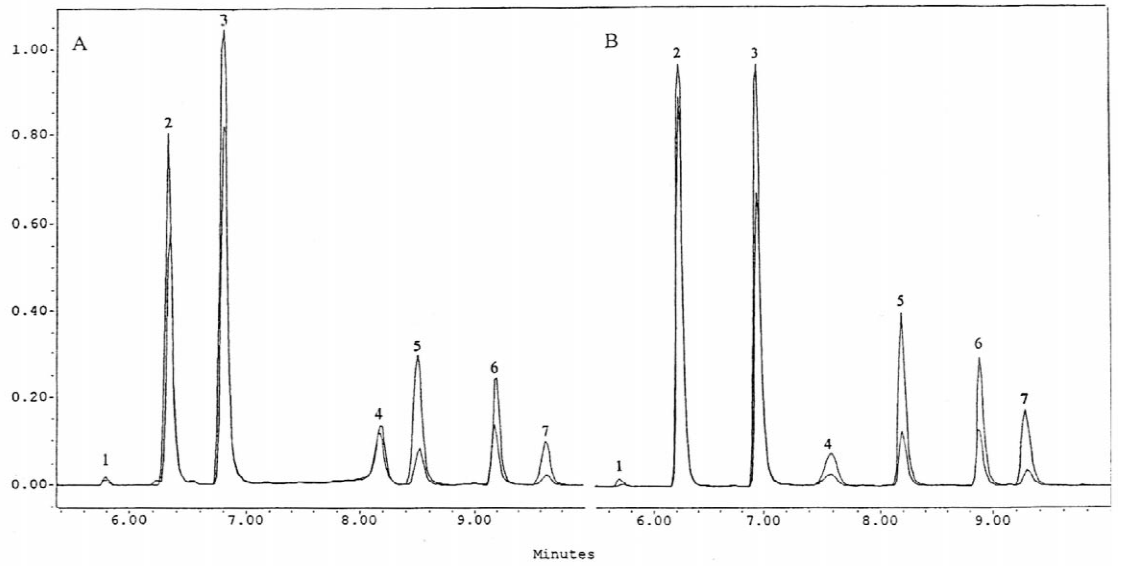

Fig. 6. Chromatograms (A, B), obtained by FL detection and DAD, applying the mol ratios of OPA–MPA–amino acid=20:60:1 ($1=1\cdot10^{-9}$ *M*) as a function of the pH value of the analyte after a total reaction time of 14 min: (A) 14 min at pH 9.3; (B) 7 min at pH 9.3 and 7 min at pH 7.2. Peaks in (A): 1, his0; 2, his1; 3, β-ala1; 4, his2; 5, β-ala2; 6, orn2; 7, orn3. Peaks in (B): 1, gly0; 2, gly1; 3, GABA1; 4, gly2; 5, GABA2; 6, lys2; 7, lys3; (detailed data in Table 5). Chromatographic conditions: column, A and B eluents, gradient program as in Ref. [5]. Reproduced with permission from Ref. [34].

Table 5
Stability of the OPA–MPA derivatives of histidine, β-alanine, ornithine (peaks 1–7 in Fig. 6A), glycine, γ-aminobutyric acid (GABA) and lysine (peaks 1–7 in Fig. 6B), as a function of the pH of solutions

Amino acid	Figure	Peak	pH of solution	
			Response (%)[a]	
			9.3[b]	7.2[c]
Histidine0	6A	1	1.4	1.3
Histidine1		2	41.7	49.1
Histidine2		4	56.9	49.6
Destruction (%)[d]=9				
β-Alanine1	6A	3	96.1	73.1
β-Alanine2		5	3.9	26.9
Destruction (%)[d]=0				
Ornithine2	6A	6	88.5	79.9
Ornithine3		7	11.5	20.1
Destruction (%)[d]=0				
Glycine0	6B	1	0.3	1.1
Glycine1		2	97.2	91.4
Glycine2		4	2.5	7.5
Destruction (%)[d]=15				
GABA1	6B	3	96.6	88.3
GABA2		5	3.4	11.7
Destruction (%)[d]=16				
Lysine2	6B	6	93.3	77.5
Lysine3		7	6.7	22.5
Destruction (%)[d]=14				

[a] Expressed in the total.
[b] Distribution of response percentages after 14-min reactions at pH 9.3.
[c] Distribution of response percentages after 7-min reaction time at pH 9.3, followed by 7-min reaction time at pH 7.2.
[d] Destruction (%) expressed in total of responses, $100\times(7.2^{c}/9.3^{b})$.

(Fig. 5A–C) reveal that the total of products do not decrease considerably, not even after 6 h reaction time (data in Table 4, detailed version in Ref. [34]).

Acidifying the solution of the OPA–MPA amino acids from pH 9.3 to pH 7.2, for 7 min, before injecting them, – subsequent to a 7-min reaction time at pH 9.3 – resulted in considerable changes in their responses. In particular, in the cases of the multiple derivatives providing amino acids (Fig. 6A and B, Table 5: data, comparing the stability after 7 min reaction time obtained at pH 7.2 to those of pH 9.3, on the basis of the total of responses). As seen the transformation rate of the first forming derivative upon acidification, became highly accelerated, the decomposition of the total of derivatives increase from 0 to 16% (Table 5: destruction, %).

Responses of the single OPA amino acids, acidifying them under the same conditions as detailed above are also decreased, in order of listing: threonine (11%), serine (10%), leucine (4%), isoleucine (3%), all others (≤2%). This experience, regarding its tendency, proved to be in accordance with that of the OPA–MCE amino acids [22]: although, under very similar pH condition the OPA–MCE amino acids showed an extremely dramatic drop in responses (Fig. 7) in comparison to our experiences with the OPA–MPA(NAC) amino acids (Fig. 6, Table 5, [34]). These observations are important because several papers suggest the acidification of the OPA amino acids containing solutions before injecting them onto the chromatographic column.

5. Trials in order to derivatize the secondary amino group

The second main disadvantage of the OPA reagents, is their inability to derivatize the secondary amino group (after the first one which is the uncertainty in the stability of derivatives).

To overcome this drawback one possibility, without any analysis data, was reported even in the pioneer work [3]: the oxidation of the secondary amino group with sodium hypochlorite or chloramine T, prior to the reaction of amino acids with the OPA reagents. Japanese researchers [54,55] elaborated the practical details of this derivatization technique of two steps. Later on, an other, a two-step requiring derivatization method was patented [56] and introduced into HPLC practice [57], based on the the reaction of the secondary amino group with 9-fluorenylmethylchloroformate (FMOC) subsequent to the reaction of the primary amino acids with the OPA–MPA reagent. Recently, a special OPA–chlornitrobenzofurazan (NBF) reagent was suggested to derivatize proline and hydroxyproline for their HPLC separation [21]. Unfortunately, the OPA–NBF reagent does not react with the primary amino group,

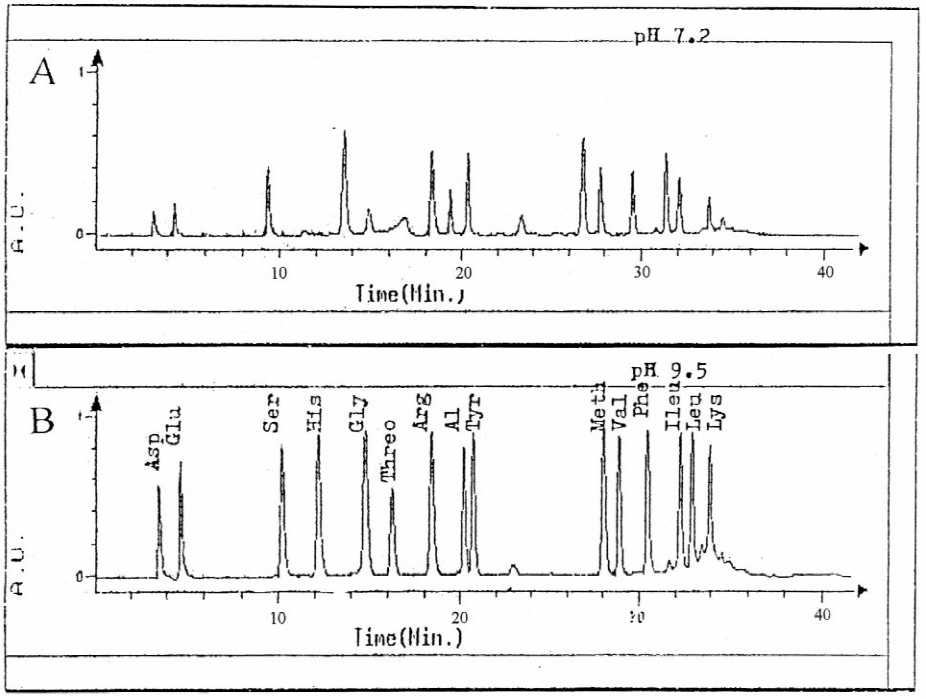

Fig. 7. Responses of OPA–MCE amino acids at pH 7.2 (A) and 9.5 (B) in phosphate buffers. Reproduced with permission from Ref. [22].

consequently two separate reactions (OPA–MCE and OPA–NBF) and elutions had to be performed [21].

5.1. The two-step method: oxidation of the secondary amino group by hypochlorite prior to the reaction of amino acids with the o-phthaldialdehyde reagent

The two step-method has been elaborated [45], improved [46,54,55] and patented also [58] in the post-column mode. Involving all of disadvantages belonging to the post-column techniques, i.e., the need of extra pumps, temperature controllable reaction coils resulting in high cost, increased time consume and decreased sensitivity. Instead of OPA–MCE–oxidized proline [54,55,58], providing low fluorescence the introduction of the OPA–NAC derivative was an advancement being as stable and as sensitive as all other OPA–NAC amino acids [45,46]. In spite of selected cases which inevitably need simultaneous quantitation of the primary and secondary amino acids, because of the above detailed disadvantages the hypochlorite oxidation has not gained wide acceptance.

5.2. Double pre-column derivatization with the o-phthaldialdehyde–3-mercaptopropionic acid–9-fluorenylmethylchloroformate reagent

The first introduction of the OPA–MPA–FMOC derivatization was patented [56] and practically described [57] applying a robotic autosampler. This pioneer work [57] contained several chromatographic proposals: the quantitation of 17 primary amino acids+proline within 12 min, 28 primary amino acids+hydroxyproline and proline within 20 min, as well as 36 primary amino acids+hydroxyproline and proline within 40 min. All separations were performed on the same column (Hypersil ODS, 20 cm×4.6 mm, 5 μm) in order to determine the amino acids of various special matrices under optimum conditions: from bovine serum albumin, wort, beer and plants with 12 min, from plasma deproteinized by acetonitrile with 20 min and by sulfosalycylic acid with 40 min run times.

The amino acid contents of β-lactoglobulin and peptides, including proline, have been determined by HPLC and identified by MS, in the 10–100 p*M* range within 12 min [58]. Further improvements have been published [51,53,59] for the quantitation amino acids in plasma [51], in potato tuber (20 amino acids/8 min) [59] and 42 amino acids in urine [53].

6. Advances in the high-performance liquid chromatography of amino acids present in various matrices with different SH-group-containing *o*-phthaldialdehyde reagents

In this section recently published applications will be reviewed, focusing on selected matrices, related to the HPLC quantitation of amino acids as their OPA derivatives [6,20,59–66].

The OPA–MCE derivatizations are still popular [20,60], in spite of the unpleasant odor of MCE and the fact that the OPA–MCE amino acids are the less stable products. Forty plasma amino acids were separated on a short column (125×3 mm, 5 μm, Spherisorb ODS) in 49 min (Fig. 8), with a good reproducibility (relative standard deviation ≤4%) [20]. The amino acid content of the cerebrospinal fluid measured as their OPA–MCE derivatives [60], is worth of mention: applying FL detection and ED, simultaneously, ensuring improved versatility and specificity in the identification of aspartic- and glutamic acids, glycine, taurine and GABA (Fig. 9). The separations of 22 amino acids in 12 min (Fig.

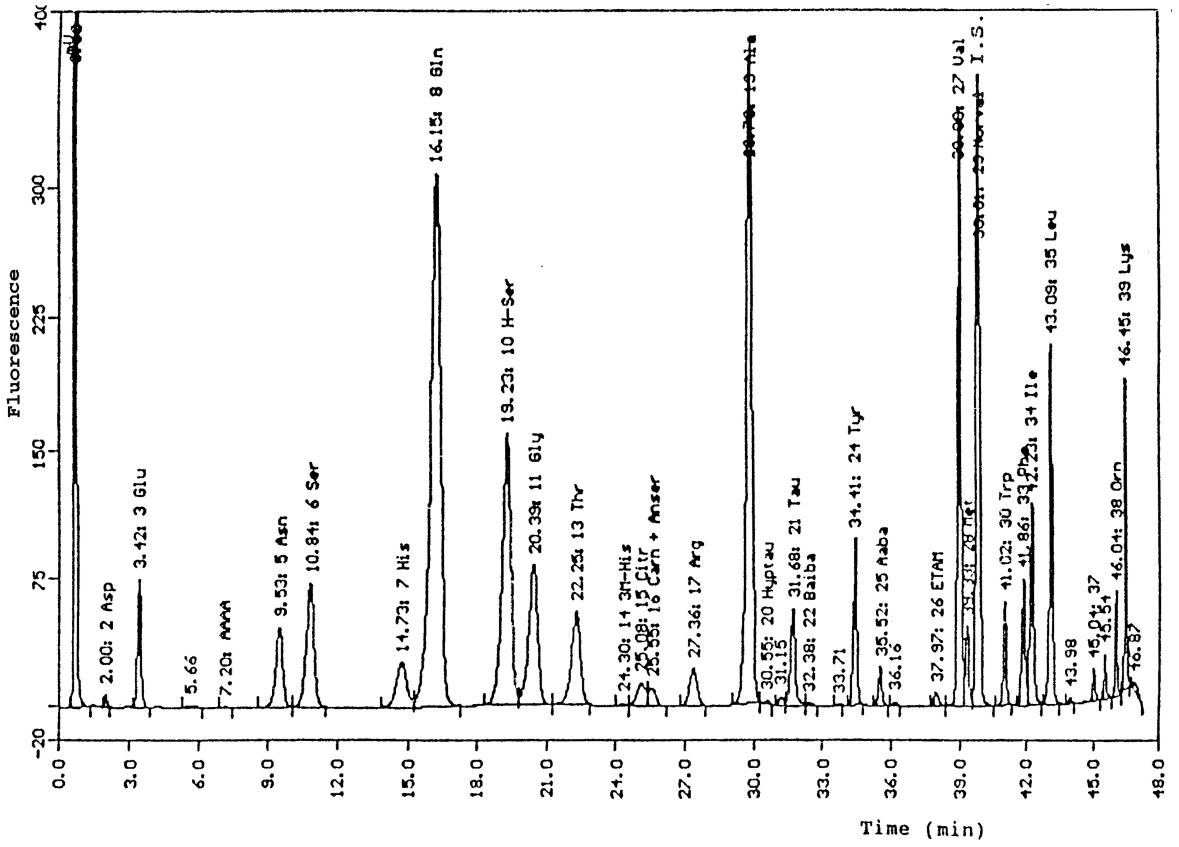

Fig. 8. Elution profile of amino acids obtained from an OPA–MCE derivatized human plasma sample. Reproduced with permission from Ref. [20].

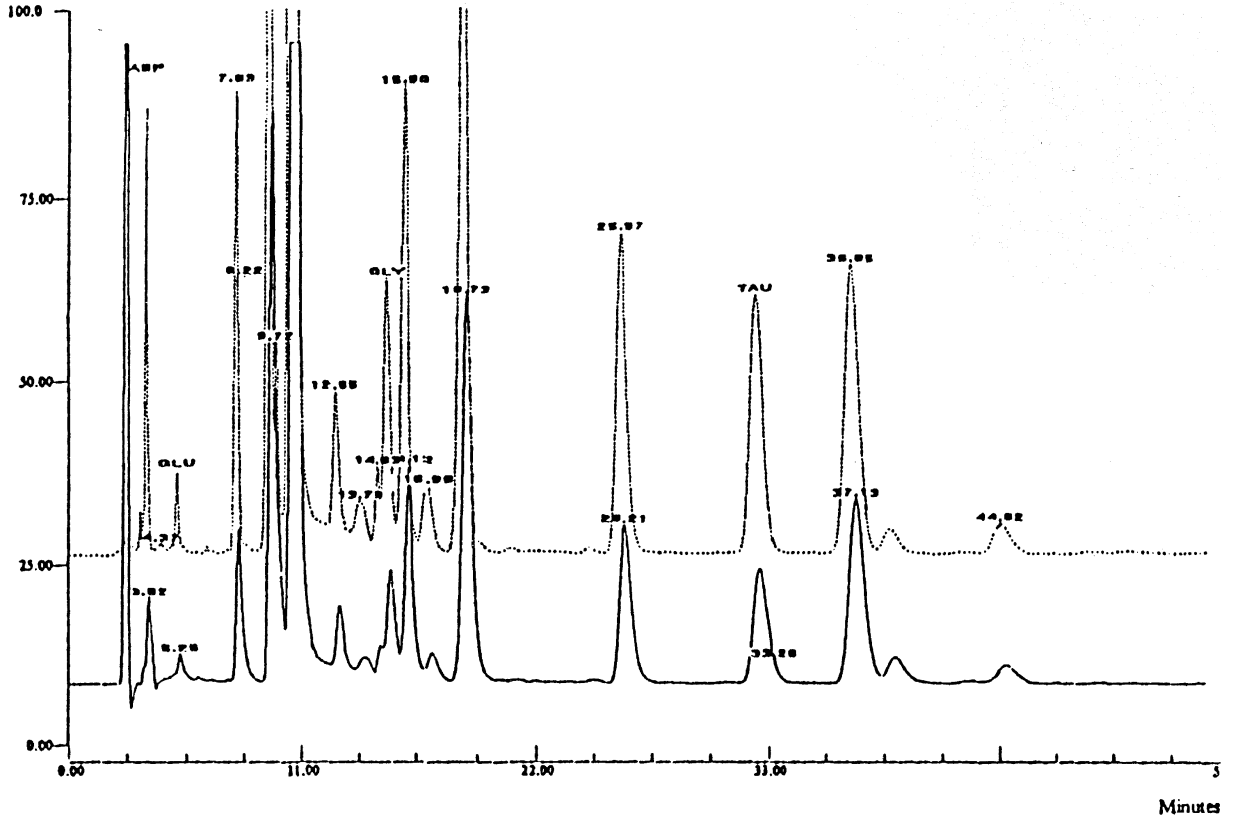

Fig. 9. Separation of aspartic acid (Asp, t_R=3.86 min), glutamic acid (Glu, t_R=5.38 min), glycine (Gly, t_R=15.24 min), taurine (Tau, t_R=32.41) and γ-aminobutyric acid (GABA, t_R=50.19 min, not present) as OPA–MCE derivatives obtained by electrochemical (solid line) and fluorometric (dashed line) detections. Reproduced with permission from Ref. [60].

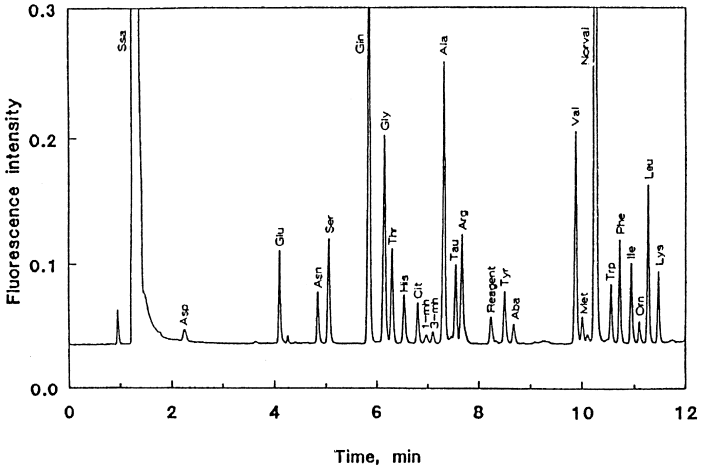

Fig. 10. OPA–MPA derivatized amino acids obtained from a human plasma sample. Reproduced with permission from Ref. [61].

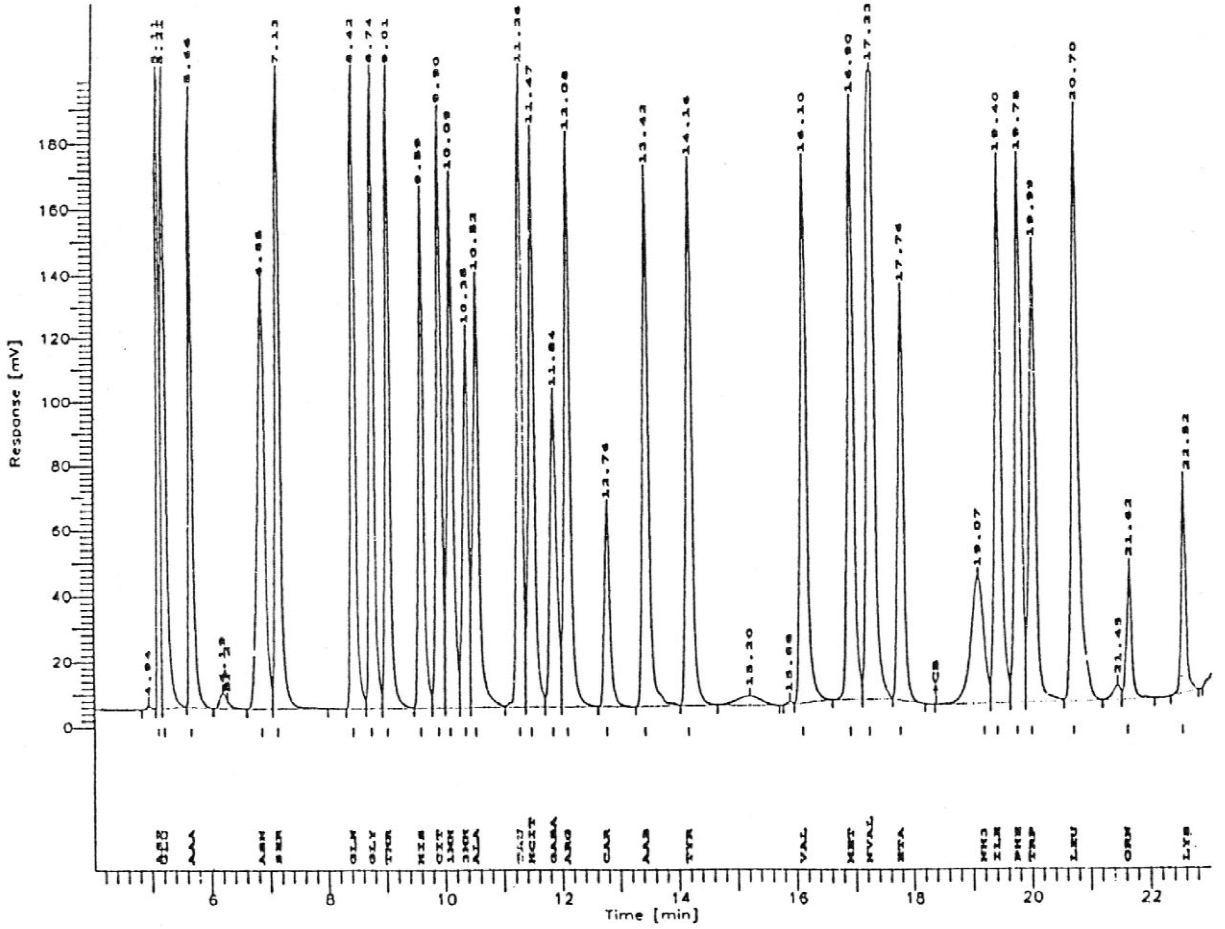

Fig. 11. Separation of 30 physiological amino acids as OPA–MPA derivatives obtained from arterial plasma of pig. Reproduced with permission from Ref. [62].

10, [61]) and 30 physiological amino acids in 28 min (Fig. 11, [62]) were performed both on short columns, from plasma samples deproteinized by sulfosalycylic acid (Figs. 10, 11). The quantitation of the amino acid content of apples [6], applying DAD and FL detection, simultaneously, was optimized with filtered samples: calculated their amino acids contents on the basis of all OPA–MPA products (Fig. 12).

The chiral recognition characteristic of the OPA–*N*-L/D-alkyl cysteine reagents [63–66] made possible in a simple and inexpensive way to determine the L- and D-amino acids, in extremely different concentrations, in the presence of each other, simultaneously. As the OPA–*N*-acetyl-L-cysteine derivatives in geological samples [63], as the OPA–*N*-isobutyryl-L(D)-cysteine derivatives in foods (Fig. 13, [64]), in the water analysis [61] as well as, as the OPA–*N*-*tert*.-butoxycarbonyl-L-cysteine derivatives in brain and in serum samples [66].

The high speed separation of 20 amino acids, including proline, as their OPA–MPA–FMOC derivatives is attractive (Fig. 14, [59]) performed with high precision (RSD≤1.13%)

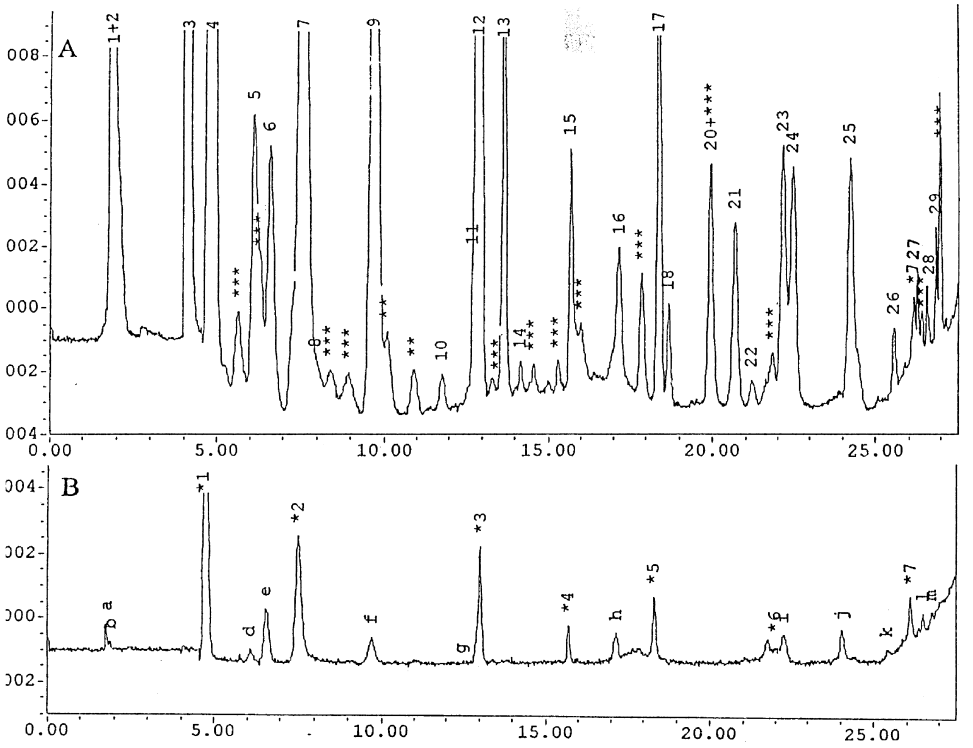

Fig. 12. Elution profile of 25+4 OPA–MPA amino acids. (A) Amino acids of Jonagored apple derivatized according to Ref. [6]; (B) blank test belongs to the A elution. Peaks: 1, aspartic acid, 2, glutamic acid; 3, asparagine; 4, serine; 5, glutamine; 6, histidine; 7, glycine; 8, homoserine; 9, threonine; 10, β-alanine1; 11, arginine; 12, alanine; 13, GABA1; 14, homoarginine; 15, tyrosine; 16, glycine2; 17, valine; 18, methionine; 19, cyst(e)ine; 20, β-alanine2; 21, GABA2; 22, tryptophan; 23, phenylalanine; 24, isoleucine; 25, *n*-leucine; 26, ornithine2; 27, lysine2; 28, ornithine3; 29, lysine3; *=system peaks, **not amino acid type impurities in apple; ***unknown apple constituents providing amino acid type spectra. Reproduced with permission from Ref. [6].

7. Conclusion

In summary, the author of this review – based on recent experiences [4–6,34] – takes the responsibility to state: OPA derivatives of amino acids are much–much more stable than they are generally believed to be, considering that some of them are providing more than one reaction product (for the time being the successively formed products have not been taken into account). Consequently, in the HPLC analysis of amino acids the following requirements are to be followed: (i) the mol ratios of the OPA–SH-group-containing additive/amino acid should be close to 20:60:1; (ii) the reagents, saved in the refrigerator at ≤4°C, should be at least 90 min and maximum 9 days old; (iii) the reaction time between the OPA reagent and the amino acids should be at least 7 min; (iv) the OPA derivatives should not be acidified before loading them onto the chromatographic column; (v) the HPLC elution procedure should be proper for the separation and quantitation of all components formed, including the more than one OPA derivative providing amino acids. (vi) Last, but not least, in order to be able to get reliable amino acid contents, in particular when working in the low p*M* level, the blank value of the reagent should be carried out at least every day and the impurity values are to be subtracted from the coeluting amino acid derivative.

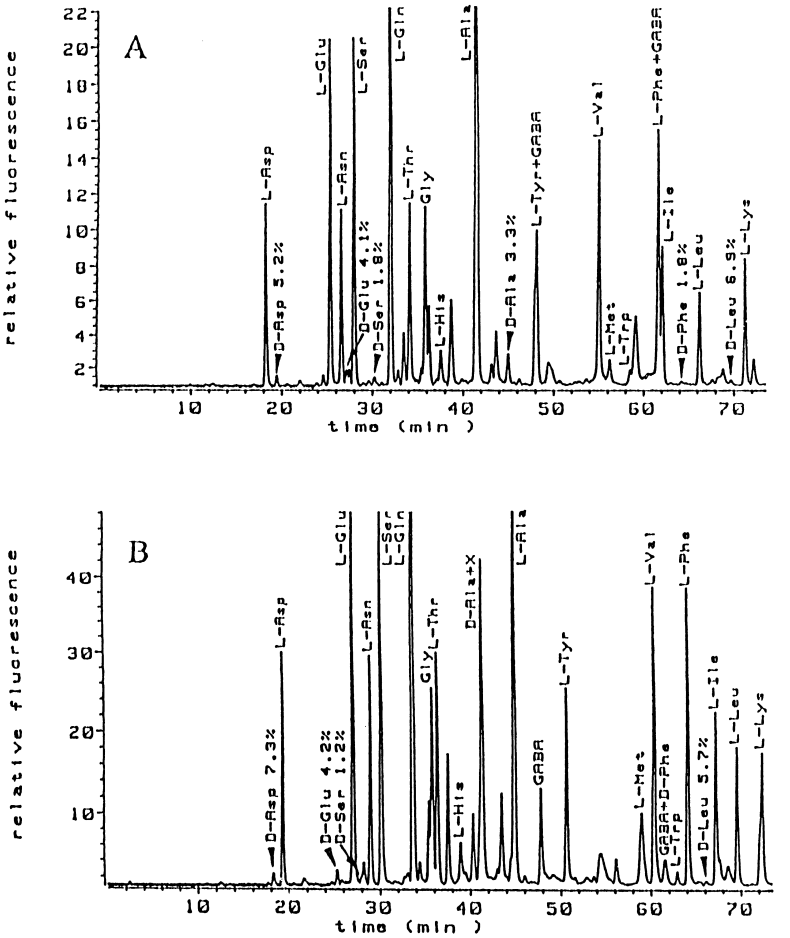

Fig. 13. Aminogram of fir honey derivatized with OPA–isobutyryl-L-cysteine (A) and OPA–isobutyryl-D-cysteine (B). Reproduced with permission from Ref. [64].

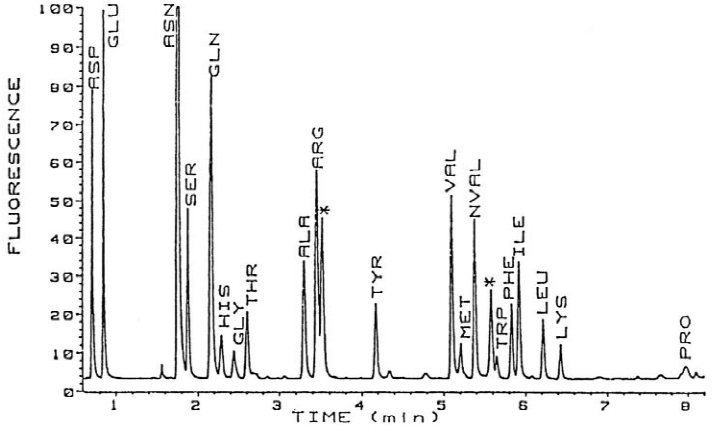

Fig. 14. Chromatogram of amino acids, extracted from potato tubers, measured as OPA–MPA–FMOC derivatives. Reproduced with permission from Ref. [59].

8. Nomenclature

BSTFA	Bis(trimethylsilyl)trifluoroacetamide
BT	*tert*.-Butylthiol
CE	Capillary electrophoresis
CEC	Capillary electrochromatography
ED	Electrochemical detection
ESP	Electrospray
ET	Ethanethiol
FL	Fluorescence
FMOC	9-Fluorenylmethyl chloroformate
GABA	γ-Aminobutyric acid
GC	Gas chromatography
γ-Glcys	γ-Glutamylcysteine
GSH	Glutathione
HPLC	High-performance liquid chromatography
IEC	Ion-exchange chromatography
MCE	3-Mercaptoethanol
MCP	3-Mercapto-1-propanol
MPA	3-Mercaptopropionic acid
MS	Mass spectrometry
NAC	*N*-acetyl-L-cysteine
NBF	Chlornitrobenzofurazan
OPA	*o*-Phthaldialdehyde
DAD	Photodiode array detection
TLC	Thin-layer chromatography
TSP	Thermospray

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References

[1] G. Lunn, L.C. Hellwig (Eds.), Handbook of Derivatization Reactions for HPLC, Amino Acids, Wiley, 1998, p. 625.
[2] I. Molnár-Perl, in: Z. Deyl et al. (Ed.), Advanced Chromatographic and Electromigration Methods in Biosciences, Elsevier, Amsterdam, 1998, p. 416.
[3] M. Roth, Anal. Chem. 43 (1971) 880.
[4] I. Molnár-Perl, I. Bozor, J. Chromatogr. A 798 (1998) 37.
[5] I. Molnár-Perl, A. Vasanits, J. Chromatogr. A 835 (1999) 73.
[6] A. Vasanits, D. Kutlán, P. Sass, I. Molnár-Perl, J. Chromatogr. A 870 (2000) 271.
[7] M.C.G. Avarez-Coque, M.J.M. Hernández, R.M.V. Camañas, C.M. Fernández, Anal. Biochem. 180 (1989) 172.
[8] J.D.H. Cooper, G. Ogden, J. McIntosh, D.C. Turnell, Anal. Biochem. 142 (1984) 98.
[9] G.L. Lookhart, B.L. Jones, Cereal Chem. 62 (1985) 97.
[10] H.W. Jarret, K.D. Cooksy, B. Ellis, J.M. Anderson, Anal. Biochem. 153 (1986) 189.
[11] H.S. Sista, J. Chromatogr. 359 (1986) 231.
[12] T.A. Durkin, G.M. Anderson, D.J. Cohen, J. Chromatogr. 428 (1988) 9.
[13] J. Kehr, U. Ungerstedt, J. Neurochem. 51 (1988) 1308.
[14] O. Orwar, S. Folestad, S. Einarsson, P. Andiné, M. Sandberg, J. Chromatogr. 566 (1991) 39.
[15] B.W. Boyd, R.T. Kennedy, Analyst 123 (1998) 2119.
[16] A.M. Uhe, G.R. Collier, E.A. McLennan, D.J. Tucker, K. O'Dea, J. Chromatogr. 564 (1991) 81.
[17] D. Tsikas, J. Sandmann, D. Holzberg, P. Pantazis, M. Raida, J.C. Fröhlich, Anal. Biochem. 273 (1999) 32.
[18] G. Noctor, C.H. Foyer, Anal. Biochem. 264 (1998) 98.
[19] R.C. Dorresteijn, L.G. Berwald, G. Zomer, C.D. de Gooijer, G. Wieten, E.C. Beuvery, J. Chromatogr. A 724 (1996) 159.
[20] D. Fekkes, A. van Dalen, M. Edelman, A. Voskuilen, J. Chromatogr. B 669 (1995) 177.
[21] B.H. Klein, J.W. Dudenhausen, J. Liq. Chromatogr. Rel. Technol. 18 (1995) 4007.
[22] G. Michael, G. Henrion, Git. Fachz. Lab. 9 (1995) 769.
[23] G. Georgi, C. Pietsch, G. Sawatzki, J. Chromatogr. 613 (1993) 35.
[24] S.S. Simons Jr., D.F. Johnson, J. Am. Chem. Soc. 98 (1976) 7098.
[25] S.S. Simons, Jr., D.F. Johnson, J. Chem. Soc., Chem. Commun. (1977) 374.
[26] S.S. Simons Jr., D.F. Johnson, Anal. Biochem. 82 (1977) 250.
[27] S.S. Simons Jr., D.F. Johnson, J. Org. Chem. 43 (1978) 2886.
[28] S.S. Simons Jr., D.F. Johnson, J. Chromatogr. 261 (1983) 407.
[29] J.F. Stobaugh, A.J. Repta, L.A. Sternson, J. Org. Chem. 49 (1984) 4306.
[30] O.S. Wong, L.A. Sternson, R.L. Schowen, J. Am. Chem. Soc. 1077 (1985) 6421.
[31] R.C. Simpson, J. Spriggle, H. Veening, J. Chromatogr. 261 (1983) 407.
[32] P.A. Metz, J. Gehas, J. Spriggle, H. Veening, J. Chromatogr. 330 (1985) 307.
[33] P.A. Tippet, B.E. Clayton, A.I. Mallet, Biomed. Environ. Mass Spectrom. 14 (1987) 737.
[34] D. Kutlán, I. Molnár-Perl, J. Chromatogr., submitted for publication.
[35] R.G.J. van Leuken, A.L.L. Duchateau, G.T.C. Kwakkenbos, J. Pharm. Biomed. Anal. 13 (1995) 1459.
[36] H.M.H. van Eijk, D.R. Rooyakkers, P.B. Soeters, E.P. Deutz, Anal. Biochem. 271 (1999) 8.
[37] V.J.K. Svedas, I.J. Galajev, I.L. Borisov, I.V. Berezin, Anal. Biochem. 101 (1980) 188.
[38] J.F. Stobaugh, A.J. Repta, L.A. Sternson, K.W. Garren, Anal. Biochem. 135 (1983) 495.

[39] J.F. Stobaugh, A.J. Repta, L.A. Sternson, J. Pharm. Biomed. Anal. 4 (1986) 341.
[40] J.R. Cronin, S. Pizarello, W.E. Gandi, Anal. Biochem. 93 (1979) 174.
[41] A.P. Halfpenny, Ph.R. Brown, J. High Resolut. Chromatogr. Chromatogr. Commun. 8 (1985) 243.
[42] D.W. Hill, F.H. Walters, T.D. Wilson, J.D. Stuart, Anal. Chem. 51 (1979) 1338.
[43] P. Kucera, H. Umgat, J. Chromatogr. 255 (1983) 563.
[44] H. Godel, T. Graser, P. Földi, P. Pfaender, P. Fürst, J. Chromatogr. 297 (1984) 49.
[45] N. Nimura, T. Kinoshita, J. Chromatogr. 352 (1986) 169.
[46] M. Fujiwara, Y. Ishida, N. Nimura, A. Toyama, T. Kinoshita, Anal. Biochem. 166 (1987) 72.
[47] P. Lindroth, K. Mopper, Anal. Chem. 51 (1979) 1667.
[48] G.A. Qureshi, L. Fohlin, J. Bergström, J. Chromatogr. 297 (1984) 91.
[49] B.J. Mixcallef, B.J. Shelp, R.O. Ball, J. Liq. Chromatogr. 12 (1989) 1281.
[50] U. Bütikofer, D. Fuchs, J.O. Bosset, W. Gmür, Chromatographia 31 (1991) 441.
[51] H.G. Worthen, H. Liu, J. Liq. Chromatogr. 15 (1991) 3323.
[52] H. Brückner, T. Westhauser, Chromatographia 39 (1994) 419.
[53] C. Carducci, M. Birarelli, V. Leuzzi, G. Santagata, P. Serafini, I. Antonozzi, J. Chromatogr. A 729 (1996) 173.
[54] Y. Ishida, T. Fujita, K. Akai, J. Chromatogr. 204 (1981) 143.
[55] J. Haginaka, J. Wakai, Anal. Biochem. 171 (1988) 398.
[56] R. Schuster, A. Apffel, Application Note 12-5954-6257, Hewlett-Packard.
[57] R. Schuster, J. Chromatogr. 431 (1988) 271.
[58] D.T. Blankenship, M.A. Krivanek, B.L. Ackermann, A.D. Cardin, Anal. Biochem. 178 (1989) 227.
[59] T. Bartók, G. Szalai, Zs. Lõrincz, G. Börcsök, F. Sági, J. Liq. Chromatogr. 17 (1994) 4391.
[60] V. Rizzo, A. Anesi, L. Montalbetti, G. Bellantoni, R. Trotti, G.V. Melzi d'Eril, J. Chromatogr. A 729 (1996) 181.
[61] T. Teerlink, P.A.M. vanLeeuwen, A. Houdijk, Clin. Chem. 40 (1994) 245.
[62] H.M.H. van Eijk, D.R. Rooyakkers, N.E.P. Deutz, J. Chromatogr. 620 (1993) 143.
[63] M. Zhao, J.L. Bada, J. Chromatogr. A 690 (1995) 55.
[64] H. Brückner, M. Langer, M. Lüpke, T. Westhauser, H. Godel, J. Chromatogr. A 697 (1995) 229.
[65] H.P. Fitznar, J.M. Lobbes, G. Kattner, J. Chromatogr. A 832 (1999) 123.
[66] A. Hashimoto, T. Nishikawa, T. Oka, K. Takahashi, T. Hayashi, J. Chromatogr. 582 (1992) 41.