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Stability and characteristics of the *o*-phthaldialdehyde/3-mercaptopropionic acid and *o*-phthaldialdehyde/*N*-acetyl-L-cysteine reagents and their amino acid derivatives measured by high-performance liquid chromatography[1]

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Abstract

The quality and quantity of impurities present in *o*-phthaldialdehyde (OPA)/3-mercaptopropionic aicd (MPA) and OPA/*N*-acetyl-L-cysteine (NAC) reagents have been measured and characterized performing fluorescence and photodiode array detection, simultaneously. The amounts of impurities determined are considerable. Consequently, they have to be deducted from the coeluting amino acid. Stability studies, carried out with 24 amino acids, including—as believed—the less stable OPA derivatives, such as glycine, γ-aminobutyric acid (GABA), β-alanine, histidine, lysine and ornithine, proved that up to 50 min their decomposition is not significant. After 6 h reaction time, the OPA/MPA amino acids manifest higher stability (≥93%) than the corresponding OPA/NAC ones (≥88%). In order to obtain quantitative interactions: (i) extended reaction time (7–28 min) is needed to achieve 100% yield for alanine, β-alanine, GABA, isoleucine, ornithine and lysine; (ii) those amino acids which furnish more than one derivative (glycine, GABA, β-alanine, histidine, lysine and ornithine) are to be quantitated on the basis of the total of their peaks; (iii) reproducibility investigations revealed that the mol ratios of the OPA reagent/amino acids should be at least 20 times larger than the total of amino acids to be determined. The probable composition of the double derivatives on the basis of their characteristic fluorescence intensities and UV absorbances were discussed: glycine, GABA, β-alanine and histidine might furnish the 1-thiosubstituted-2-alkyl- and the 1,3-dithiosubstituted-2-alkylisoindoles, while lysine and ornithine are eluting as their mono- and dimer isoindoles.

Keywords: Derivatization, LC; Amino acids; Phthaldialdehyde; Mercaptopropionic acid; Acetylcysteine

1. Introduction

The instability of the isoindoles formed from amino acids and *o*-phthaldialdehyde (OPA), in the presence of different SH-group containing compounds, as well as the instability of the reagent itself are known as two of the main disadvantages of this very welcomed principle [1]: suitable for the fast derivatization of amino acids in aqueous media prior to their HPLC.

To overcome the instability of the amino acid

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derivatives the use of automatic derivatization devices were suggested. To extend the lifetime of the primarily introduced OPA/β-mercaptoethanol (MCE) reagent it has been reactivated by completing it with the MCE [2–12] continuously (daily [2], in every second- [3–5], or third days [6–8] and weekly [9–12]).

The instability of the amino acid derivatives obtained with any of the OPA reagents was the object of several investigations [3,12,13,20,22,23,28–40]. Primarily the stability of the OPA/MCE [3,12,28–33] and the OPA/ethanethiol (ET) [34,35] amino acids was studied. The special UV and fluorescence (Fl) characteristics, as well as the stabilities of the corresponding OPA/3-mercaptopropionic acid (MPA) and OPA/*N*-acetyl-L-cysteine (NAC) isoindoles have not been determined and compared to each other, with the only exception of our work performed outside the chromatographic system [19]. Stability comparisons carried out by HPLC [13,20,22,23], were reported in qualitative manner only [13,22,23] or without any experimental data [20] dealing one by one with the stability of the OPA/MCE versus OPA/NAC [20,22,23] and OPA/MCE versus OPA/MPA derivatives [13].

Because of the intrinsic instability of the OPA/MCE isoindoles, as SH-group containing alternatives, MPA [13–19] and *N*-alkyl-L-cysteines [19–27] were preferred: the common advantage of the OPA/MPA and OPA/NAC reagents, can be characterized by the fact that they provide more stable isoindoles, compared to those formed with the OPA/MCE reagent. In addition the optical resolution of enantiomeric amino acids obtained by the OPA/NAC and other *N*-alkyl-L-cysteine reagents could be also extensively utilized.

Due to an exhaustive literature overview with the OPA/MPA(NAC) derivatives (Table 1) primarily, the wide diversity of the derivatization conditions, in particular, that of the lifetime of the OPA/MPA and the OPA/NAC reagents proved to be confusing and

Table 1
Conditions in the derivatization of amino acids with the OPA/MPA and OPA/NAC reagents: literature data

Related to the final volume						Storage of the reagent		Reaction time	Detection			Remarks			Ref.
OPA (mg/ml)	MPA[a] (μl/ml)	Buffer		Molar ratios OPA/		a,r	Time	(min)	UV (nm)	Fluorescence		A	B	C	
		M/L	pH	MPA	Amino acid					λ_{ex}	λ_{em}				
5.0[b]	5.0[b]	0.37[b]	9.4	0.65	—	—	—	—	—	330	418		+	+	[13]
0.5	0.5	0.45	9.5	0.65	1.7×10^3	r	1w	5	334	334	445	+		+	[14]
3.0	5.0	0.22	10.2	0.39	31	r	1w	2.5	338	230	450		+	+	[15]
1.05	1.09	0.45	9.5	0.63	42	r	1w	2.5	—	330	450		+	0	[16]
3.0	3.0	0.45	10.4	0.65	5.9	—	—	2	—	335	440		+		[17]
0.18	0.18	0.08	10.0	0.65	3.0×10^4	a	3d	3	—	230	389		+		[18]
0.25	0.5	0.16	9.4	0.32	19–236	a/r	4d	1–240	334	337	454		+	+	[19]
2.7[b]	3.3[b]	0.05[b]	—	0.97	—	r	1w	2	—	360	405		+	+	[20]
1.0	1.0	0.31	10.0	0.82	<1	a	2d	10	280	344	443	+	+	+	[21]
1.6[b]	2.0[b]	0.38[b]	10.0	0.97	—	a	1m	1	—	348	450		+	+	[22]
2.1	2.5	0.03	9.5	1.0	5–200	a	1w	<1	340	340	445	+		+	[23]
0.80	0.49	0.1	9.4	2.0	74–400	r	3w	15	—	340	450		+	+	[24]
0.27	0.33	0.1	9.5	1.0	≥10	r	—	1	336	—	—		+		[25]
2.0	2.0	0.28	9.0	1.65	139	a	1d	2	—	344	443		+		[26]
2.9	6.2	0.25	10.4	0.65	12	—	—	2	—	230	445		+		[27]
0.25	0.93	0.16	9.4	0.32	19–236	a/r	4d	1–240	334	337	442		+	+	[19]

a, ambient temperature; r, refrigerator; d, day; w, week; m, months; A, B and C, stability studies under various conditions; A, outside the chromatographic system; B, under chromatographic conditions; C, papers devoted primarily to stability studies.

[a]NAC (mg/ml) were used in papers [13–19]; MPA in papers [19–25]; NAC in paper [26]; *N-tert*.-butyloxycarbonyl-L-cysteine and *N*-isobutyryl-L-cysteine in paper [27].

[b]Relating to the reagent volume, final volume of reaction mixture was not given.

needed explanation: the OPA, MPA and NAC concentrations varied, in order of listing, between 0.18 and 5 mg/ml (Table 1, first and second vertical columns). Similarly, also the buffer concentrations reported were extremely different (Table 1, third column: 0.03—0.45 M/L). However, the mol ratios of the OPA/SH-group containing agent varied only in a relatively narrow range (Table 1, fifth vertical column: [OPA]/[MPA]=0.32—0.65 and [OPA]/[NAC]=0.32—2), the mol ratios of the reagents to amino acids show extreme differences (Table 1, sixth vertical column: [OPA]/[amino acids]=$<1–3\times10^4$).

As to the lifetime of the reagents they have been used from 1 day [26] to 1 month [22], including the proposals also for 2 days [21], 3 days [18] and 1 week [23], applying their storage at ambient temperature. Others, saving these reagents in the refrigerator reported 1 week [14–16,20] and 3 weeks [24] until they proved to be utilizable. Detailed tests, proving the initial activity of these reagent until their well-defined lifetimes, were not given. The only study [19] relates to the existence and extent of the self fluorescence and UV absorbance of both reagents, measured as the total of blank values, outside the chromatographic system, in the period of 1–240 min.

In this paper, on the basis of our earlier experiences [19], and, on the basis of all those uncertainties reported in the literature [13–35], including the pioneer work [1] and the studies of the fluorescent reaction products [36–40] the following questions were intended to be answered:

(1) Do have the OPA/MPA(NAC) reagents self fluorescence and/or self absorbance which are to be taken into account in the quantitation of the amino acid derivatives, under HPLC conditions, applying fluorescence and photodiode array (PDA) detection, simultaneously?

(2) Must this phenomenon, i.e., the reagent's self fluorescence/absorbance, be associated with the extremely different reagents's storage conditions?

(3) What is the lifetime of the OPA/MPA(NAC) reagents until providing optimum responses?

(4) What is the reason (if any) of the believed lower stability of the glycine, β-alanine, γ-aminobutyric acid (GABA), histidine, ornithine and lysine derivatives, in comparison to the rest of amino acids?

(5) What are the optimum UV/fluorescence responses of the OPA/MPA and OPA/NAC isoindoles, also in comparison to each other, performing various mol ratios of the reagents to the amino acids to be determined?

(6) What is the correlation between the OPA–amino acid interactions in the presence and in the absence of the SH-group containing compound?

2. Experimental

2.1. Materials

OPA, MPA, NAC and amino acids were obtained from Sigma (St. Louis, MO, USA) and from Serva (Heidelberg, Germany). HPLC-grade methanol, acetonitrile were purchased from Romil (Leics., UK). All other reagents were of the highest purity available.

2.2. Standard solutions

Stock solutions of free amino acids have been prepared with distilled water [17], one by one and/or in two stock solutions containing 2.5×10^{-4} M/L of each and were further diluted to obtain [OPA]/[MPA]([NAC]):[amino acid]^T=4.23:1, 8.46:1, 21.2:1, 42.3:1 and 105.8:1 (stock solution1 (ST-1) contained: aspartic and glutamic acids, serine, glycine, alanine, GABA, tyrosine, valine, phenylalanine, leucine and lysine; stock solution2 (ST-2) contained: asparagine, glutamine, β-alanine, threonine, homoserine, arginine, histidine, methionine, isoleucine, cysteine, tryptophan, ornithine, homoarginine).

Stock solution of OPA contained 0.256 g OPA (weighed with analytical precision) in 50 ml methanol (further on: methanolic OPA solution).

2.3. Buffer solution

Borate buffer was mixed in 50:50 (v/v) ratios from 0.2 *M* boric acid (dissolved in 0.2 *M* potassium chloride)/0.2 *M* sodium hydroxide (pH 9.9±0.05).

2.4. Reagent solutions

OPA/MPA reagent was obtained by mixing, in

order of listing, 5.0 ml methanolic OPA, 20.0 ml borate buffer and 50 μl MPA solutions: final pH 9.3±0.05.

OPA/NAC reagent was prepared from 5 ml methanolic OPA solution, 20.0 ml borate buffer containing 0.1 g NAC: final pH 9.3±0.05. The mol ratios of OPA to the MPA and NAC were, without exception, OPA/MPA(NAC)=1/3.

2.5. Derivatization

2.5.1. Stability study and characterization of the reagent solutions

Blank measurements and/or derivatizations were performed with freshly prepared (reagent age, ≥90 min [19]) reagent solutions, saved in the refrigerator, at ~4°C, for various period of times and injected by the robotic autosampler (Waters 717, thermostated for ~4°C).

2.5.2. Stability study and characterization of the OPA/MPA(NAC)-amino acid solutions

Derivatizations were performed with reagents prepared at least 90 min earlier before use, and saved no longer than ≤9 days. The same amount of reagent solutions (120 μl) was mixed with 380 μl ST-1 or ST-2 of amino acids, as well as with selected amino acid solutions one by one, and left to react for 7 min before injection.

2.5.3. Reproducibility study of the isoindole derivatives, at pH 9.3±0.05

Derivatizations were carried out with reagent solutions prepared at least 90 min before use, and retained no longer than ≤9 days. The same amount of reagent solutions (120 μl) was mixed with variously diluted (380 μl) ST-1 and ST-2 of amino acids and injected as detailed above.

2.6. Reaction between amino acids and OPA, in the absence of the SH-group containing agent, followed by the addition of MPA

Reactions were performed as a function of the time and temperature conditions. ST-1 were reacted with the OPA in the mol ratios of [OPA]/[amino acid]=20.9/1, at ~20 and ~4°C, for 25 and 75 min, as well as for 60 h, at pH 9.8±0.05. After the indicated reaction times the mixtures of [OPA]/[amino acids] were subjected to chromatography, and the remaining mixture completed immediately with the corresponding amount of MPA providing the mol ratios of [OPA]/[MPA]/[amino acid] as described in Section 2.5 (pH 9.3±0.05). After 7 min reaction times the mixtures were subjected again to chromatography.

2.7. Chromatography

The system was a Waters HPLC instrument (Waters Pharmaceutical Division, Milford, MA, USA), consisting of Waters 996 PDA and Waters 274 fluorescence detectors, a Waters 600 Controller quaternary pump with thermostattable column area, a Waters 717 Autosampler, operating with the Millennium Software (version 2010, 1992-95, validated by ISO 9002). The column was a Hypersil ODS bonded phase, 5 μm (150×4+20×4 mm guard column).

Detections have been performed simultaneously applying the PDA (Waters 996) and fluorescence (FL) (Waters 274) detectors, connected in order of listing. Blank tests, concentration dependence and stability values have been taken between 190 and 400 nm (PDA), evaluated at 334 nm (OPA/MPA(NAC)-amino acids) and at 240 nm (OPA/amino acids), as well as at the optimum excitation and emission wavelengths: at $\lambda_{ex}/\lambda_{em}$=337/454 nm.

The eluent system consisted of two components: (A) eluent was 0.05 *M* sodium acetate, pH 7.2, while (B) eluent was prepared from 0.1 *M* sodium acetate–acetonitrile–methanol (46:44:10) (mixed in volume ratios and titrated with glacial acetic acid or 1 *M* sodium hydroxide to pH 7.2). A simple gradient program was followed (Table 2), at 30°C, the eluent flow-rate was 1.8 ml/min.

Table 2
Gradient program

Step	Time (min)	A (%)	B (%)
1	0.00	100	0.0
2	10.00	0.0	100.0
3	13.00	0.0	100.0
4	15.00	100.0	0.0
5	23.00	100.0	0.0

3. Results and discussion

3.1. Impurities in the OPA/MPA and OPA/NAC reagents

3.1.1. Quantitative evaluation of the impurities

On the basis of our recent experiences which revealed that considerable self fluorescences are to be taken into account in the cases of both reagents [19], the present investigations were intended to prove the consequences of this peculiarity of OPA reagents on the quantitative evaluation of the corresponding amino acid derivatives under chromatographic conditions. The quantity of the reagent peaks have been followed by simultaneous PDA and FL detections from 50 min until 22 days (Tables 3 and 4) while the quality of the impurities in comparison to the corresponding amino acid derivatives has been evaluated on the basis of their spectral characteristics (Figs. 1–4).

Selected impurities shown in Tables 3 and 4 and in Figs. 1–4, eluted together with amino acids (before 9.33 min, in the case of OPA/MPA derivatives: Table 3, Figs. 1 and 2, indicated by *2–*5 and *7, or before 9.68 min, in the case of the OPA/NAC derivatives: Table 4, Figs. 3 and 4, indicated by *1–*5, and *9–*11), are to be regarded as blank values which should be subtracted from the coeluting amino acid derivative. Certainly, it is a matter of both the gradient and the amino acid in question, which of the impurities are the coeluting ones, i.e., which of them should be taken into consideration in

Table 3
Impurities in the OPA/MPA reagent

Impurity peaks			Integrator units[a]/impurity peaks: as a function of reagent's age[b]							
No.	Retention time (min)	Maxima (nm)	50 min	240 min	1 day	2 days	3 days	7 days	10 days	22 days
*1 UV	3.13	219, 252	1.4	5.5	14	23	37	77	112	160
*2 FL	5.84		53	93	61	112	60	45	20	11
UV		228, 333	2.8	5.2	3.2	6.2	3.2	1.9	0.8	0.4
*3 FL	6.56		38	62	24	62	20	14	7.5	0.5
UV		214, 252	7.1	16	22	23	21	19	15	14
*4 FL	7.14		9.5	21	13	25	11	7.3	4.3	1.4
UV		219, 342	2.5	3.2	0.7	1.3	0.6	<0.3	<0.3	<0.3
*5 FL	7.68		4.9	6.0	5.3	7.8	5.2	5.1	3.4	1.6
UV		224, 266	1.6	1.7	2.1	2.7	2.5	3.5	1.3	2.5
*6 FL	8.78		18	29	46	38	43	31	5.3	<0.3
UV		233, 276	0.3	0.4	0.6	0.4	0.4	<0.3	<0.3	<0.3
*7 FL	9.33		43	47	56	50	46	30	12	3.0
UV		224, 261	23	19	13	17	17	12	9.5	1.3
*8 FL	11.24		13	17	21	24	27	25	14	1.0
UV		233, 276	2.8	3.5	4.5	5.2	6.6	6.9	5.3	<0.3
*9 UV	11.53	243, 333	25	29	24	15	12	6.3	2.9	0.4
*10 FL	12.29		72	72	83	76	77	73	26	1.5
UV		228, 333	84	84	92	85	84	78	23	1.0
*11 FL	13.08		50	42	31	22	17	11	21	110
UV		228, 252	6.9	3.6	1.3	0.5	<0.3	<0.3	0.4	3.5
Total:										
FL	⇒⇒	⇒⇒	302–135 =167; (67)	389–131 =258; (103)	340–135 =205; (82)	417–122 =295; (118)	306–121 =185; (74)	241–109 =132; (53)	113–61 =52; (21)	130–113 =17; (7)
UV	⇒⇒	⇒⇒	158–119 =39; (234)	171–120 =51; (306)	177–122 =55; (320)	180–105 =75; (450)	185–103 =82; (442)	206–91 =115 (690)	170–31 =139; (834)	187–6 =181; (1086)

[a] ~2.5 integrator units correspond to 1 p*M* amino acid derivative performing FL detection and ~15 p*M* performing UV detection. Values in parentheses represent the total of impurities, in the range of amino acids elution, expressed in p*M*; detection limit <0.3 integration unit; impurities *1 and *9 do not have FL intensities.

[b] Averages of four injections.

Table 4
Impurities in the OPA/NAC reagent

Impurity peaks			Integrator units/impurity peaks: as a function of reagent's age							
No.	Retention time (min)	Maxima (nm)	50 min	240 min	1 day	2 days	3 days	6 days	10 days	22 days
*1 UV	3.13	214, 252	0.6	0.8	5.4	5.5	7.2	8.6	8.4	12
*2 FL	5.77		152	84	57	42	28	15.4	17.4	25
UV		328	11	5.9	4.6	3.5	2.3	1.7	2.5	1.6
*3 FL	6.33		128	62	28	20	11	5.8	7.3	4.5
UV		238, 338	28	18	10	8.3	6.7	4.4	5.5	4.8
*4 FL	6.97		48	27	23	20	12	7	5.9	6.0
UV		224, 276	2.6	1.5	1.4	1.4	1.0	0.6	0.3	0.5
*5 FL	7.50		7.3	4.4	3.7	3.6	2.7	2.1	2.2	2.7
UV		224, 266	1.0	1.2	1.3	1.4	1.6	2.3	12	3.5
*6 FL	7.80		21	22	9.2	2.4	2.1	0.5	1.3	6.0
*7 FL	8.00		12	7.2	7.6	7.7	6.6	6.6	6.1	4.6
*8 FL	8.50		49	46	16	6.0	3.3	2.1	1.5	1.3
UV		214, 252	2.4	3.2	2.9	3.6	2.9	2.8	2.9	2.3
*9 FL	8.73		106	61	28	22	13	9.9	8.3	4.6
UV		247, 290	1.0	0.3	2.4	4.4	5.4	6.5	7.9	6.1
*10 FL	9.10		8.8	5.8	5.8	4.9	3.9	3.8	3.9	4.3
UV		224, 261	21	19	20	20	20	17	10	10
*11 FL	9.68		10	6.9	1.6	<0.3	<0.3	<0.3	<0.3	8
UV		219, 266	<0.3	0.7	3.2	7.2	8.7	11	13	13
*12 FL	10.30		92	68	50	31	19	11	5	2.6
UV		228, 338	19	15	13	8.3	5.9	2.6	0.6	<0.3
*13 UV	10.72	338	8.3	8.8	7.5	4.0	2.4	0.7	<0.3	<0.3
*14 FL	12.65		6.6	12	81	231	379	553	552	300
UV		228, 299	<0.3	<0.3	1.7	5.5	10	15	0.6	<0.3
Total										
FL	⇒⇒	⇒⇒	641–99 =547; (219)	406–80 =326; (130)	311–94 =217; (87)	390–262 =128; (51)	481–398 =83; (33)	617–564 =53; (21)	611–557 =54; (22)	370–303 =67; (27)
UV	⇒⇒	⇒⇒	84–26 =58; (348)	74–16 =58; (348)	73–15 =58; (348)	73–14 =59; (354)	74–18=56; (336)	73–18 =55; (330)	64–1 =63; (378)	54 (324)

Indications as in Table 3, as well as: impurities *1 and *13 do not have FL intensities, while impurities *6 and *7 do not provide UV absorbances.

the quantitative evaluation of the given amino acid derivative. (In order to control this study we performed a short gradient (Table 2) studying the constituents of ST-1 and ST-2, separately.)

As to the quantity of the impurities, those which are eluting in the range of amino acids are of primary importance. Therefore, the total impurities (as a function of the age of the reagents), have been calculated in two separate parts (Tables 3 and 4, last two rows). Namely, without the knowledge of the exact amount of blank values, applying either FL or UV detection (Tables 3 and 4, one but last rows, values in parentheses), the amounts of amino acids can be substantially overestimated: in our present cases, in total, by equivalents of 68, 103, 82, 118, 74, 53, 21 and 7 p*M*, for the OPA/MPA derivatives (Table 3), or, in total by equivalents of 219, 130, 87, 51, 33, 21, 22 and 27 p*M*, for the OPA/NAC derivatives (Table 4), depending on the condition of the reagents. (Note: It is worthy of mention that, however, the tendency in respect of the increase and decrease of the single impurities, as a function of reagent age and quality are very similar but, the amount of the single impurities is different from reagent to reagent. Consequently, the knowledge of impurities is obligatory and the reagent blank is to be

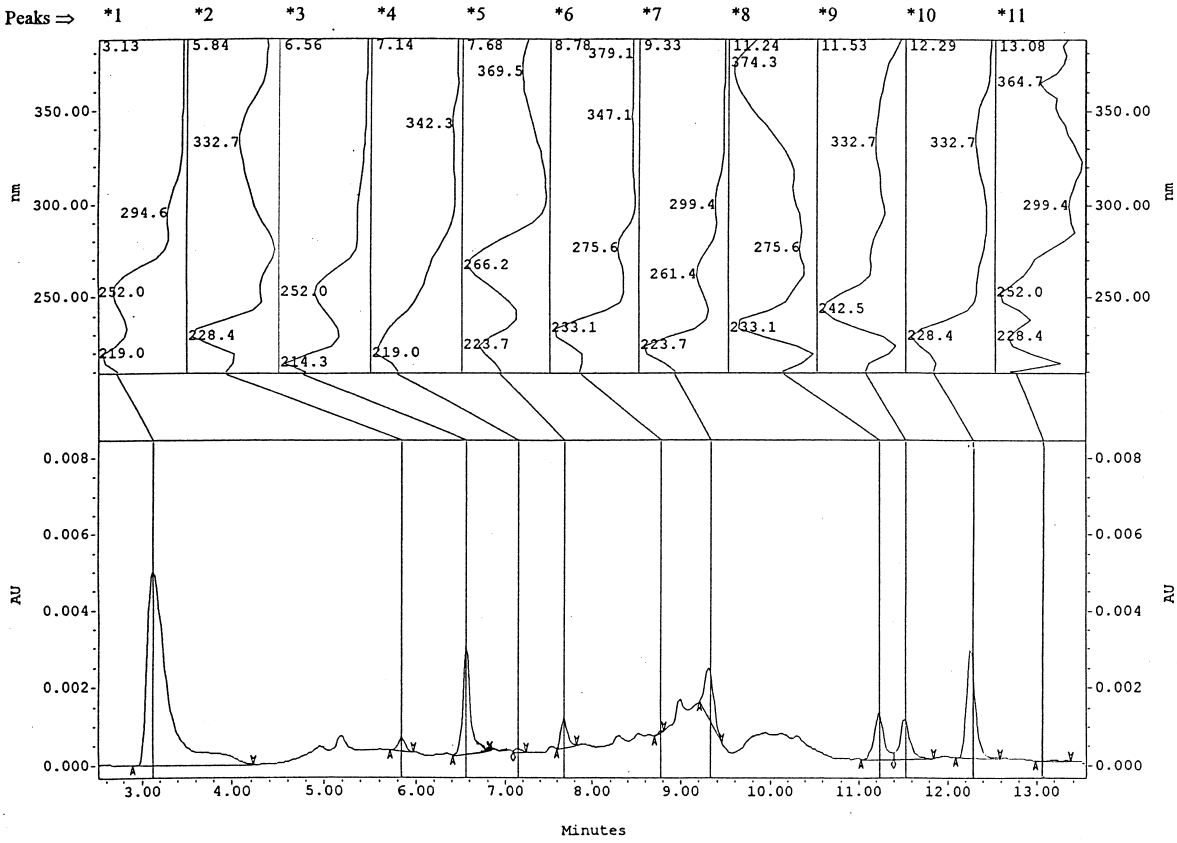

Fig. 1. Chromatogram (shown at 334 nm) and PDA spectra of the impurities (*1–*11) measured in the [OPA]/[MPA]=$9.2\times10^{-7}/2.8\times10^{-6}$ reagent of 7 days.

measured every day. Reproducible quantitation of amino acids cannot be performed without the deduction of the blank values, in particular not in reproducibility studies of amino acids in low p*M* concentrations (Tables 5–8).)

It is worth mentioning 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 used until 9 days, only, remaining on the safe side, in order to obtain quantitative derivatization. Derivatization study performed with reagents of various ages (Table 5) proved that the utilizability of the reagents are strongly limited by their age. Consequently, after 9 days the old reagents, the OPA/MPA and the OPA/NAC ones equally, should be discarded.

3.1.2. Spectral characterization of the impurities

Evaluating the spectral characteristics both of the impurities and those of the amino acid derivatives (Figs. 1–4) the following lessons can be drawn.

(1) Reagent peaks of unknown structure manifest different maxima: however, some of them might be of amino acid origin furnishing two special maxima, characteristic to both types of the amino acid derivatives, at 228 and at 332–337 nm (Fig. 1, impurities *2, *10; Fig. 3, impurity *12; and amino acid spectra in Figs. 2 and 4).

(2) Comparing the maxima of the impurities obtained with the two different reagents it is obvious that very likely the same impurities are present in both reagents, taking into account that their retention times can be shifted. The corresponding pairs are, in

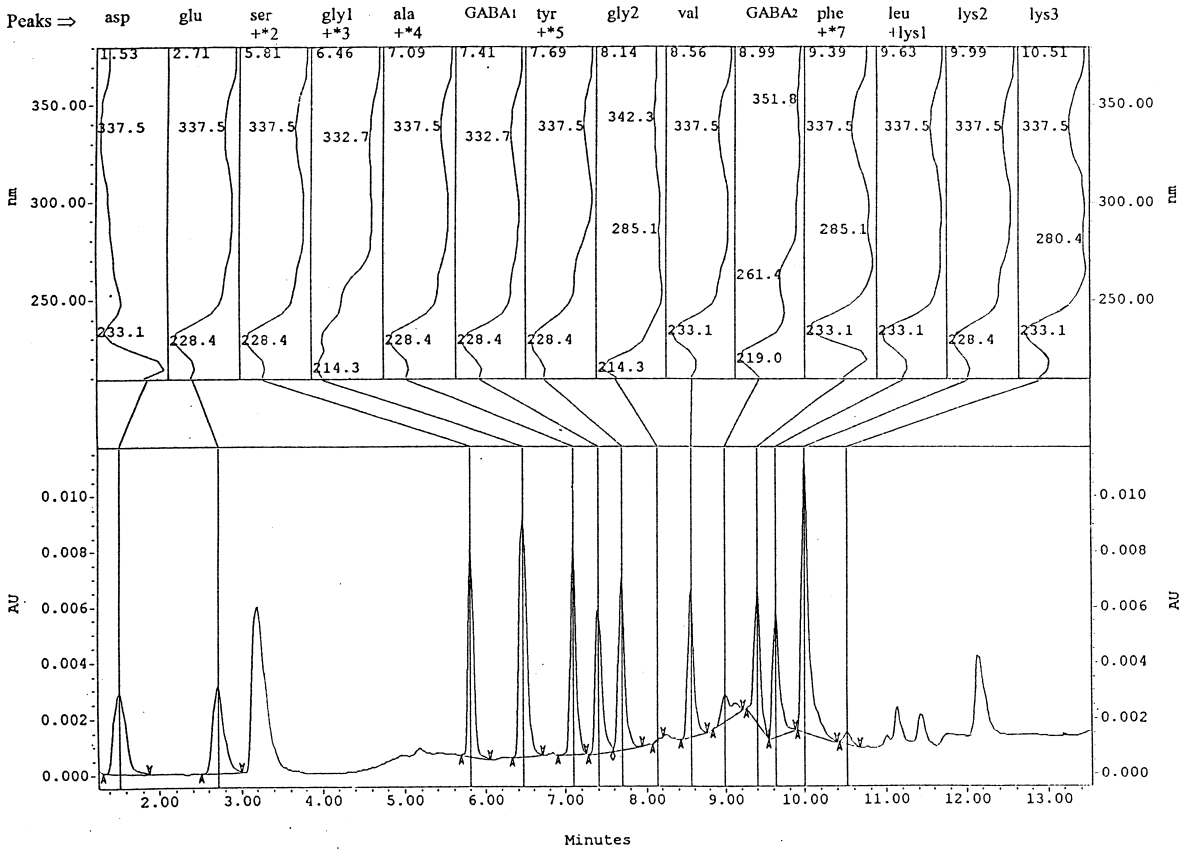

Fig. 2. Chromatogram (shown at 334 nm) and PDA spectra of the amino acids obtained from the ST-1 with the OPA/MPA reagent of 7 days ([OPA]/[MPA]/[amino acid]^{T}=9.2×10^{-7}/2.8×10^{-6}/4.35×10^{-8}; [OPA]/[amino acid]=21.2:1).

order of Figs. 1 and 3 as follows: *1–*1, *2–*2, *5–*5, *7–*10 and *11–*14.

3.2. Reactivation of the OPA/MPA(NAC) reagents

Both type of reagent can be renewed by completing them again by the initial amount of the SH-group-containing agent. The reactivated reagents provide the same amino acid responses as those aged 1–10 days. Although, as a result of completion, the ratios of the OPA/SH-group-containing agent became uncertain and, since the pH of the completed reagents must be repeatedly adjusted, their concentration also will be altered. Thus, the reactivation of the old reagents is only of theoretical importance: proving the fact that the decreased activity of the OPA/MPA and the OPA/NAC reagents can be of the same character as reported for the OPA/MEC and OPA/ET ones [1–12]. Very likely, due to the same deactivation/oxidation pathway of the SH-group moiety of any of these molecules.

3.3. Stability study and derivatization peculiarities of the OPA/MPA and OPA/NAC isoindoles

Preliminary investigations carried out with both derivatives, applying 7-min reaction times (Figs. 2 and 4) revealed that those amino acids which are believed to be the less stable ones (glycine, GABA, β-alanine, histidine, ornithine and lysine) are eluting in more than one derivatives.

The spectra of amino acids, independently of their SH-group constituents, i.e., in cases of the OPA/MPA and OPA/NAC amino acid derivatives equally, provide two maxima at ~228 and at ~332–339 nm (Figs. 2 and 4). The same results have been obtained

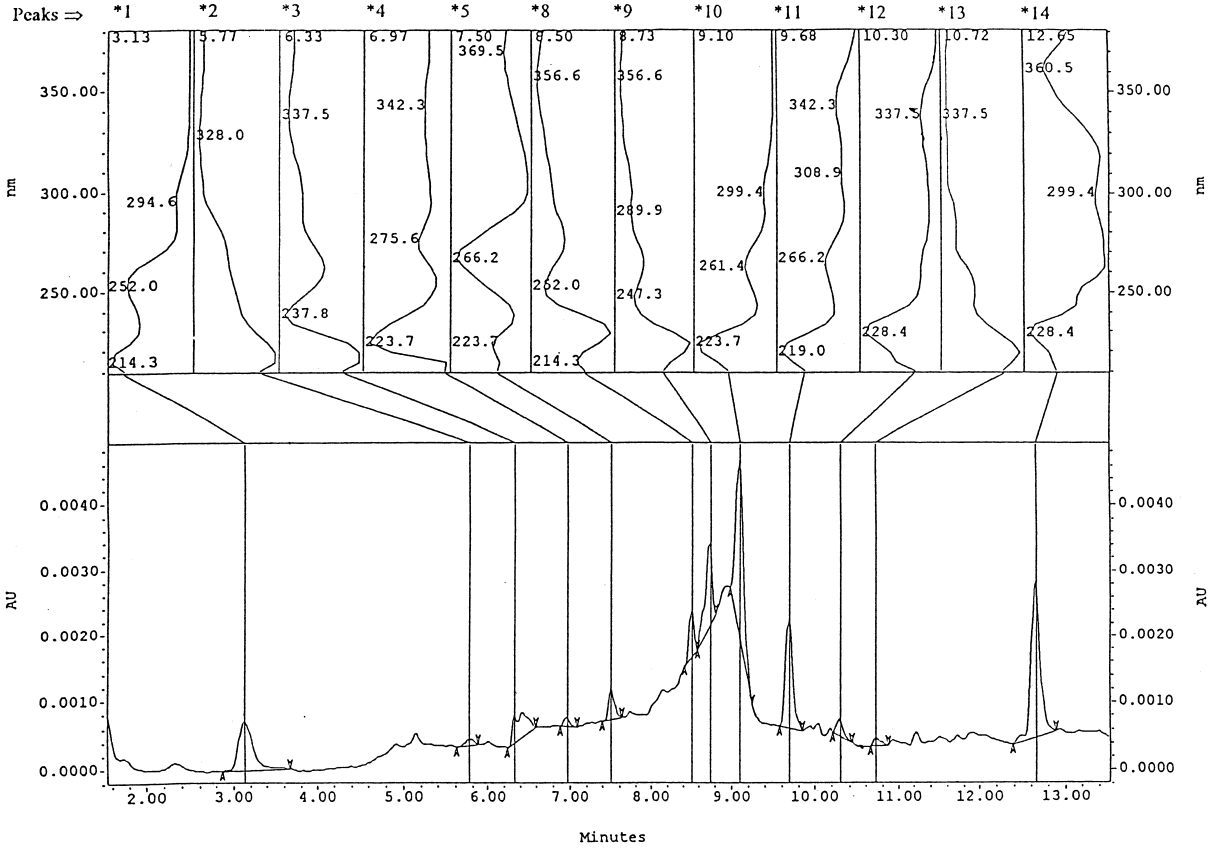

Fig. 3. Chromatogram (shown at 334 nm) and PDA spectra of the impurities (*1–*14) measured in the OPA/NAC reagent of 7 days; concentrations as in Fig. 1.

with both amino acid derivatives analyzing the compounds of ST-2 solution.

On the basis of this experience exhaustive study was performed with both derivatives of constituents of ST-1 and ST-2, as a function of the reaction time (7, 28 and 51 min, and 3, 4 and 6 h), at ~4°C. Results proved (Table 6, Figs. 5 and 6) that:

(i) in the cases of those amino acid derivatives which are providing single derivatives, and are not listed in Table 6, significant losses (<4%) have not been detected, not even after 6 h reaction time.

(ii) Maximum FL intensities and UV absorbances for the OPA/MPA derivatives of aspartic acid, glutamic acid, β-alanine, alanine, GABA, isoleucine, lysine and ornithine, as well as for the OPA/NAC derivatives of threonine, were measured after 28 min reaction time.

(iii) Derivatives of serine, threonine, tyrosine, methionine, tryptophan, isoleucine and leucine furnished, not significantly, but detectable loss of responses (Table 6), showing, after 6 h reaction times, slightly higher for the OPA/NAC derivatives (88–94%) in comparison to the corresponding OPA/MPA ones (93–97%).

(iv) Experiences obtained with glycine, GABA, β-alanine, histidine, lysine and ornithine are of particular importance, furnishing substantially new principles in the understanding of the characteristics of—for the time being believed—not stable derivatives. On the basis of our results it became evident that the stabilities of these derivatives are similar to the others in so far as being aware of the fact that they are eluting in more than one peak. Consequently, evaluating the total of peaks (Table 6):

(1) glycine proved to be one of the most stable derivatives providing amino acids: the total of

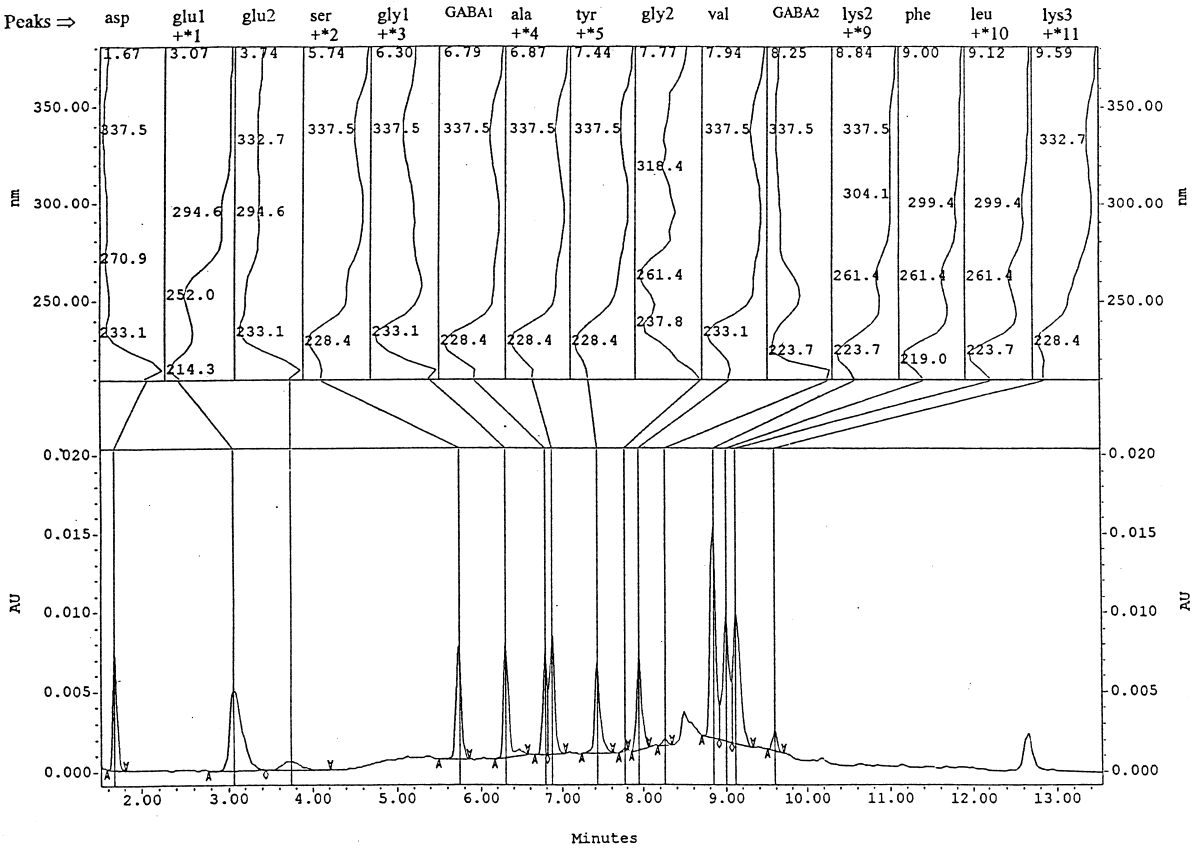

Fig. 4. Chromatogram (shown at 334 nm) and PDA spectra of the OPA/NAC-amino acid derivatives obtained from the ST-1 with the OPA/NAC reagent of 7 days; concentrations as in Fig. 2.

Table 5
Reproducibility of the OPA/MPA amino acid derivatives obtained with reagents saved for various periods of time in the refrigerator

Amino acid	Arbitrary units/1 p*M* amino acid; reagent saved at ~4°C for 1–28 days										
	1	4	7	8	9	10	*A*[a]	R.S.D. (%)	15 (%)[b]	21 (%)[b]	28 (%)[b]
Aspartic acid	2.81	3.01	2.86	2.86	2.96	2.83	2.90	2.5	1.86 (64)	0.17 (5.9)	0.033 (1.0)
Glutamic acid	3.28	3.32	3.26	3.26	3.30	3.33	3.29	0.91	2.20 (67)	0.05 (1.5)	0.007 (0.2)
Serine-*2	3.81	3.86	3.79	3.87	4.04	4.07	3.91	3.1	2.68 (69)	0.10 (2.6)	0.104 (2.7)
Gly1-*3+Gly2	4.12	4.16	4.16	4.30	4.37	4.18	4.22	2.3	3.02 (72)	0.04 (0.9)	0.027 (0.6)
Alanine-*4	3.56	3.76	3.74	3.75	3.83	3.61	3.71	2.7	2.63 (71)	0.07 (1.9)	0.022 (0.6)
GABA1+GABA2	4.04	3.98	4.15	4.16	4.24	3.94	4.09	2.9	3.39 (83)	0.04 (1.0)	0.031 (0.8)
Tyrosine-*5	3.59	3.60	3.56	3.63	3.64	3.49	3.59	1.5	2.53 (70)	0.06 (1.7)	0.019 (0.5)
Valine-*6	3.81	3.79	3.80	3.77	3.82	3.75	3.79	0.70	2.64 (70)	0.15 (4.0)	0.057 (1.5)
Phenylalanine-*7	3.60	3.67	3.58	3.82	3.92	3.87	3.74	3.9	2.88 (77)	0.10 (2.7)	0.028 (0.7)
Leucine-Lys1	3.66	3.76	3.79	3.80	3.85	3.76	3.77	1.7	2.80 (74)	0.10 (2.7)	0.013 (0.3)
Lys1+Lys2+Lys3	1.57	1.58	1.74	1.75	1.70	1.59	1.65	5.0	1.28(77)	0.01 (0.6)	0.001 (<0.1)

[a] *A*, average of fluorescence intensities.
[b] (%), expressed as the average of 1–10 days.

Table 6
Stability and characteristics of the OPA/MPA and OPA/NAC amino acid derivatives as a function of the reaction time

Amino acid	OPA/MPA amino acids					OPA/NAC amino acids				
	Retention time (min)	Response (%)[a]			RR	Retention time (min)	Response (%)[a]			RR
		7 min	3 h	6 h			7 min	3 h	6 h	
Serine	5.81	100	95	93	16.4	5.74	100	93	92	12.9
Threonine	6.48	100	98	96	14.0	6.42	98[b]	99	90	13.2
Tyrosine	7.65	100	97	95	16.1	7.74	100	95	89	11.1
Methionine	8.45	100	99	97	19.2	8.28	100	96	94	11.6
Tryptophan	8.98	100	100	97	16.0	8.83	100	95	94	11.9
Isoleucine	9.08	97[b]	99	96	16.0	8.67	100	95	94	14.7
Leucine	9.63	100	98	97	18.8	9.12	100	88	88	9.0
Derivatives eluting in two or three (OPA/MPA-Lys+Orn) peaks										
Glycine1	6.48	95	67	58	15.9	6.43	97	75	62	15.5
Glycine2	8.09	5	33	42	24.6	7.87	3	25	38	17.5
Total		100	100	100			100	100	100	
Histidine1	6.47	73	63	42	15.8	6.35	57	55	44	10.8
Histidine2	8.24	27	23	21	22.8[c]	8.33	43	36	38	18.4[c]
Total		100	86	63			100	91	82	
β-Alanine1	6.92	85[b]	52	39	17.2	6.63	89[b]	73	56	15.1
β-Alanine2	8.65	10[b]	48	61	25.6	8.39	6[b]	27	42	16.9
Total		95	100	100			95	100	98	
GABA1	7.43	73[b]	39	26	18.1	6.98	88[b]	72	59	14.6
GABA2	8.93	16[b]	61	64	23.5	8.43	5[b]	28	41	16.6
Total		89	100	90			93	100	100	
Ornithine1	8.82	<1[b]	3	7	1	—				
Ornithine2	9.42	71[b]	27	12	2.4	8.65	79[b]	49[b]	35	2.2
Ornithine3	9.89	5[b]	70	66	4.9	9.23	5[b]	46[b]	65	3.9
Total		77	100	95			84	95	100	
Lysine1	9.45	<1[b]	3	6	5.0	—				
Lysine2	9.93	54[b]	19	10	3.8	8.92	67[b]	48	31	2.8
Lysine3	10.40	13[b]	78	84	8.0	9.48	6[b]	52	69	5.5
Total		68	100	100			73	100	100	

[a]Expressed in the total; RR=FL/UV represents the response ratios of the fluorescence intensities versus the UV ones; indications as given in Figs. 2 and 4, values correspond to the peaks in Figs. 6 and 7.
[b]Maximum FL was measured after extended reaction time, mainly after 28 min.
[c]Measured at their maxima, at 353 nm.

glycine1 and glycine2 does not change even after 6 h reaction time.

(2) In the cases of β-alanine, GABA, ornithine and lysine, according to our earlier experiences [19], the maximum fluorescences were measured after extended reaction times (7–28 min).

(3) The less stable OPA/MPA/histidine—after 6 h—provided 63%, and the OPA/NAC/histidine 82% responses. The loss in the cases of OPA/MPA-GABA (90%), OPA/MPA-ornithine (95%) and OPA/NAC/β-alanine (98%) were as low as manifested by any others.

3.4. Assumption on the composition of reaction products of double peaks

Two possibilities would stand to reason:

(1) in the cases of glycine, β-alanine, GABA and histidine the formation of the 1,3-dithio-substituted-2-alkylisoindoles described for the product of OPA/MCE-*n*-propylamine would be obvious [38]; while,

(2) in the cases of the two basic groups containing lysine and ornithine the formation of the monomer and dimer derivatives seem to be the most simple approach. Those phenomena that have been attribu-

Table 7
Reproducibility of the quantitation of different amounts of OPA/MPA amino acids on the basis of their fluorescence intensities

Amino acids	Arbitrary units/1 p*M* amino acid[a]					Average[b]	R.S.D. (%)
Injected (p*M*):	~800	~400	~160	~80	~32		
[OPA/(MPA)]:[amino acid]^T:	4.23:1	8.46:1	21.2:1	42.3:1	105.8:1		
Aspartic acid	*2.76*	*2.76*	2.90	2.88	2.97	2.92	1.6
Glutamic acid	3.33	3.28	3.29	3.32	3.27	3.30	0.78
Serine-*2	*3.54*	*3.46*	3.83	3.91	3.92	3.89	1.3
Gly1-*3+Gly2	*3.93*	*3.81*	4.16	4.14	4.34	4.21	2.6
Alanine-*4	3.68	3.59	3.76	3.70	3.76	3.70	1.9
GABA1+GABA2	4.12	4.01	4.12	3.97	4.09	4.06	1.7
Tyrosine-*5	3.59	3.53	3.60	3.54	3.62	3.58	1.1
Valine-*6	3.81	3.72	3.82	3.73	3.80	3.78	1.3
Phenylalanine-*7	3.79	3.64	3.75	3.69	3.74	3.72	1.6
Leucine-Lys1	3.74	3.64	3.78	3.77	3.93	3.78	2.8
Lys1+Lys2+Lys3	1.73	1.64	1.65	1.63	1.63	1.66	2.5

[a]Obtained from at least three separate tests carried out with ST-1.
[b]Averages of tests obtained with mol ratios of [OPA/(MPA)]:[amino acid]^T=4.23:1, 8.46:1, 21.2:1, 42.3:1 and 105.8:1, with the exceptions of the italicized data.

ted to the 1,3-dithiosubstituted-2-alkylisoindoles [38,39], i.e., the shift in their UV maxima [38] and the decreased difference in their FL and UV responses (Ref. [39], ‘‘ . . . the extinction coefficient for the 1,3-disubstituted isoindole is approximately twice that of the 1-substituted derivative [38], the fluorescence yield of this disubstituted derivative must have been decreased by a factor of two.’’) have been evaluated in all six cases.

We found that the UV maxima of the derivatives of longer retention times, with the only exception being histidine, have been insignificantly shifted (Figs. 5 and 6: from 333.9 to 338.7 nm for glycine, GABA, β-alanine, lysine and ornithine, and from 333.9 to 353.0 nm for histidine). However, the response ratio (RR) values measured in the fluorescence/UV intensities (RR=FL/UV) proved to be essentially different (Table 6, RR values in the fifth and tenth vertical columns).

In particular characteristic differences have been detected comparing:

(i) the RR values of the earlier eluting derivatives to the later eluting ones in the case of all six amino acid derivatives, and

Table 8
Reproducibility of the quantitation of different amounts of OPA/NAC amino acids on the basis of their fluorescence intensities

Amino acid	Arbitrary units/1 p*M* amino acid					Average	R.S.D. (%)
Injected (p*M*):	~800	~400	~160	~80	~32		
[OPA/NAC]:[amino acid]^T:	4.23:1	8.35:1	20.9:1	41.7:1	105.8:1		
Aspartic acid	*2.84*	*2.84*	2.97	2.95	3.05	2.99	1.8
Glu_1-*1+Glu_2	2.94	2.86	2.86	2.72	2.76	2.83	3.1
Serine-*2	*2.86*	*2.87*	3.24	3.25	3.47	3.32	3.9
Glye1-*3+Gly2	3.70	3.69	4.04	3.89	*4.54*	3.83	4.4
GABA1+GABA2	2.79	2.75	3.01	*2.54*	2.75	2.83	4.4
Alanine-*4	3.71	3.72	3.75	4.02	3.99	3.84	4.0
Tyrosine-*5	2.54	2.52	2.61	2.56	2.65	2.58	2.1
Valine-*7	2.92	2.89	3.00	2.91	3.01	2.95	1.9
Lys2-*9+Lys3-*11	1.26	1.26	1.30	1.27	1.29	1.30	1.4
Phenylalanine	2.13	2.09	2.09	2.12	2.14	2.11	1.1
Leucine-*10	2.42	2.40	2.54	2.35	2.43	2.43	2.9

Indications as in Table 7.

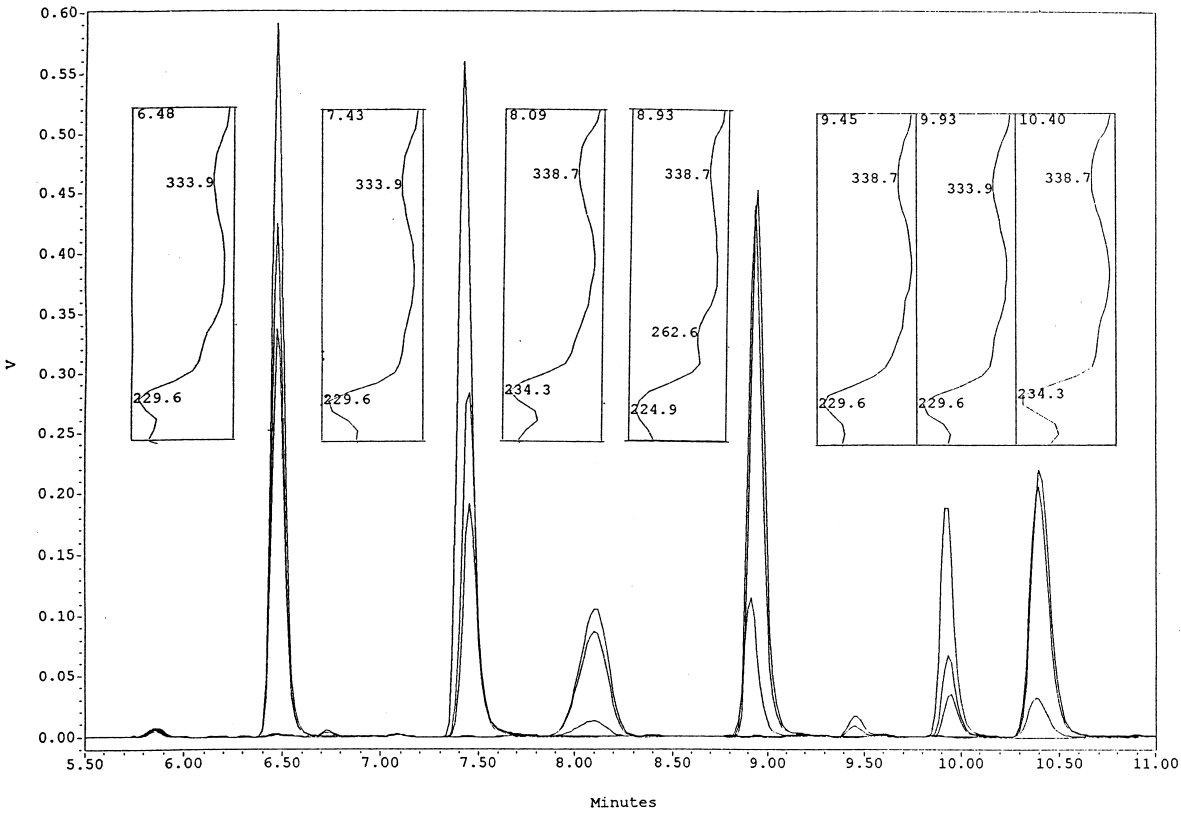

Fig. 5. Simultaneous FL chromatogram and PDA spectra of the OPA/MPA derivatives of glycine, GABA and lysine, as a function of the reaction time (7 min, and 3 and 6 h; detailed results in Table 6).

(ii) the RR values of the group of glycine1,2, GABA1,2, β-alanine1,2 and histidine1,2, to the group of lysine2,3 and ornitine2,3. It is unambiguous

(iii) that the RR differences of amino acid derivatives of shorter retention times manifest significantly lower RR values than their corresponding longer retention time ones, and

(iv) that these differences are outstandingly higher in the cases of lysine2,3 and ornitine2,3 than in the other group of amino acids providing the two derivatives (glycine, GABA, β-alanine and histidine).

Accepting that the FL intensities of the 1,3-thiodisubstituted-2-alkylindoles are the same as their corresponding 1-thiosubstituted-2-alkylindole ones, while their UV absorbances are approximately twice that of the 1-substituted derivative [39]. Thus, glycine1, GABA1, β-alanine1 and histidine1, all of shorter retention times and lower RR values, are presumed to be the 1,3-thiodisubstituted-2-alkylindoles, while their corresponding derivatives of longer retention times were probably the 1-thiosubstituted-2-alkylindoles.

These consequences would be in contradiction with the facts that:

(i) all single OPA derivatives manifest the lower RR values,

(ii) these derivatives are the fast forming ones which do not transform, and,

(iii) in the cases of those amino acids that provide more than one derivative their second or third derivatives are forming from the first ones, assuming this origin earlier for the disubstituted derivatives [38,39]. These derivatives, according to our experiences, proved to be the products of longer retention times and those of larger RR values. Thus, these derivatives of longer retention times, on the basis of their production/condition [38,39], should

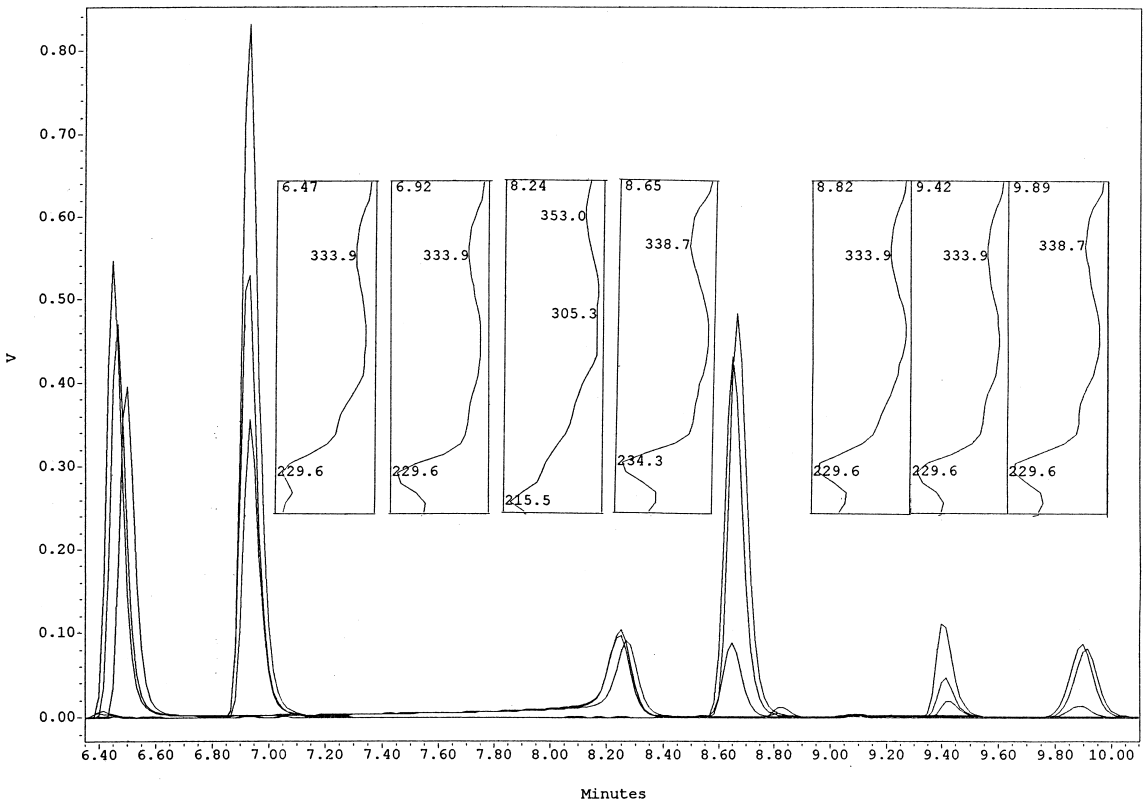

Fig. 6. Simultaneous FL chromatogram and PDA spectra of the OPA/MPA derivatives of histidine, β-alanine and ornithine, as a function of the reaction time (7 min, and 3 and 6 h; detailed results in Table 6).

have been the 1,3-thiodisubstituted-2-alkylindoles; while, according to their fluorescence/absorbance characteristics, should have been the 1-thiosubstituted-2-alkylindoles.

The two basic functions containing ornithine and lysine furnished considerably larger differences in their RR values (Table 6, for the OPA/MPA and OPA/NAC derivatives, in order of listing: ornithine2/ornithine3=2.4/4.9 and 2.2/3.9, as well as lysine2/lysine3=3.8/8.0 and 2.8/5.5, respectively). These correlations of RR values seem to be very likely in accordance with earlier experiences [39] obtained with the dimer isoindol adduct of (OPA/*n*-propylamine)$_2$/ethanedithiol which revealed that the ''second isoindole moiety has very little effect on ϵ per isoindole ring''. This means that two molecules of OPA/MPA(NAC) are reacting with one molecule of lysine or ornithine, resulting in the doubled UV intensities, and in the unchanged FL intensities of the dimers.

To prove both of these speculations further NMR and MS studies are needed. These investigations would be certainly of theoretical importance, but they are not inevitably necessary from the analytical point of view.

Experiences discussed above raise the question: what is the composition of all those OPA/MPA(NAC) amino acid derivatives which are providing a single derivative, taking into account that their RR values (Table 6) vary between 14.0 and 19.2 (average RR=16.7) for the OPA/MPA and between 9.0 and 14.7 (average RR=11.8) for the OPA/NAC amino acids (including also all constituents of ST-1 and ST-2). (Note: the only observation concerning the existence of a double peak for the lysine and ornithine relates to their OPA/ET [4]

and OPA/MCE [6,7] derivatives. Recently [40], even these experiences could not be confirmed with the OPA/MPA/9-fluorenylmethyl chloroformate derivatives, while two peaks were found in the case of hydroxylysine.)

3.5. Reproducibility study of the OPA/MPA/(NAC) amino acid derivatives

The quantitation of different amounts of amino acids obtained with the constituents of ST-1, applying the same amount of the OPA reagent, i.e., with various mol ratios of the reactants are listed in Tables 7 and 8.

On the basis of our results it can be stated that:

(i) the reproducibility of quantitations—including glycine, GABA and lysine—in the cases of the OPA/MPA derivatives is excellent (R.S.D.%≤2.8), while also for the OPA/NAC ones it is acceptable (R.S.D.%≤4.4).

(ii) Comparing the FL intensity of the OPA/MPA derivatives to the OPA/NAC ones the tendency in respect of the corresponding amino acids proved to be the same. On average, the FL values for the OPA/MPA derivatives were higher by ~20%, i.e., 3.48 and 2.82 arbitrary units/1 p*M* for the OPA/MPA and OPA/NAC amino acids, respectively.

(iii) In order to obtain quantitative interactions the mol ratios of the reagents to the total of amino acids should be ≥20, the coeluting impurities of the reagent are to be deducted from the corresponding derivatives and, last but not least, those amino acids which elute in more than one peak should be evaluated on the basis of the total of their derivatives.

3.6. Study on the stability and characteristics of the OPA amino acid derivatives

The qualitative remark given in the pioneering work [1], i.e., "... in the absence of a reducing

Table 9
Yield of the OPA/MPA amino acid derivatives followed by the OPA amino acid reaction performed for different times

Amino acid	Yield of the OPA/MPA amino acid isoindoles (%)[a]; reaction time/condition of OPA amino acid interaction				
	25 min/~4°C	75 min/~4°C	75 min/~20°C	60 h/~4°C	60 h/~20°C
Aspartic acid	89	70	41	1.5	1.4
Glutamic acid	75	41	9	0.9	0.8
Asparagine	23	6	2	1	0.5
Serine-*2	98	95	83	37	7
Glutamine	59	49	8	0.7	0.01
Gly1-*3+Gly2	71	36	7	9	6
Hist1+Homoser	85	66	32	7	0.4
Threonine	94	87	75	63	24
Arg+β-Ala1+β-Ala2	14	2	04	05	03
Alanine	68	30	5	3	2
Homoarginine	54	20	0.7	0.3	0.08
GABA1+GABA2	2.9	0.3	0.2	0.1	0.2
Tyrosine-*5	68	32	8	0.7	0.7
Valine-*6	68	29	6	0.7	0.4
Histidine2	62	28	2	3	0.8
Methionine	63	43	10	1	0.7
Tryptophan	12	0.9	2	1	0.1
Phenylalanine-*7	75	33	10	2	2
Isoleucine	62	35	2	1	0.2
Leucine	50	10	06	3	3
Orn2+Orn3	7	3	2	0.4	0.4
Lys2+Lys3	3	0.6	0.5	0.5	1

[a](%) on the basis of their FL intensities, expressed in their quantitative reactions.

agent, *o*-phthalaldehyde is capable of reacting otherwise with amino acids with the formation of non-fluorescent products'', was accepted. Consequently, without any detailed data, everybody followed the requirement that the OPA reagent should contain the SH group bearing additives prior to its reaction with the amino acid.

3.6.1. Stability studies

In order to clarify this tentative remark in a more defined manner we reacted the amino acids with the buffer containing OPA reagent at 4°C for 25 min, 75 min and 60 h and at 20°C for 75 min and 60 h, prior to completing their mixtures with the corresponding amount of MPA resulting in mol ratios of [OPA/(MPA)]:[amino acid]^{T}=21.2:1. Data obtained with FL detection (Table 9) revealed that the rate of the interactions between OPA and the various amino acids are different and characteristic, and they can be increased by the time and temperature of reactions.

(i) Serine and threonine, both of α-amino-β-hydroxy structure, are the less reactive amino acids towards the OPA, while

(ii) GABA, ornithine, lysine, β-alanine and histidine—five amino acids out of those six which provide more than one derivative with the OPA/MPA(NAC) reagents (Table 6, Figs. 5 and 6)—form, in relatively fast reactions, stable OPA derivatives. Glycine proved to be an exception: reacting relatively slowly with the OPA, furnishing a relatively stable OPA derivative.

(iii) The rest of the amino acids manifested a slow reaction rate with the OPA. However, after 60 h reaction time, at 20°C only threonine, serine, glycine and leucine provided fluorescent derivatives, in order of listing in amounts of 24, 7, 6 and 3%, expressed in their initial concentrations.

Table 10
Peculiarities of the OPA amino acid derivatives

Amino acid	Retention time (min)[a]	No. of peaks	1 day[b]	3 days[b]
Aspartic acid	1.86	1	0.227	—
Glutamic Acid	2.94/3.11	2	0.248	—
Asparagine	5.52/7.39	5	0.271	0.221
Serine	5.59	1	0208	0.233
Glutamine	5.86/7.06	5	0.199	0.285
Glycine	6.03	1	0.205	0.207
Homoserine	6.13/	4	0.298	0.305
Threonine	6.36	1	0.209	0.208
β-Alanine	6.63	1	0.227	0.370
Alanine	6.65	1	0.234	—
GABA	6.69	1	0.234	—
Homoarginine	6.92	1	0.201	0.226
Histidine	7.00/8.37	8	0.225	0.236
Tyrosine	7.33	1	0.200	—
Valine	7.73	1	0.197	—
Methionine	8.33	1	0.209	0.208
Arginine	9.60	1	0.355	0.171
Tryptophan	9.95/10.16	7	0.225	0.212
Isoleucine	8.52	1	0.218	—
Leucine	8.92	1	0.296	—
Phenylalanine	9.07	1	0.177	—
Ornithine	9.62/11.43	2	0.196	0.206
Lysine	10.28/11.66	2	0.259	0.299
Cysteine	12.48/14.52	2	0.117	0.295

[a]Correspond to the main peak(s).
[b]Integrator units/1 p*M* amino acid measured at 240 nm, expressed as the total of the peaks.

3.6.2. Spectral characteristics and response values of the OPA amino acid derivatives

To become more involved in the features of the OPA-amino acid derivatives we investigated their spectral characteristics and response values after 1 and 3 days reaction times applying PDA detection (Table 10, Fig. 7). Their UV maxima, shown by the spectra of the ST-1 solution (Fig. 7), have been obtained at ~247 nm, with the exceptions of phenylalanine (261, 299 nm), lysine1 and lysine2 (252.0, 256.7 nm). The retention orders of the OPA derivatives are not considerably different comparing them to the corresponding OPA/MPA(NAC) derivatives, with the only exception being the OPA/cysteines, which proved to be the last eluting ones. The retention times of the OPA amino acid derivatives are slightly higher than their corresponding OPA/MPA(NAC) ones (OPA/MPA-aspartic acid, 1.53 min; OPA/NAC-aspartic acid, 1.67 min; OPA-aspartic acid, 1.86 min). Of the 24 OPA amino acid derivatives nine provided more than one peak (Table 10, two to eight peaks). Their response values, calculated on the basis of the total of their UV intensities, measured at 240 nm (Table 10, values in the last two vertical columns), vary between 0.171 and 0.370 arbitrary units/1 p*M* amino acid. These responses proved to be very similar to those obtained with the OPA/MPA(NAC) derivatives, which proved to be, on average, expressed in arbitrary units/1 p*M*, in order of listing 0.208 and 0.238 for the OPA/MPA

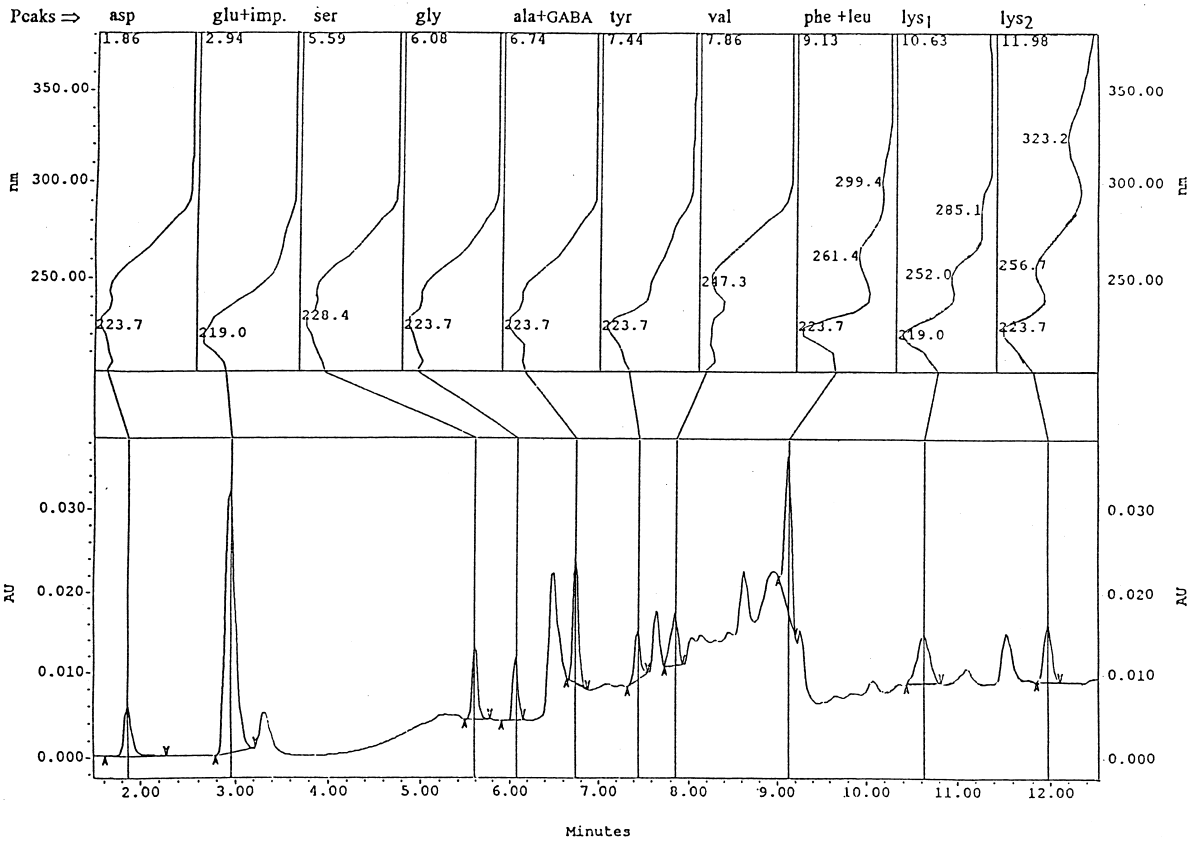

Fig. 7. Chromatogram (shown at 240 nm) and PDA spectra of the OPA amino acid derivatives obtained from the ST-1; [OPA]/[amino acid]^{T}=9.2×10^{-7}/4.35×10^{-8}=21.2:1); Detailed results in Table 10.

and OPA/NAC amino acids, respectively (measured at 334 nm).

4. Conclusions

On the basis of our investigations performed with the OPA/MPA and OPA/NAC reagents and with their derivatives of 24 amino acids it has been proved that:

(1) the OPA reagents containing the buffer and the SH-group agent can be used no longer than 9 days, presuming that it is stored in the refrigerator at ~4°C.

(2) The reaction time between the reactants should be at least 7 min.

(3) To perform the elutions of blank measurements and to deduct the responses of the impurities from the corresponding amino acid derivatives is obligatory, in particular when the constituents of the analyte are present in the low p*M* range.

(4) Glycine, β-alanine, GABA, histidine, lysine and ornithine are to be quantitated on the basis of the total of their derivatives.

(5) The mol ratios of the OPA/MPA(NAC):amino acid are ≥20:1.

(6) OPA reagent should contain the SH-group agent prior to its reaction with the amino acid(s).

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