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Review

Advancement in the derivatizations of the amino groups with the *o*-phthaldehyde-thiol and with the 9-fluorenylmethyloxycarbonyl chloride reagents☆

Ibolya Molnár-Perl

Institute of Chemistry, Department of Analytical Chemistry, L. Eötvös University, P.O. Box 32, H-1518 Budapest 112, Hungary

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ABSTRACT

An overview is presented on the advancement of the two most frequently used derivatization protocols applying the *o*-phthalaldehyde (OPA)-thiol and the fluorenylmethyloxycarbonyl (FMOC) chloride reagents, prior to the high performance liquid chromatographic analysis of amino acids. This review pays special attention (i) to the blank value, to the composition, to the stability and to the life time of the reagents, (ii) to the optimum pH conditions for the interactions and derivatives' elutions, (iii) to the characteristics and behavior of those amino acids which might provide more than one derivative correlating with the molar ratios of the reagent to the reactants, and (iv) on the practical applicability of the optimized protocols.

Contents

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E-mail address: perlne@chem.elte.hu

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1. Introduction

Before going into details with a literature overview from the pioneer works (OPA-amino acids (AAs) [1]; FMOC-AAs [2]) up to the present state of the art of these derivatization protocols [3–73], it is worth noting that based on their extensive use, these two techniques, the reaction with OPA/thiol or FMOC are the most frequently applied derivatizations in the analysis of AAs (Table 1). This is not surprising since:

(1) both reagents furnish fast reactions,
(2) both derivatives can be obtained in aqueous solutions, at ambient temperature, and,
(3) both reagents are providing derivatives of high selectivity and sensitivity, being both the OPA and the FMOC derivatives, fluorescent ones.

Certainly these two derivatization protocols did have and they still have their uncertainties, limitations, shortages and advantages: however, in the last decade the overwhelming part of uncertainties were examined and cleared up: resulting in the trouble-free, conscious and reliable use the OPA [3–19] and that of the FMOC [20,21] techniques, as well.

The goal of this review is to show the advancement of both derivatization protocols separately, on the basis of the same, strictly analytical viewpoint, thereafter, in the knowledge of their intrinsic characteristics to suggest the use of either one for special practical purposes.

1.1. HPLC of amino acids as o-phthalaldehyde derivatives

1.1.1. Literature review

The instability of the isoindoles formed from AAs and OPA, in the presence of different SH-groups containing compounds, as well as the instability of the reagent itself are known as two of the main disadvantages of this worldwide common principle, introduced forty years ago [1].

On the basis of all those uncertainties reported in the literature [22–73], including the pioneer work [1], and our own experiences [3–19] the following questions had to be answered:

(1) What is the correlation between the extremely different and contradictory stability values of OPA-AAs, i.e., can be this phenomenon associated with the extremely different preparation and storage conditions of the reagents?
(2) Do provide the OPA/MPA (NAC, MCE, ET) reagents self fluorescence and/or self absorbance which are to be taken into account in the quantitation of the AA derivatives, under HPLC conditions, applying the most common fluorescence (FL) and photo diode array (PDA) detections, simultaneously?
(3) What is the storage time of the OPA reagents until providing optimum responses?
(4) What is the reason of the virtual instability of the glycine, β-alanine, γ-aminobutyric acid (GABA), histidine, ornithine, lysine and primary amine derivatives, in comparison to the rest of AAs?
(5) What is the UV/FL optimum of the isoindoles and how are these affected by the molar ratios of OPA/thiol?
(6) What is the structure of the OPA derivatives and reaction mechanism for formation?

Table 1
Popularity of derivatization protocols prior to the HPLC of amino acids.*

Key words: HPLC +	Number pf papers	
	All years to present	From 2000 to present
Dabsyl amino acid	8 (2.3)	3 (1.7)
Dansyl amino acid	48 (13.6)	23 (11.3)
FMOC amino acid	166 (47)	92 (51.9)
OPA amino acid	98 (28)	47 (22.6)
PTC amino acid	33 (8.5)	6 (3.4)
Amino acid in total	353 (100)	177 (100)

* Indications: Based on data obtained from Science Direct, taken on the 12th July 2010, selected on the key words, given in the first column, like, HPLC + Dabsyl amino acid, etc.; ( ) = in parentheses percentages, expressed in the number of the total, given in the last two horizontal line.

In order to get answer to these questions in author's laboratory two approaches were followed: stoichiometric investigations were carried out with simultaneous PDA and FL detections [3–19] while structure elucidation were confirmed by on line HPLC/PDA/mass spectrometry (MS) applying electro spray ionization (ESI) in the positive mode [7,12,13,15].

1.2. Composition, blank value, life time and optimum storage condition of reagents

1.2.1. Composition of the reagent; derivatization conditions

The literature contains a variety of contradictory conditions regarding derivatization conditions. The controversies relate the molar ratio of OPA/thiol, effect of AA structure on reaction rate, optimal pH of the reaction, proper buffer concentrations and reaction time.

In the case of the OPA/MCE, OPA/MPA, OPA/NAC and OPA/ET derivatives have been examined and summarized, in detail [3–19]: they proved to be extremely different. All these uncertainties result in basically incomparable analytical data and consequences drown from them; in particular in the cases of the so called less stable AAs, such glycine, GABA, β-alanine, histidine, ornithine, lysine and primary amines.

According to recent experiences carried out in author's laboratory, the composition of the reagent, i.e., the molar ratio of the OPA to the SH additive, the pH and the reaction time of derivatizations proved to be of primary importance.

Consequently, in all derivatizations of AAs to be comparable (if not otherwise stated), the OPA/SH-additive was applied in the 1/3 (in cases of the OPA/MPA, OPA/NAC and OPA/MCE products), or in 1/10 (regarding the OPA/ET derivatives) molar ratios, while the OPA was used in 20–120 times molar excess to AAs to be derivatized. The reagent was saved in the refrigerator at 4 °C, and used for nine days (except OPA/ET reagent for two days), only. The pH of the reagent was 9.3 ± 0.05 ensured by 0.2 M borate buffer, and the elution temperature was controlled by column thermostat.

1.2.2. Blank value of the reagent

OPA reagents with SH-group containing additives, in general [1,22–31], are regarded as . . .“nonfluorescent itself, and when present in excess does not break down, or react to form fluorescent byproducts” [22]. Although, selected observations [23–31], including the pioneer work [1] 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 [23–31]. In the pioneer work [1] the author's warning is unambiguous: . . .“the blank fluorescence is the main factor limiting the sensitivity of the method”. By observation of others [23,24] 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 pM of each OPA/MCE AA derivative . . .” [23]. According to an other experience [24] “. . . when working with amounts of material less than about 100 pM, appropriate control must be run to compensate for the minute amounts of AAs found in even the best of commercially available reagents.” Further experiences [25–30] were mainly qualitative, advising to discard the OPA/MCE reagent solution if contaminant peaks appear, earlier, if necessary, also as two months [25], or one week [26]: due to the extremely different, believed life time of the same reagent (note of the author). The impurity peaks proved to be extremely disturbing by using electrochemical detection (ED) [27–30]. In order to improve sensitivity either special chromatographic condition [28] or the elimination of the excess reagent was proposed [29,30]. Scavenging the excess of the OPA/MCE reagent by adding iodoacetamide or N-ethylmaleimide resulted in limited success only [29]. Scavenging the excess of the OPA/*tert*.-butylthiol (BT) reagent with the excess of glycine and iodoacetamide [30] resulted in the quantitative elimination of interfering reagent peaks and in a detection limit of 50 aM AA. The stabilizing effect of nitrilotriacetic acid acting as a metal chelating agent was found to increase the OPA/MCE reagent stability at least four fold in the sample vial of an automated system [31].

Recently, an exhaustive study was carried out in author's laboratory in order to examine the characteristics of the OPA/MPA, OPA/NAC, OPA/MCE and OPA/ET reagents [3–19].

These studies performed with PDA and FL detections, simultaneously confirmed that all four reagents do have blank values both in the UV- and in the FL regions (Table 2).

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 (detailed description in the Experimental sections of papers [3–19]): the amount of the single impurities is 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 similar, and characteristic to it).

Consequently, to take into consideration the reagent peaks in order to subtract them from the coeluting AAs is obligatory. In particular in those cases when a relatively higher reagent concentration is needed, because AAs are measured in extremely different concentrations, in the presence of their matrix [3–19]. 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.

In the cases of OPA/MPA, OPA/NAC, OPA/MCE OPA/ET and storage times of ranging from 50 min to 23 days, the concentrations of the impurity peaks change with time. These co-eluting interferences correspond to concentrations of 7–473 pM for FL and 33–1086 pmol for PDA detection. The elution times of the interferences will certainly depend on the chromatographic systems.

1.2.3. Life time and optimum storage condition of reagents: depending on the SH group additive

According to literature data the OPA/MCE and the OPA/*tert*.butylthiol (BT) reagents were reported to be appropriate for derivatization up to 1 day [33], 1 week [37,38], 2 weeks [35] or 1 month [3], usually saved in the refrigerator at 4 °C [32–35,37], or at room temperature [30,37]. Others [30,32,36] do not attach substantial importance to these phenomena; as a basis of quantitative and reproducible derivatizations. Concerning the reliability and reproducibility ensuring conditions, the most desirable pH (pH = 8–11.5) and/or the applied molar ratios of the reagent to the analyte ($[OPA]/[AAs] = 5.5–1.8 \times 10^4$) proved to be extremely different [6].

To extend the lifetime of the primarily introduced OPA/MCE reagent it has been reactivated by completing it with the MCE [22–27] continuously (daily [6], in every second- [1,5,54], or third days [4,22,23] and weekly [24–27]), resulted in uncontrolled molar ratios of the reactants.

The behavior of the OPA/MPA, OPA/NAC, OPA/MCE and OPA/ET reagents, – stored in the refrigerator at 4 °C, – has been followed with UV/PDA and FL detections, simultaneously, with storage from 50 min to 27 days (Table 2) and have been characterized also on the basis of their derivatization capability.

It is noteworthy that in the case of the OPA/MPA similarly to the OPA/NAC reagents the older the reagents the smaller the amount of impurities found [3]. Thus, the best solution of the problem might has been the use of aged reagents. Unfortunately, the OPA/MPA, the OPA/NAC and the OPA/MCE reagents can be applied until 9 days, while the OPA/ET one until two days only: remaining on the safe side, in order to obtain quantitative derivatizations [3–19].

Table 2
Impurities of the OPA/MCE = 1/3, OPA/MPA = 1/3 and OPA/ET = 1/10 reagents.

Impurity peaks	pmol*/impurity peaks: as a function of the OPA/MCE = 1/3 reagent's age#										
	Reagent1							Reagent2			
	90 m	230 m	270 m	370 m	7 d	11 d	27 d	90 m	4 d	21 d	23 d
Fl in total (4 impurities)	30	20	20	25	14	50	6.3	40	11	**473**	45
UV in total (7 impurities)	58	40	**33**	45	91	357	370	201	252	783	520

Impurity peaks	pmol*/impurity peaks: as a function of the OPA/MPA = 1/3 reagent's age#							
	50 m	240 m	1 d	2 d	3 d	7 d	10 d	22 d
Fl in total (11 impurities)	67	103	82	118	74	53	21	**7**
UV in total (10 impurities)	234	306	320	450	442	690	834	**1086**

Impurity peaks	pmol*/impurity peaks: as a function of the OPA/ET = 1/10 reagent's age#					
	Reagent1		Reagent2			
	60 m	2 d	60 m	1 d	2 d	4 d
Fl in total (9 impurities)	83	63	140	148	141	124
UV in total (11impurities)	138	165	372	543	562	515

Indications: Reagent1, Reagent2 = prepared strictly under the same conditions at different time; m = min; d = day; pmol*/impurity peaks = found in the reagents, calculated on the basis of the average integrator units (obtained from 15 amino acids both from UV and from fluorescence (FL) detections; [OPA]/[amino acid] = 20–70/1.

1.3. Stability of the AA derivatives

The stability dilemma and the use of the OPA method are inseparable. Special studies devoted primarily to the HPLC of the OPA-AAs, somehow, without exception, became 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, GABA, β-alanine, histidine, ornithine and lysine have been selected as the less stable ones: in shortage of knowledge of their intrinsic characteristics, clarified recently [3–19]. Since (i) these six AAs provide more than one OPA derivative, (ii) resulting in products of similar high stability as any other AAs: so long as, the total of their FL or UV responses, i.e., the sum of derivatives obtained from the given AA were taken into account in their quantitation.

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

1.3.1.1. Early literature observations. Investigations in the 80 s attempted to clarify the factors affecting the stability of the primary

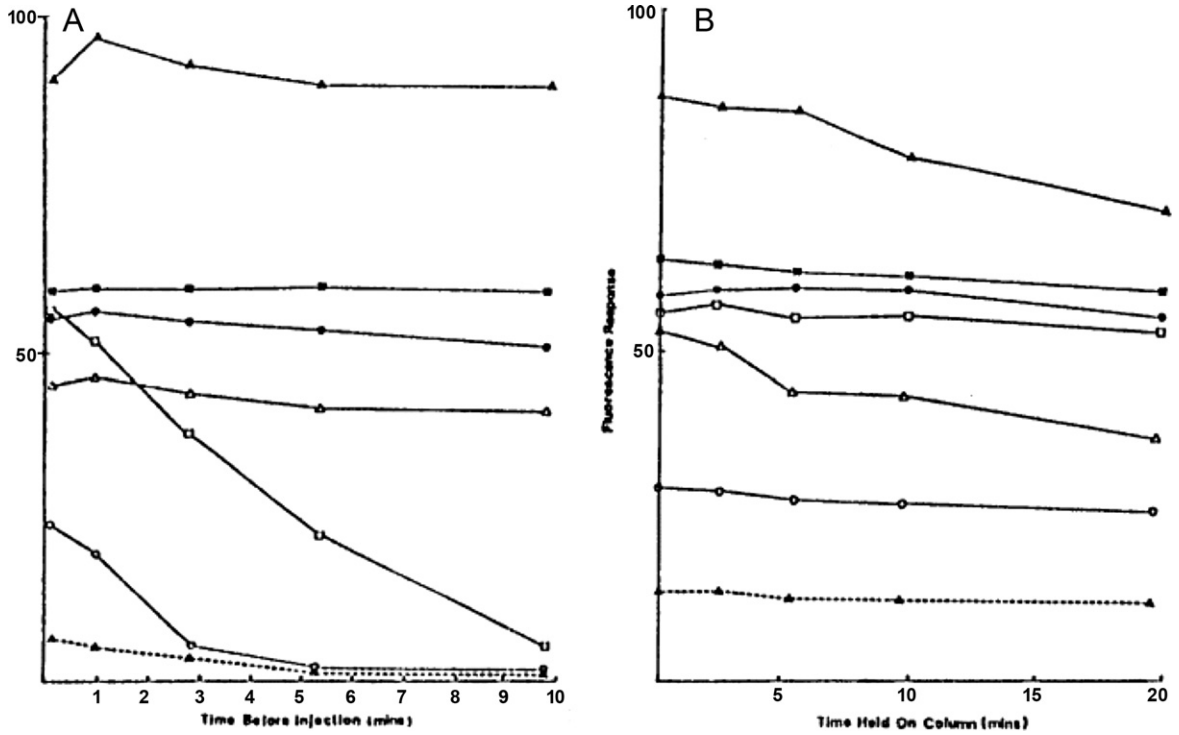

Fig. 1. Stabilities of the OPA/MCE amino acids tested with different reaction times before injections. (A) In the presence, (B) in the absence of the reagent's excess, the time held on column when the solvent flow was stopped. Symbols: ▲, glutamic acid; △, aspartic acid; □, glycine; ●, serine; △, valine; ○, lysine; ···▲···, ornithine (*Note:* the pH of solutions were pH = 6.5, the temperature was not given). Reproduced with permission from Ref. [6].

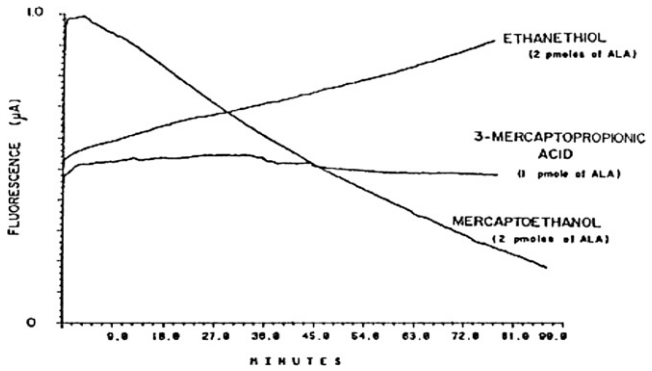

Fig. 2. The effect of various mercapto reagents on the stability of OPA-alanine. Reproduced with permission from Ref. [6].

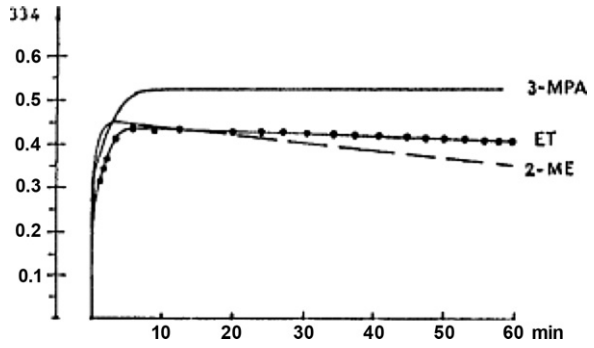

Fig. 3. The stability of the 17 OPA-AA adducts illustrated as the UV response (334 nm) versus reaction time for the three mercapto reagents: UV absorbencies of the total of 17 AAs (2.5 nM/ml of each) obtained as the OPA/MCE, OPA/ET and OPA/MPA derivatives. Reproduced with permission from Ref. [6].

amino group with the OPA/MCE [39], OPA/3-mercapto-1-propanol (MCP) [39] and with the OPA/ET [40] reagents.

The kinetic stability of the isoindole products of n-propylamine, GABA, β-alanine, glycine, β-aminobutyric acid and alanine has been characterized with the 50% decomposition ($T/2$) of the corresponding species, at 40.5 °C and at pH = 8.95 [39,40]. Varying the molar ratios of OPA/MCE/AA = 1430/149/1, (1 = 1 × 10−6 M), $T/2$ values in order of listing proved to be 18.2, 21.1, 24.9, 30.8, 57.8 and 98.9 min: it means the decomposition of β-alanine was 4 times faster than that of alanine (98.9/24.9 = 3.97). Selecting as model compound the isoindole of β-alanine, its kinetic stability was evaluated at increasing and at decreasing molar ratios of the OPA/MCE/AA reactants, as well as applying instead of MCE the 3-mercaptopropionic acid (3-MCP). As crucial points of these results it turned out that decreasing the OPA concentration in the reaction mixture of OPA/MCE/β-alanine the $T/2$ values became increased, while increasing the OPA concentration the rate of decomposition decreased. Substituting the MCE with 3-MCP leaded to considerably increased stability of the product. Consequently, these investigations, in correlation with our recent experiences [7], pointed ahead how to improve OPA derivatizations: by changing of reactant ratios, by taking into account that the high excess of OPA accelerates decomposition, and, by altering the SH-group component.

A convincing approach, regarding the destructive effect of the reagent-excess, was confirmed [23], by holding the OPA/MCE-AAs for various time intervals in the injection loop before injecting them onto the HPLC column (Fig. 1A) and compared them to those 2 min after injection of derivatives onto the HPLC column, the solvent flow rate was stopped for different time intervals to examine the stability of the OPA/MCE AA derivatives on the column (Fig. 1B).

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 four-fold stability [31]. The increased methyl alcohol concentration of the reagent improved the stability of the OPA/MCE- [41] and OPA/ET-AAs [42].

The stability of the OPA/MPA-AAs was 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, calculated on the UV responses (334 nm) of the total of 17 AAs (Fig. 3, [44]) proved the constant stability of the OPA/MPA derivatives.

The advantage of the OPA/N-alkyl-cysteine reagents over the OPA/MCE and OPA/ET ones, due to their chiral recognition characteristics, attracted only few scientists [22,45,46]. The introduction of NAC as SH-group containing alternative was explained mainly in qualitative manner [45].

1.3.1.2. Recent exhaustive study on the stability of the OPA/MPA, OPA/NAC, OPA/MCE and OPA/ET-AAs, with particular attention to the multiple derivatives providing compounds.

1.3.1.2.1. Stability of the single derivatives providing AAs. The comparison of the stability of 26 OPA/MPA, OPA/NAC, OPA/MCE and OPA/ET AA derivatives recently [13–19], proved that all of them are significantly more stable than they are generally believed to be: (i) the single derivatives providing 20 AAs [11], as OPA/MPA or OPA/NAC derivatives exhibited <4% degradation, even after 6 h, the OPA/MCE ones after 75 min reaction time, while the OPA/ET derivatives remain stable even after 6 h (ii) The AAs 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 derivative furnishing OPA-AAs [3–6,18].

1.3.1.2.2. Stability of the multiple derivatives providing AAs. The principle of the multiple derivatives providing AAs proved to be the crucial point of their stability issue [3,4]. Earlier observations [33,47–53] 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 additional five, in total of six ornithine derivatives. The double peaks of the OPA/MPA-lysine were reported as the basis of its quantification [50], while in an other work [51] the presence of the 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 OH-lysine 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 after 15 min reaction time in extremely high concentrations [33].

Our first approach [4] related to the main contradiction of OPA derivatizations: in order to get clear answer to the background of the 'believed to be extreme low stability' of glycine, β-alanine, GABA, histidine, ornithine and lysine, these six AAs have been derivatized in two groups as OPA/MPA derivatives (Fig. 4A and B). Changes in responses, have been followed by UV and fluorescence detections, simultaneously, as a function of the reaction time, strictly under the same, well defined practical conditions (detailed optimum derivatization conditions in the Experimental section of [4]).

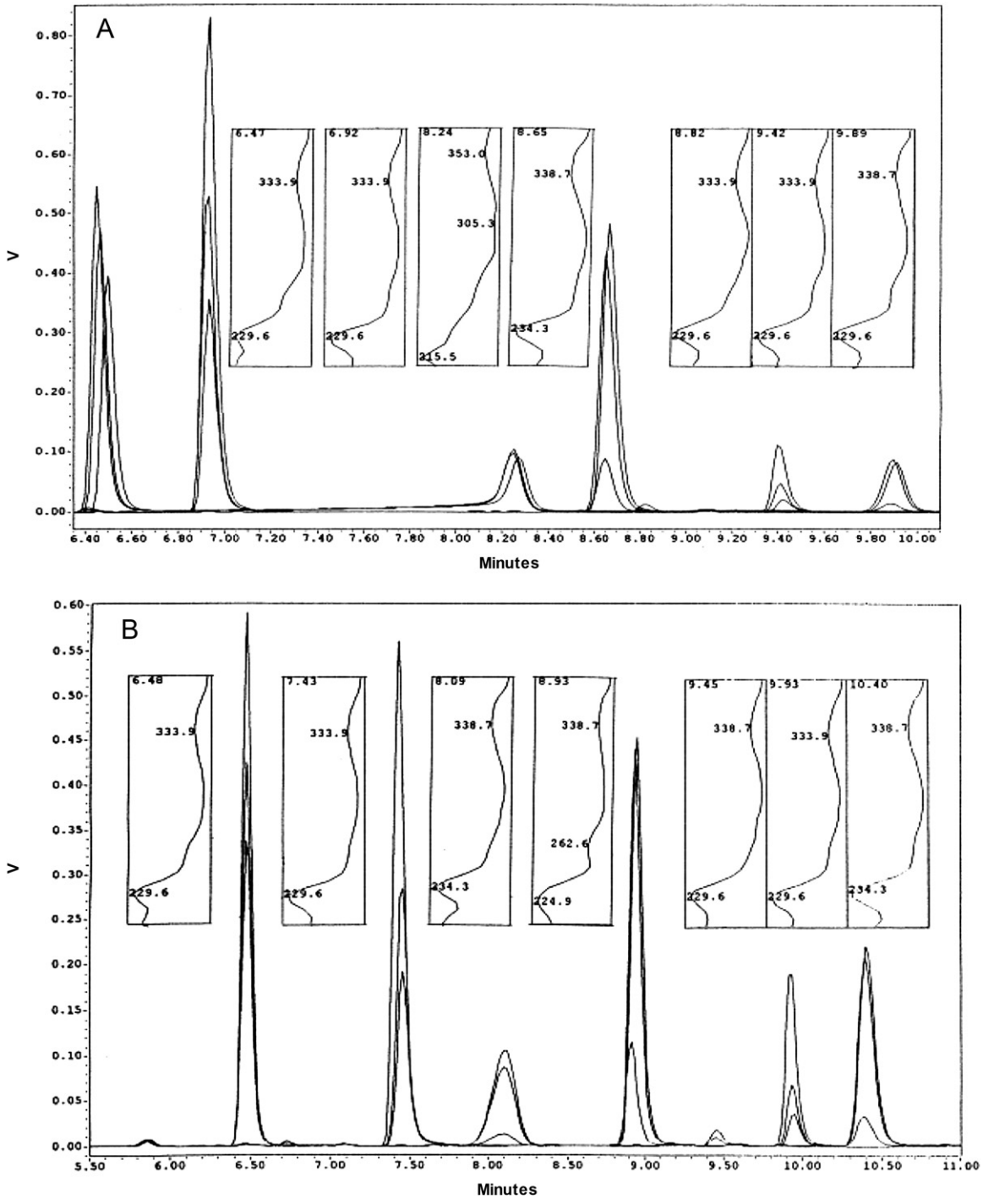

Fig. 4. Simultaneous FL chromatogram and PDA spectra of the OPA/MPA derivatives of glycine, GABA and lysine (A) and histidine, β-alanine and ornithine, (B) as a function of the reaction time (7 min, 3 h and 6 h; detailed results in Table 3). Reproduced with permission from Ref. [3].

On the basis of our results it has been proved (Fig. 4A and B and Table 3) that:

(i) In the cases of those AA derivatives which are providing single derivatives, and, are not listed in Table 3, significant losses (<4%) have not been detected, even not after 6 h reaction time.
(ii) Maximum FL intensities and UV absorbencies for the OPA/MPA derivatives of aspartic acid, glutamic acid, β-alanine, alanine, GABA, isoleucine, lysine and ornitine, 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 significant, but detectable loss of responses (Table 3) which proved to be 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 principle in the understanding of the characteristics of – for the time being believed – not stable derivatives. On the basis of our stoichiometric studies it became evident

Table 3
Stability and characteristics of the OPA/MPA and OPA/NAC-AA derivatives as a function of the reaction time.

Amino Acid	OPA/MPA-AAs					OPA/NAC-AAs				
	t_R	Response %*			RR	t_R:	Response %			RR
	min	7 m	3 h	6 h		min	7 m	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#	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#	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
In 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&	8.33	43	36	38	18.4&
In total		100	86	63			100	91	82	
β-Alanine1	6.92	85#	52	39	17.2	6.63	89#	73	56	15.1
β-Alanine2	8.65	10#	48	61	25.6	8.39	6#	27	42	16.9
In total		95	100	100			95	100	98	
GABA1	7.43	73#	39	26	18.1	6.98	88#	72	59	14.6
GABA2	8.93	16#	61	64	23.5	8.43	5#	28	41	16.6
In total		89	100	90			93	100	100	
Ornithine1	8.82	<1#	3	7	1	-				
Ornithine2	9.42	71#	27	12	2.4	8.65	79#	49#	35	2.2
Ornithine3	9.89	5#	70	66	4.9	9.23	5#	46#	65	3.9
In total		77	100	95			84	95	100	
Lysine1	9.45	<1#	3	6	5.0	-				
Lysine2	9.93	54#	19	10	3.8	8.92	67#	48	31	2.8
Lysine3	10.40	13#	78	84	8.0	9.48	6#	52	69	5.5
In total		68	100	100			73	100	100	

%* = expressed in the total; t_R = retention time; h = hrs; RR = Fl/UV represent the response ratios of the fluorescence intensities versus the UV ones; values are corresponding to the peaks in Fig. 4AB; # = maximum FL was measured after extended reaction time, mainly after 28 min; & = measured at their maxima, at 353 nm.

that the stability of these derivatives is 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 3):

- Glycine proved to be one of the most stable derivatives providing AA: the total of glycine1 and glycine2 do not change even after 6 h reaction time.
- In the cases of β-alanine, GABA, ornithine and lysine, accordingly our earlier experiences [3] the maximum fluorescences were measured after extended reaction times (7–28 min).
- The less stable OPA/MPA-histidine – after 6 h – provided 63%, the OPA/NAC-histidine 82% responses. The loss in the cases of OPA/MPA-GABA (90%), OPA/MPA-ornithine (95%) and OPA/NAC-alanine (98%) were as low as manifested any others.

Already, in these introductory investigations we assumed that the molar ratio of the OPA/SH-group additive plays a special and key role in the transformation of the initially formed OPA derivative: therefore we varied these ratios from 1/0.5 through 1/3 to 1/10 (Fig. 5A–C).

As seen in Fig. 5A–C, proved also by exact data [5], decreasing the molar ratios of OPA/MPA in the reagent from 1/0.5 (Fig. 5A) through 1/3 (Fig. 5B) to 1/10 (Fig. 5C), led to a significant rate reduction regarding the transformation of the first products to the forthcoming ones. For example, in all six cases, for both derivatives (OPA/MPA, OPA/NAC), the peak ratios of glycine (Fig. 5A–C) similarly to those of GABA, β-alanine, histidine, ornithine and lysine (not shown) reveal that the total of products do not decrease considerably, not even after 6 h reaction time (data in Table 3, detailed data in paper [5]).

These phenomena proved to be a very important indication, pointing ahead to solve the issue of the formation/transformation of the multiple OPA-derivatives.

Evaluating these experiences from analytical point of view, they proved to be really promising. Namely: taking into account the total of responses of these more than one derivative providing AAs, we could prove that these amino acids manifest the same stability than all others. Consequently, the only requirement was to develop a proper chromatographic technique that ensured the quantitation of total of derivatives formed [4].

1.3.2. The role of pH in the stability of the OPA derivatives of AAs

The two selected factors affecting the transformation of the primarily formed products to their transformed ones are (i) the molar ratios of OPA to the SH-group additive (detailed above) and (ii) the pH of solution containing the OPA-AAs [5,54].

Acidifying the solution of the OPA/MPA-AAs from pH = 9.3 to pH = 7.2, for 7 min, before injecting them (subsequently to 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 AAs: 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%.

Responses of the single OPA-AAs, acidifying them under the same condition 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-AAs [37].

Table 4
Fragmentation patterns of the OPA/MPA and OPA/NAC derivatives of amino acids.

Amino acids	Mw	Ions								
		First OPA/MPA derivative's				Transformed OPA/MPA derivative's ions				
		MH^+ calc.	MH^+ obt.	$MH^+ - COO$	$MH^+ - m/z = 105$	$MH^+ + OPA$	$MH^+ + OPA - H_2O$	MH^+	$2OPA + MPA - 2H_2O$	$OPAH^+$
Glycine	75.1	**280.3**	280.0		175.0	**414.0**	396.0	280.0		–
β-Alanine	89.1	**294.3**	294.0	–	189.1	**428.0**	410.0	294.0	338.0	–
Lysine	146.2	**555.6**	555.2	511.0	405.1#	**689.0**	671.0	555.0	338.0	
∈-NH_2-caproic	131.2	**336.4**	336.1	292.0	231.1	470.1	**452.2**	336.1		135.1
		Ions								
Amino	Mw	First OPA/NAC derivative's				Transformed OPA/NAC derivative's ions				
acids		MH^+calc.	MH^+obt.	MNa^+	$MH^+ - m/z = 129$	$MH^+ + OPA$	$MNa^+ + OPA$	$MH^+ + OPA\text{-}H_2O$	$MNa^+ + OPA+Na$	$MNa^+ + OPA + 2Na$
Glycine	75.1	**337.4**	337.1	359.1	208.0	471.2	**493.1**	453.0	–	–
β-Alanine	89.1	**351.4**	351.1	373.0	222.0	485.1	**507.2**	467.2	–	–
GABA	103.1	**365.4**	365.1	387.1	236.1	528.6	**521.2**	481.2	543.1	564.8
Histidine	155.2	417.5	417.3	**461.4**	310.3	–	–	–	–	–
Ornithine	132.1	**655.7**	655.3	677.2	–	789.2	–	–	–	–
Lysine	146.2	**669.8**	669.3	691.4	–	803.3	**825.3**	785.3	847.3	869.3

Indications: Mw = molecular weight; (Mw_{OPA} = 134.1; Mw_{MPA} = 106.1; Mw_{NAC} = 163.2); concentrations: [OPA]/[MPA]([NAC])/[AA]([A]) = 20/60/1, 1 = ∼lxlO∼[9]; bold printed data = abundant ions.

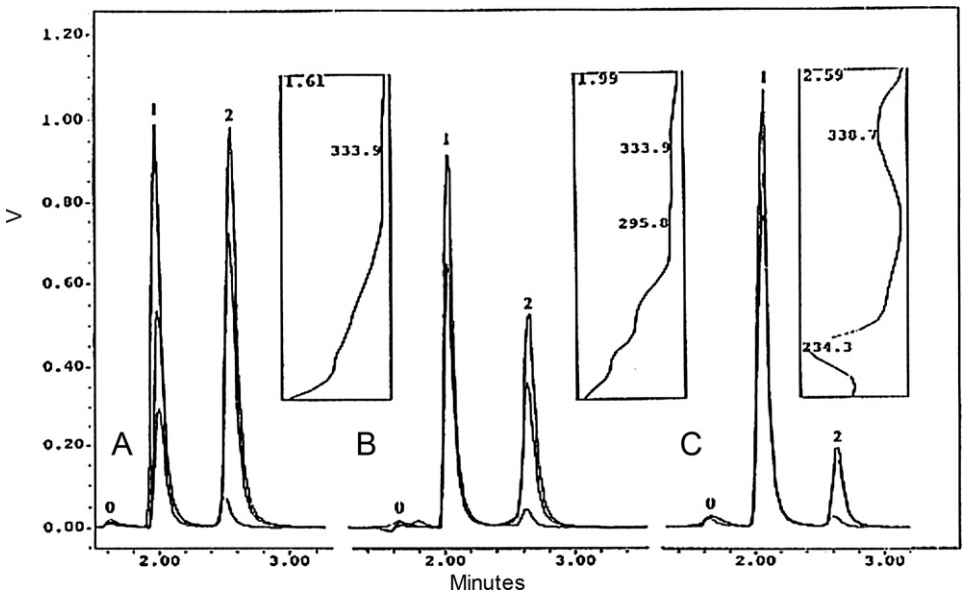

Fig. 5. Chromatograms (A–C), obtained by Fl/DAD detections, applying the molar ratios of OPA/MPA/AA = 20/10/1 (A), OPA/MPA/AA = 20/60/1 (B), OPA/MPA/AA = 20/200/1 (C) (1 = 1×10^{-9} M) as a function of the reaction time (7 min, 3 h, 6 h). Peaks: 1, gly0; 2, gly1; 3, gly2; reproduced with permission from Ref. [5].

1.4. Studies on reaction mechanism

1.4.1. Earlier literature data

The formation and composition of the fluorescent adduct, the 'main' reaction product proved to be 1-alkylthio-2-alkyl-substituted isoindoles [55–64]: obtained from 1-1-1 molecules of OPA, NH2- and SH-group containing compounds with the elimination of 2 moles of H_2O: based on spectral and NMR [55–59], kinetic [60,61], as well as on those of mass spectrometric (MS) evidences [62–66]. Although, beside the 'main' product, very likely as a result of its decomposition (disproportionation/autooxidation), the presence of the 1,3 dithio-substituted-2-alkyliso-indoles were also identified, based on NMR evidence [58], supported also by kinetic data [60,61].

Earlier HPLC–MS studies [65,66] related to special tasks. The thermo-spray (TSP)-HPLC–MS was applied to (i) determine S-nitrosoglutathione in the presence of an enormous excess of reduced glutathione as their OPA/MCE derivatives [32], while (ii) the diastereomeric isoindoles of alanine, valine, leucine phenylalanine, phenylglycine and their corresponding amides have been separated and identified as OPA/NAC derivatives [65].

The isotope enrichment of citrulline, arginine, tyrosine, valine and leucine has been measured by HPLC–MS (ESI) as their OPA/MPA derivatives [66] working in the negative ion mode.

1.4.2. Mass spectrometric studies of the initially formed derivatives

All of our experiences detailed above raised the questions to be answered:

(i) What is the composition of the transformed derivatives, consequently, what is the unknown reaction mechanism they are originating from?
(ii) What is the common and determinative peculiarity of all those compounds that providing more than one OPA derivatives?
(iii) Is it possible and in what way to inhibit or at least to decrease the transformation of the initially formed derivatives?

The answers to these questions have been given one by one in the following manner.

At first, the composition of the OPA/MPA and OPA/NAC derivatives of glycine, β-alanine, GABA, histidine, ornithine, lysine and ε-amino caproic acid, applying on line HPLC–PDA–MS (ESI), were measured in the positive mode (Table 4 and Fig. 6A and B). Data revealed unambiguously, that the initially formed products of all of these AAs were the well-known isoindoles while their transformed versions, without exception, contain an additional molecule of OPA.

Spectra of the individual derivatives proved to be particularly informative (Table 4 and Fig. 6A and B). They served as unambiguous evidence both to earlier results with OPA derivatives of –NH–CH_2-bonding (Table 5), and, to the suggested reaction scheme (Fig. 7). Evaluating the MS spectra the following conclusions can be drown.

1.4.2.1. Mass spectrometric studies of the initially formed derivatives: fragments of the initially formed derivatives. Table 4: initially formed

Table 5
Structural properties of the multiple/single OPA derivatives providing amino acids and amines.

More than one OPA derivative	
Glycine	NH_2CH_2–COOH
β-Alanine	NH_2CH_2–CH_2)–COOH
γ-Aminobutyric acid	NH_2CH_2–$(CH_2)_2$–COOH
Ornithine	NH_2CH_2–$(CH_2)_2$–$CHNH_2$–COOH
Lysine	NH_2CH_2–$(CH_2)_3$–$CHNH_2$–COOH
N-α-Acetyl-L-lysine	NH_2CH_2–$(CH_2)_3$–$CHNH(CH_3CO)$–COOH
N-α-Benzyloxy-L-lysine	NH_2CH_2–$(CH_2)_3$–$CHNH(C_6H_5CH_2O)$–COOH
ε-Aminocaproic acid	NH_2CH_2–$(CH_2)_4$–COOH
Histidine	$C_3N_2H_3$–CH_2–*CH*NH_2–COOH *(irregular)*
Ethanolamine	NH_2CH_2–CH_2–OH
n-C_1–C_5, i-C_4 amines	NH_2CH_3, NH_2–$(CH_2)_{2\text{-}5}$
C_2–C_5 diamines	NH_2CH_2–$(CH_2)_{1\text{–}4}$–NH_2
Agmatine	NH_2CH_2–$(CH_2)_3$–NHC(=NH)NH_2
Tyramine	NH_2CH_2–CH_2–C_6H_4–OH
Bis(hexamethylene)triamine	NH_2CH_2–$(CH_2)_5$–NH–$(CH_2)_5$–CH_2NH_2
Spermine	NH_2CH_2–$(CH_2)_2$–NH–$(CH_2)_4$–NH)–$(CH_2)_2$–CH_2NH_2
Spermidine	NH_2CH_2–$(CH_2)_3$–NH–$(CH_2)_2$–CH_2NH_2
Single OPA derivative	
α-Amino Acids (all)*	R-*CH*NH_2–COOH, *except the above ones
N-ε-acetyl-L-lysine	$NH(CH_3CO)$–$(CH_2)_2$–*CH*NH_2COOH
N-ε-formyl-L-lysine	NH(CHCO)–$(CH_2)_2$–*CH*NH_2COOH
i-Propylamine	CH_3–*CH*NH_2–CH_3
sec.-Butylamine	CH_3–*CH*NH_2–CH_2CH_3
tert.-Butylamine	CH_3–CNH_2CH_3–CH_3

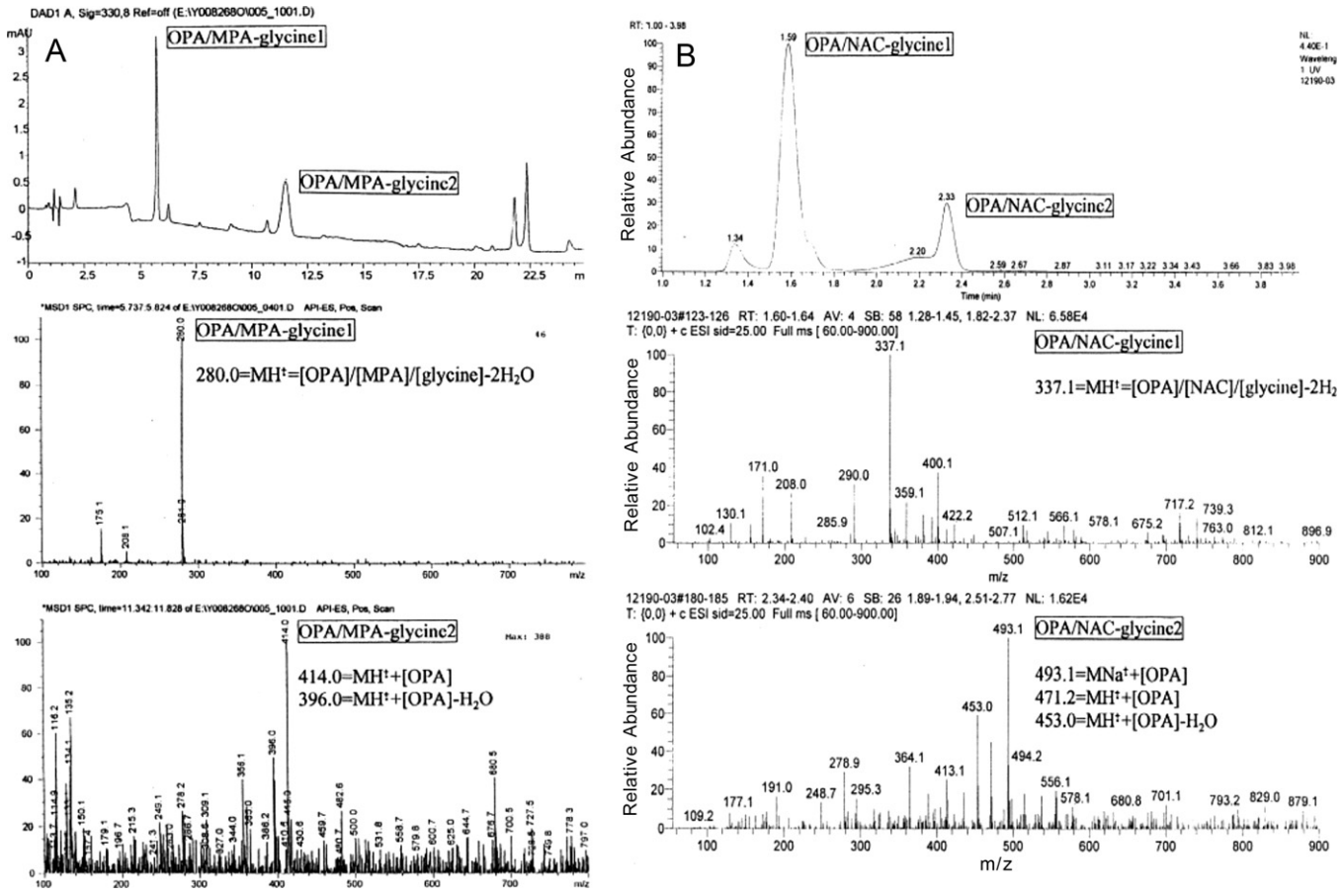

Fig. 6. PDA/UV chromatograms (first lines: glycine1, glycine2), and MS spectra of the initially obtained (glycine1: spectra in second lines) and transformed (glycine2: spectra in third lines) OPA/MPA- (A) and OPA/NAC-glycine (B) derivatives; (detailed composition of fragments in Table 4). With permission from Ref. [7].

OPA/MPA and OPA/NAC derivatives, Fig. 6A and B), compounds indicated by number1, as well as ornithine2 and lysine2 (the tiny amounts of ornithine1 and lysine1 could not be ionized.

(i) The protonated molecular ions (MH^+) and/or their cationized versions (MNa^+) were detected, as abundant fragments, in spectra of all initially formed derivatives, with the exception (OPA/NAC derivatives of histidine.

(ii) Further selective fragment ions (SFI) were obtained by partial loss of the aliphatic chain of both SH-group additives (by losses of $m/z = 72$ instead of $m/z = 73$ in case of OPA/MPA-glycine1: Fig. 6A, $m/z = (280–72) = 208$ and those of $m/z = 129$ instead of $m/z = 130$ in eight spectra of the initially formed OPA/NAC-derivatives: m/z values in Table 4, seventh vertical column). The elimination of the MPA anion with the mass of $m/z = 105$ was obtained in the spectra of the initially formed glycine1, β-alanine1 and ε-aminocaproic acid1 while from lysine2 the fragment of $m/z = 405.1$ was formed by the simultaneous loss of one molecule CO_2 and MPA (Fig. 6A) spectra of glycine1, β-alanine1, ε-aminocaproic acid1 and lysine2).

1.4.2.2. Mass spectrometric studies of the initially formed derivatives: fragments of the transformed derivatives. Table 4: transformed OPA/MPA and OPA/NAC derivatives, Fig. 6A and B, compounds indicated by number2, {with exceptions of ornithine2 and lysine2 which are initially formed OPA species}, as well as ornithine3, lysine3).

(i) The protonated molecular ions ($MH^+ + OPA$) and/or their cationized version(s) ($MNa^+ + OPA$) were detected in all transformed species (the only exception was histidine: its transformed derivative could not be identified, only under specified conditions [12]: Section 1.6).

(ii) Dehydrated species, obtained from the transformed molecular ions by elimination of one molecule of water ($MH^+ + OPA - H_2O$), were masses of high intensity. The presence of these fragments is of primary importance corresponding to the stabilized end-product of our proposed mechanism-scheme (Fig. 7: compounds VII and VIII).

(iii) Fragments with the same masses, i.e., those of $m/z = 278.2–279.1$ are of particular importance. The existence of these ions ($MW = C_{17}H_{12}NOS = 278$, its protonated form: $m/z = 279$) proved to be independent of the reagent's SH-group containing component and that of the NH_2-group containing compound. Consequently, it served as a strong evidence to our proposed mechanism, being formed by the complete elimination of the aliphatic-chains of the transformed end-product, belonging both to the SH-group and to the NH_2 containing parts [7].

1.4.3. Basic molecular structure of the multiple derivatives furnishing primary amino-group containing compounds

As to the initial molecular structure of all of those derivatives examined in our laboratory [3–19] we distinguished them according to their common structural characteristics, i.e., to the more than one and to the single OPA derivative providing species (Table 5). According to our data and its explanations it is clear that the substituents of the neighboring C atom to the primary amino group play a key role. Referring to Fig. 7 the mobilizable H atom of the CH_2 group next to the isoindole Nitrogen initializes the reaction

Table 6
Responses and stabilities of the single OPA/ET-derivative providing amino acids as a function of reaction time and the reactants' molar ratios, based on UV and fluorescence (FL) detections (OPA–ET = 1/10 reagent of 80 % (v/v) methanol content; pH = 9.30).

Amino acids⇓	t_R, min⇓	Integrator unit/pmol								
		Fluorescence detection								
Reaction time, min ⇒		1	3	7	10	26	50	360	Average	RSD%
[OPA]/[amino acid] = 120/1; 1 = 2 × 10^{-10} M										
Aspartic a.	4.48	*3.04*	*3.41*	*3.88*	*3.94*	**4.33**	**4.43**	**4.53**	**4.43**	2.26
Glutamic a.	5.67	*3.76*	*4.08*	**4.46**	**4.50**	**4.71**	**4.75**	**4.91**	**4.67**	3.99
Asparagine	10.03	*3.70*	*4.04*	**4.32**	**4.37**	**4.51**	**4.46**	**4.72**	**4.48**	3.47
Glutamine	11.00	*5.10*	**5.21**	**5.27**	**5.32**	**5.33**	**5.37**	**5.57**	**5.35**	2.31
Threonine	13.9	*4.11*	*4.53*	**4.91**	**4.96**	**5.07**	**5.10**	**5.11**	**5.03**	1.78
Methionine	16.85	**5.33**	**5.48**	**5.36**	**5.45**	**5.34**	**5.68**	**5.42**	**5.44**	2.23
Tryptophan	17.02	**5.60**	**5.86**	**5.98**	**6.01**	**6.02**	**5.89**	*6.46*	**5.89**	2.67
Isoleucine	17.43	*5.39*	*5.86*	**6.01**	**6.10**	**6.18**	**6.33**	**6.63**	**6.25**	3.88
[OPA]/[amino acid] = 67/1; 1 = 2 × 10^{-10} M										
Aspartic a.	4.00	*3.35*	*3.57*	*3.95*	**4.12**	**4.45**	**4.64**	**4.69**	**4.48**	5.8
Glutamic a.	4.93	*4.10*	*4.30*	**4.65**	**4.70**	**4.93**	**4.97**	**5.07**	**4.86**	3.72
Asparagine	9.57	*3.90*	*4.25*	**4.54**	**4.64**	**4.70**	**4.67**	**4.89**	**4.69**	2.73
Glutamine	10.35	*5.70*	**5.82**	**5.90**	**5.70**	**5.80**	**5.83**	**6.04**	**5.85**	1.95
Serine	11.19	**4.56**	**4.58**	**4.56**	–	**4.54**	**4.53**	*3.72*	**4.55**	0.42
Threonine	12.17	*4.46*	*4.89*	**5.29**	**5.25**	**5.51**	**5.50**	**5.67**	**5.44**	3.18
Arginine	13.67	**5.02**	**5.09**	**5.08**	–	**5.06**	**5.07**	*4.89*	**5.06**	0.53
Tyrosine	14.90	**5.20**	**5.22**	**5.28**	–	**5.23**	**5.21**	**5.30**	**5.24**	0.77
Alanine	15.08	**5.05**	**5.11**	**5.31**	–	**5.34**	**5.30**	*4.78*	**5.22**	2.53
Methionine	16.63	**6.42**	**6.62**	**6.92**	**6.87**	**6.63**	**6.69**	**6.62**	**6.68**	2.52
Valine	16.80	**5.62**	**5.72**	**5.87**	–	**5.88**	**5.85**	**5.63**	**5.76**	2.09
Tryptophan	16.83	**5.26**	**5.37**	**5.51**	**5.49**	**5.22**	**5.45**	**5.67**	**5.42**	2.85
Isoleucine	17.23	**6.30**	**6.36**	**6.80**	**6.79**	**6.69**	**6.93**	**6.86**	**6.67**	3.71
Phenylalanine	17.39	**5.35**	**5.33**	**5.40**	–	**5.48**	**5.49**	**5.44**	**5.41**	1.22
Leucine	17.57	**5.51**	**5.59**	**5.75**	–	**5.77**	**5.74**	**5.57**	**5.65**	1.96
[OPA]/[amino acid]=15/1; 1=2 × 10^{-10} M										
Aspartic a.	4.00	*2.37*	*2.72*	*3.35*	*3.46*	*4.08*	**4.54**	**4.54**	**4.54**	–
Glutamic a.	4.93	*3.11*	*3.52*	*4.11*	*4.23*	**4.75**	**4.99**	**4.92**	**4.88**	2.52
Asparagine	9.57	*2.98*	*3.62*	*4.28*	*4.38*	**4.66**	**4.78**	**4.78**	**4.74**	1.46
Glutamine	10.35	*4.76*	***5.65***	***5.73***	***5.92***	***5.69***	***5.80***	***5.74***	**5.75**	1.65
Threonine	12.17	*3.34*	*4.21*	*4.72*	*5.00*	**5.24**	**5.40**	**5.25**	**5.29**	1.69
Methionine	16.90	*5.77*	*6.09*	**6.51**	**6.38**	**6.47**	**6.58**	**6.32**	**6.45**	1.60
Tryptophan	17.10	*4.56*	**4.93**	**5.12**	**5.15**	**5.21**	**5.13**	**5.41**	**5.15**	3.00
Isoleucine	17.50	*4.87*	*5.47*	*6.05*	**6.27**	**6.57**	**6.59**	**6.74**	**6.54**	3.00

Amino acid⇓	Integrator unit/pmol								
	UV detection								
	1	3	7	10	26	50	360	ave-rage	RSD%
[OPA]/[amino acid] = 120/1; 1 = 2 × 10^{-10} M									
Aspartic a.	*0.22*	*0.29*	*0.32*	**0.34**	**0.35**	**0.38**	**0.34**	**0.35**	5.4
Glutamic a.	*0.31*	*0.33*	**0.36**	**0.37**	**0.38**	**0.40**	**0.40**	**0.38**	4.68
Asparagine	*0.26*	*0.29*	**0.31**	**0.31**	**0.32**	**0.31**	**0.32**	**0.31**	1.74
Glutamine	**0.37**	**0.38**	**0.39**	**0.39**	**0.39**	**0.37**	**0.36**	**0.38**	3.21
Threonine	*0.27*	*0.30*	**0.33**	**0.34**	**0.34**	**0.35**	**0.34**	**0.34**	2.08
Methionine	**0.35**	**0.36**	**0.37**	**0.37**	**0.35**	**0.37**	**0.37**	**0.36**	2.62
Tryptophan	**0.33**	**0.35**	**0.34**	**0.35**	**0.35**	**0.35**	*0.38*	**0.35**	2.42
Isoleucine	*0.34*	*0.36*	**0.38**	**0.37**	**0.39**	**0.38**	**0.41**	**0.39**	3.93
[OPA]/[amino acid] = 67/1; 1 = 2 × 10^{-10} M									
Aspartic a.	*0.27*	*0.31*	*0.33*	**0.36**	**0.38**	**0.39**	**0.41**	**0.38**	5.4
Glutamic a.	*0.36*	*0.36*	**0.37**	**0.39**	**0.40**	**0.39**	**0.41**	**0.39**	3.78
Asparagine	*0.28*	**0.33**	**0.34**	**0.35**	**0.35**	**0.35**	**0.35**	**0.34**	1.28
Glutamine	*0.36*	**0.38**	**0.38**	**0.40**	**0.37**	**0.38**	**0.38**	**0.38**	2.57
Serine	**0.33**	**0.33**	**0.32**	–	**0.32**	**0.32**	*0.26*	**0.32**	1.69
Threonine	*0.31*	*0.33*	**0.35**	**0.36**	**0.38**	**0.38**	**0.38**	**0.37**	3.82
Arginine	**0.33**	**0.34**	**0.34**	–	**0.33**	**0.34**	**0.33**	**0.33**	1.63
Tyrosine	**0.32**	**0.33**	**0.33**	–	**0.33**	**0.32**	**0.32**	**0.32**	1.68
Alanine	**0.34**	**0.35**	**0.37**	–	**0.37**	**0.37**	**0.33**	**0.35**	4.95
Methionine	**0.40**	**0.40**	**0.42**	**0.41**	**0.41**	**0.40**	**0.42**	**0.40**	2.20
Valine	**0.33**	**0.34**	**0.34**	–	**0.35**	**0.34**	**0.34**	**0.34**	1.86
Tryptophan	**0.33**	**0.30**	**0.33**	**0.31**	**0.32**	**0.31**	**0.33**	**0.31**	3.81
Isoleucine	*0.34*	*0.34*	**0.37**	**0.37**	**0.37**	**0.37**	**0.39**	**0.37**	2.39
Phenylalanine	**0.33**	**0.34**	**0.35**	–	**0.35**	**0.34**	**0.33**	**0.34**	2.63
Leucine	**0.36**	**0.37**	**0.36**	–	**0.36**	**0.36**	**0.34**	**0.36**	2.74
[OPA]/[amino acid]=15/1; 1=2 × 10^{-10} M									
Aspartic a.	*0.20*	*0.25*	*0.29*	*0.31*	*0.35*	**0.41**	**0.38**	**0.39**	5.4
Glutamic a.	*0.25*	*0.29*	*0.34*	*0.35*	**0.38**	**0.41**	**0.39**	**0.39**	3.88
Asparagine	*0.23*	*0.27*	*0.32*	*0.33*	**0.35**	**0.36**	**0.35**	**0.35**	1.63
Glutamine	*0.33*	**0.38**	**0.39**	**0.41**	**0.39**	**0.40**	**0.40**	**0.39**	2.65
Threonine	*0.24*	*0.30*	*0.33*	*0.36*	**0.38**	**0.39**	**0.38**	**0.38**	1.50
Methionine	*0.34*	**0.39**	**0.41**	**0.40**	**0.40**	**0.41**	**0.39**	**0.40**	2.23
Tryptophan	*0.26*	*0.28*	**0.30**	**0.30**	**0.29**	**0.30**	**0.30**	**0.29**	1.50
Isoleucine	*0.27*	*0.31*	*0.34*	**0.35**	**0.35**	**0.36**	**0.37**	**0.35**	2.67

Indications as in Tables 1–5, as well as: italic printed values have been omitted from averages; a = acid; Elution conditions: 50 °C, 1 ml/min. 0 min: 70 % eluent A + 30 % eluent B; 1–6 min: 50 % eluent A + 50 % eluent B; 11–16 min: 70 % eluent B + 30 % acetonitrile; 17–25 min: 70 % eluent A + 30 % eluent B.

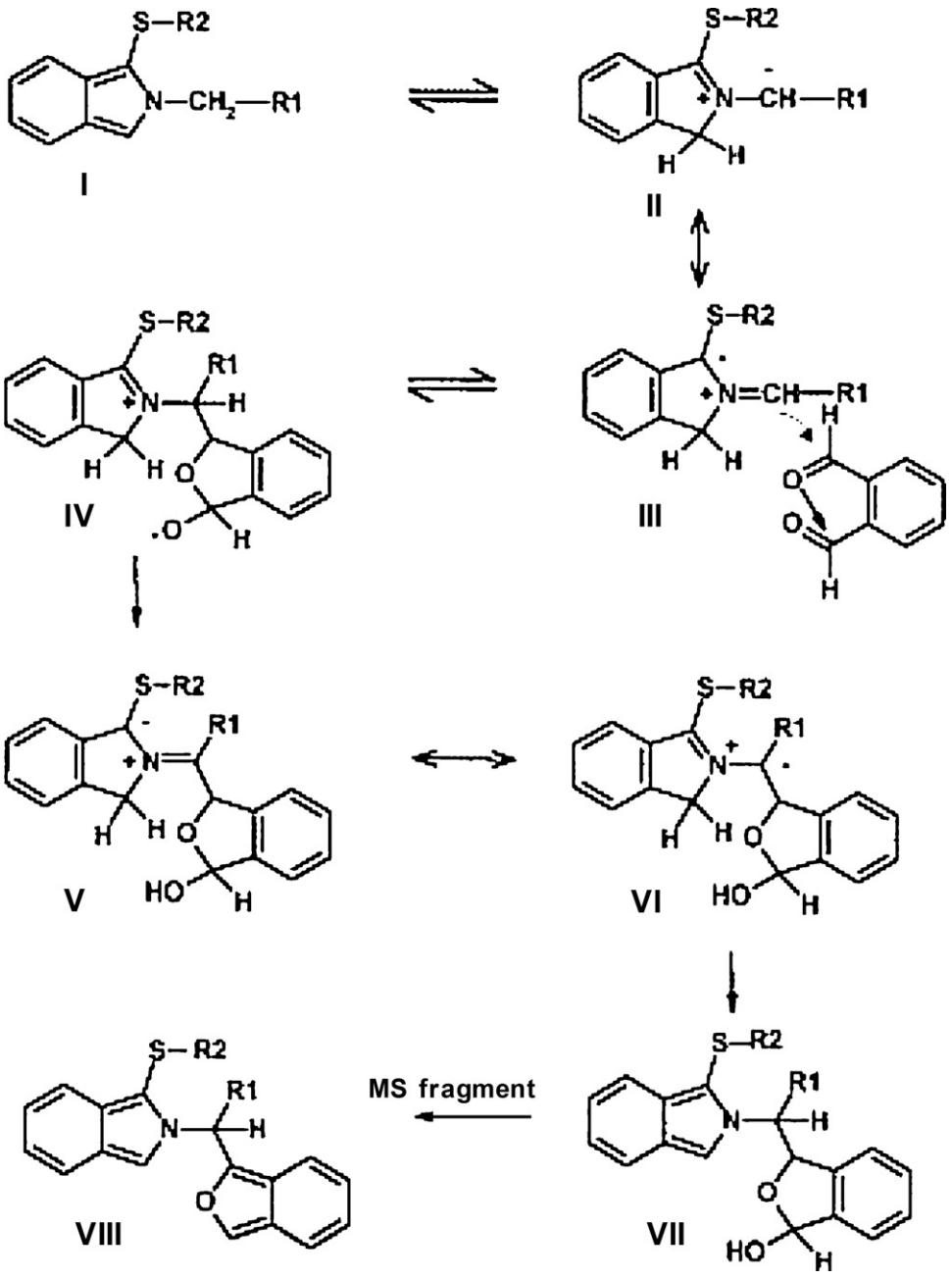

Fig. 7. Reaction pathway of the formation/transformation of the multiple derivative providing amino acids and amines. With permission from Ref. [7].

with an additional OPA molecule (Fig. 7 compound III), i.e., this is the first step leading to the transformation of the initially formed OPA species. Consequently, the nucleophile reaction of OPA with the isoindole must start at the CH_2 group only, following the pathway shown in Fig. 7. This assumed mechanism was supported by MS data (Fig. 6A and B and Table 4) and further completed by analytical results.

1.4.4. Considerations of analytical consequences

In the light of our stoichiometric mass spectrometric experiences [3–19] i.e., in the knowledge of the composition of the transformed derivatives, and that of the reaction pathway they are originating from (Fig. 7), – we decided to change the composition of the reagent. Molar ratios of OPA to the SH additive were altered for OPA/MPA and for OPA/NAC = 1/50. This observation implies that the free OPA concentration favors the transformation of the initially formed isoindole that is needed for the 'ready to react' state, it means those of free OPA molecules (OPA and the SH additive reacts as a preformed species with the amino group [7–11]). Thus, we expected that by further increasing, in a considerable manner, the concentration of the SH-group additive in the reagent, should result in two benefits, simultaneously: (i) in a decrease of the transformation rate of the initially formed derivative, and, (ii) in an increase of the overall stability of the total of derivatives.

The realization of this assumption leaded to the expected results in the case of C_1–C_4 aliphatic monoamines [8]. However in the case of the multiple derivative providing AAs resulted in increasing number of transformed species. Consequently, for analytical purposes, the use of the OPA/MPA or the OPA/NAC, or, as a reagent of choice, the OPA/ET one, all, had to be applied under strictly the same, optimized analytical conditions (OPA/MPA (NAC) = 1/3; OPA/ET = 1/10).

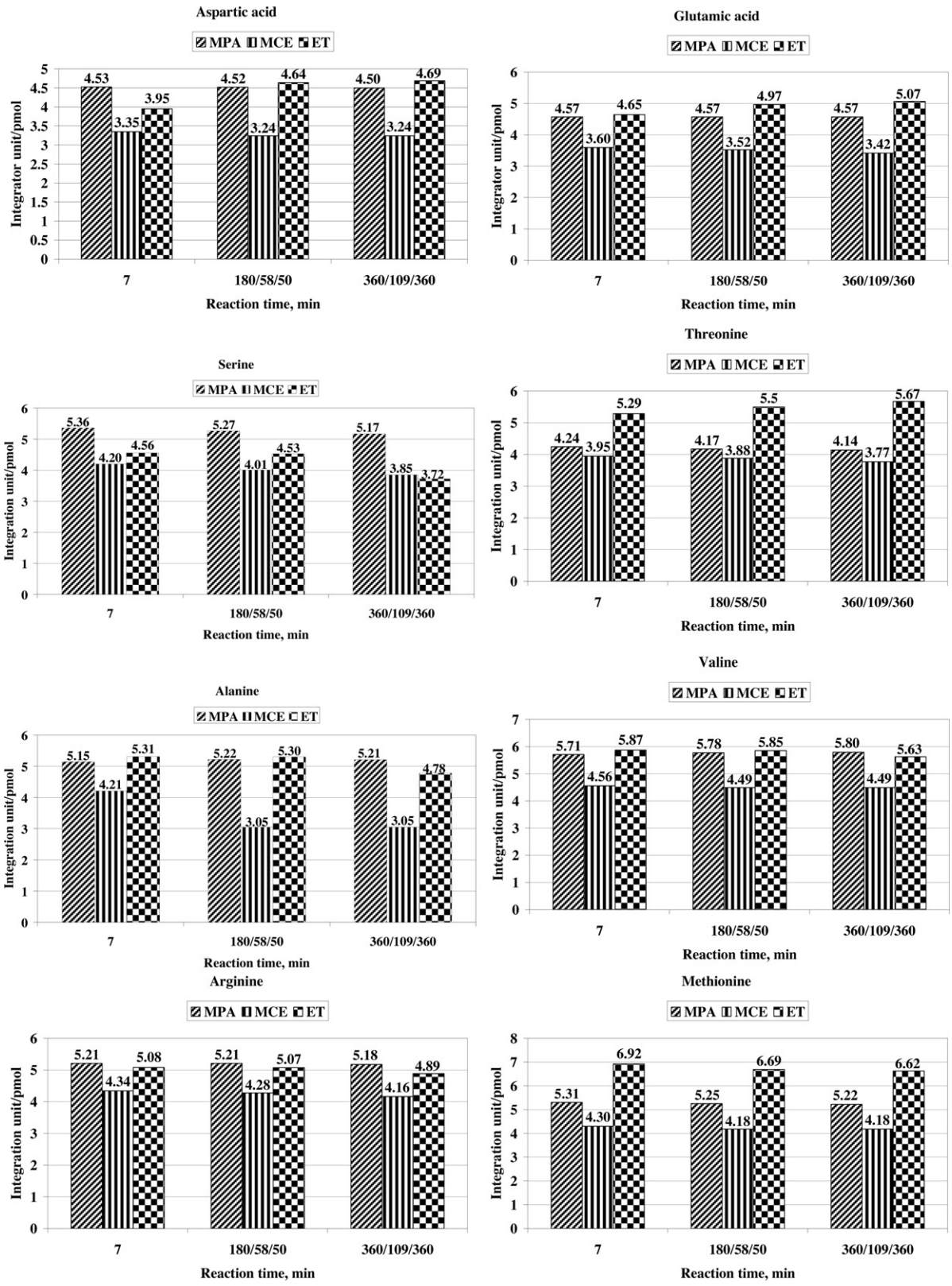

Fig. 8. Comparison of the fluorescence response values of the single derivative providing amino acids as a function of the *o*-phthalaldehyde reagent's SH-additive and the reaction time. With permission from Ref. [18].

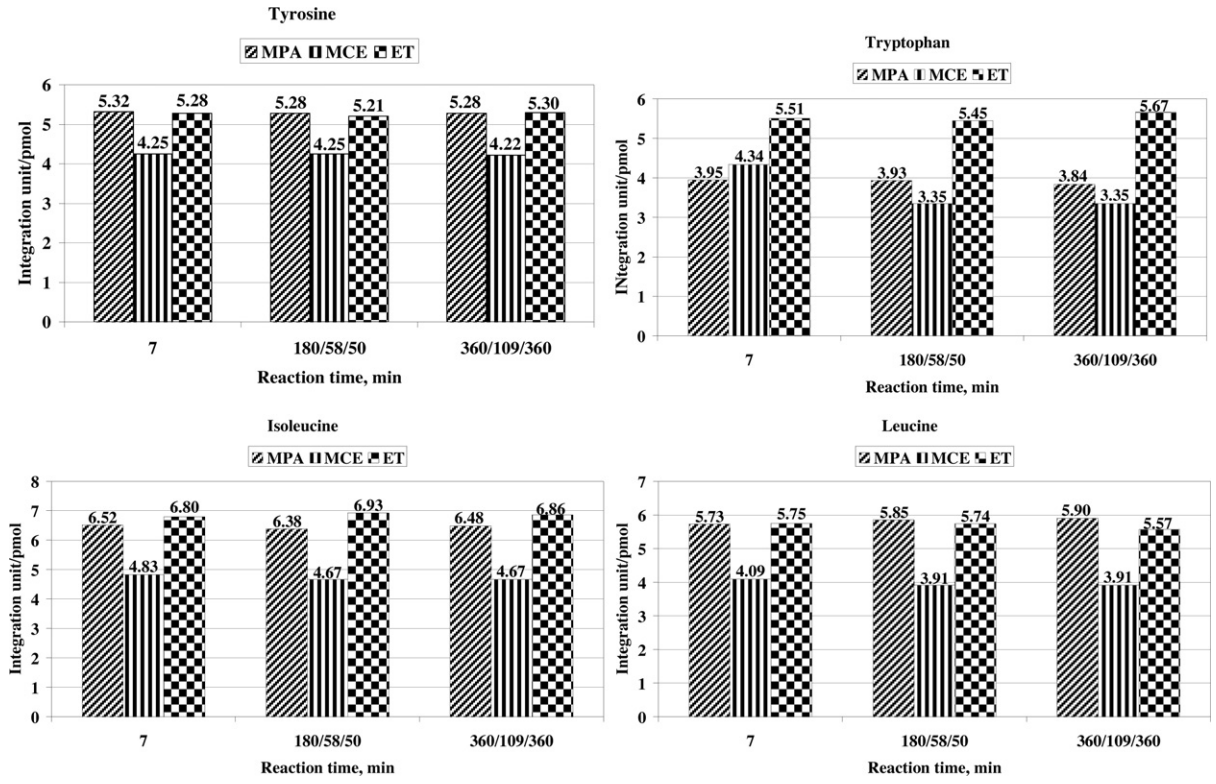

Fig. 8. (*Continued*).

1.5. Comparison of responses and stability of AA derivatives as a function of the SH group containing additive

1.5.1. Stability and characteristics of the single OPA/ET-derivative providing amino acids as a function of the reaction time and the molar ratios of reactants; Comparison of stabilities depending on the OPA reagent's SH additive

As expected, the '=CH–NH_2' moiety containing amino acids provide single OPA/ET derivatives (Table 6) of long term stability.

The impact of the OPA/ET/amino acid molar ratios on their derivatization rate was a new experience. In contrary to data:

(a) obtained with the OPA/MPA derivatives [3–12], they manifest the same molar responses for the OPA/MPA/amino acid ratios from 4/1 up to 105/1,
(b) in cases of the OPA/ET derivatives considerable differences were obtained comparing quantitative interactions depending on the molar excess of the OPA reagent (Table 6: [OPA]/[amino acids] = 15/1–120/1). Thus, in order to increase the reaction rates at least the [OPA]/[amino acid] = 50–70/1, or higher molar excess of OPA is to be used.

Comparing fluorescence responses of the single derivative providing amino acids depending on the OPA reagent' SH-additive they were derivatized with (Fig. 8), it is clear that,

a. stabilities – independently on their composition – are the same,
b. the lowest response values were provided by the OPA/MCE derivatives [11], while,
c. the responses of the OPA/MPA and OPA/ET derivatives proved to be comparable: with the few exceptions of the outstanding responses of the OPA/ET glutamine, threonine, methionine and tryptophan derivatives (Fig. 8).

In conclusion, it has been confirmed that independent on the OPA-thiol reagents all the '=CH–NH_2' moiety containing, isoindoles are providing single and stable products, suitable for reliable and reproducible analytical purposes.

1.5.2. Stability of derivatives as a function of the thiol additive

The '–CH_2–NH_2' moiety containing amino acids such as glycine, β-alanine, γ-aminobutyric acid, ornithine, lysine and also histidine after its rearrangement [12] furnish OPA/ET derivatives of outstanding stability and intensity in their FL and UV responses, equally (Tables 6 and 7 and Fig. 9).

Due to the peculiarity of the '–CH_2–NH_2' moiety containing amino acids/amines the expected transformed products [3–19] – containing one additional OPA molecule (UV_{max}: 339 nm), and/or one additional SH-compound (UV_{max}: 343 nm), – are formed also as OPA/ET derivatives (Tables 7 and 8 and Fig. 9). However – and this is the outstanding advantage of the OPA/ET derivatization – these transformation processes are quite negligible: in particular in a medium of high alcohol content, after short derivatization times of analytical interest (Table 7, derivatives obtained with the ~80% methanol containing OPA/ET reagent).

Histidine is a special exception [12]: its structure readily undergoes intramolecular re-arrangement resulting in the formation of the '–CH_2–NH_2' – moiety containing tautomer. Histidine initially provides two stable derivatives, independently of the reagent's composition it was labeled with [12,18]. Histidine's exceptional tautomer equilibrium cannot be quantitatively avoided, only influenced by the temperature: thus, its amount is to be calculated on the basis of its two derivatives [12,18].

The excellent stability of the OPA/ET derivatives of these very important amino acids, in comparison to all corresponding investigated species (OPA/MCE, OPA/MPA, OPA/NAC), confirms the doubtless requirement of coming back to the OPA/ET derivatization (Fig. 9 and Table 8). This consideration needs the scientific conviction of the community of analytical chemists (involved in the

Table 7
Responses and stabilities of the multiple OPA/ET-derivative providing amino acids as a function of reaction time and the reagents' methanol content, based on UV and fluorescence (FL) detections (OPA/ET = 1/10 reagent; pH = 9.30).

Amino acid ⇓	t_R, min⇓	UV_{max}, nm ⇓	Response, %													
			Fluorescence detection													
Reaction time, min ⇒			1		3		7		23		43		220		360	
Methanol (v/v)% ⇒			80	20	80	20	80	20	80	20	80	20	80	20	80	20
Histidine1	3.25	353	40.80	35.00	42.20	35.20	43.10	33.80	41.40	33.40	41.20	34.10	44.70	32.20	40,40	32,80
Histidine2	3.72	339	59.20	65.00	57.80	64.80	56.90	66.20	58.60	66.60	58.80	65.90	55.30	67.80	59,60	67,20
Integrator unit/pmol			**4.59**	**4.44**	**4.78**	**4.95**	**5.29**	**5.87**	**6.07**	**5.91**	**6.25**	**6.00**	**5.88**	**5.91**	**6.31**	**5.68**
Glycine1	4.28	334	99.60	98.60	99.60	98.40	99.60	97.20	99.60	95.40	99.50	93.20	99.10	81.60	99,00	69,60
Glycine 2	5.63	339	–	1.10	–	1.30	–	2.40	–	3.70	–	5.20	–	10.20	–	15,90
Glycine 3	8.28	343	0.40	0.20	0.40	0.30	0.40	0.40	0.40	0.90	0.50	1.60	0.90	8.20	1,00	14,50
Integrator unit/pmol			**6.30**	**5.90**	**6.25**	**5.97**	**6.27**	**6.00**	**6.26**	**5.92**	**6.30**	**5.90**	**6.16**	**4.71**	**6.18**	**3.87**
β-Alanine1	5.57	334	99.50	98.10	99.60	96.90	99.50	95.80	99.50	94.30	99.30	88.70	98.30	77.60	97,00	69,40
β-Alanine2	9.02	339	0.10	1.80	0.10	3.00	0.10	4.00	0.20	5.20	0.30	10.20	1.20	17.50	2,50	21,90
β-Alanine3	14.15	343	0.40	0.10	0.30	0.10	0.30	0.20	0.30	0.50	0.40	1.20	0.50	4.90	0,50	8,80
Integrator unit/pmol			**6.39**	**6.09**	**6.48**	**6.16**	**6.57**	**6.15**	**6.43**	**6.11**	**6.53**	**5.72**	**6.69**	**4.61**	**6.69**	**3.20**
GABA1[n]	7.23	334	100	98.50	99.90	97.40	100	96.00	99.90	94.90	99.80	88.40	99.00	76.30	98,50	90,30
GABA2	10.87	339	–	1.50	0.10	2.60	–	4.00	0.10	5.10	0.20	11.60	1.00	23.70	1,60	9,70
Integrator unit/pmol			**6.25**	**6.11**	**6.25**	**6.11**	**6.30**	**6.18**	**6.23**	**6.10**	**6.28**	**5.87**	**6.35**	**4.36**	**6.53**	**3.25**
Ornithine1	4.23	334	99.90	99.90	100	99.80	99.90	99.70	99.80	99.60	99.70	99.10	99.50	96.60	99,30	91,20
Ornithine2	6.07	339	–	–	–	0.10	–	0.20	–	0.30	–	0.80	–	3.30	–	8,70
Ornithine3	7.42	343	0.10	0.10	0.0	0.10	0.10	0.10	0.20	0.10	0.30	0.10	0.50	0.10	0,80	0,10
Integrator unit/pmol			**3.15**	**2.84**	**3.34**	**3.18**	**3.41**	**3.26**	**3.53**	**3.33**	**3.55**	**3.20**	**3.57**	**2.41**	**3.61**	**1.87**
Lysine1	4.65	334	99.90	99.60	9.80	99.70	99.90	99.40	99.80	98.80	99.80	97.30	99.20	90.50	98,50	86,00
Lysine2	6.97	339	–	0.37	–	0.27	–	0.58	–	1.17	–	2.18	0.20	7.87	0,40	11,50
Lysine3	8.25	343	0.10	0.03	0.20	0.03	0.10	0.02	0.20	0.03	0.20	0.52	0.60	1.63	1,1	2,50
Integrator unit/pmol			**5.07**	**5.02**	**5.39**	**5.13**	**5.62**	**5.29**	**5.61**	**5.28**	**5.66**	**4.84**	**5.67**	**3.00**	**5.75**	**2.12**
			UV													
Histidine1			32.60	29.80	33.20	28.00	37.00	26.40	33.10	27.60	32.40	27.30	37.30	26.90	32.60	26.70
Histidine2			67.40	70.20	66.80	72.00	63.00	73.60	66.90	72.40	67.60	72.70	62.70	73.10	67.40	73.30
Integrator unit/pmol			**0.17**	**0.16**	**0.18**	**016**	**0.20**	**0.20**	**0.22**	**0.21**	**0.23**	**0.21**	**0.23**	**0.20**	**0.22**	**0.19**
Glycine1			9.70	99.50	99.60	99.30	99.50	98.60	99.50	97.40	99.40	95.20	99.10	83.50	98.80	71.90
Glycine2			–	0.30	–	0.40	–	1.00	–	2.00	–	2.70	–	5.70	–	9.10
Glycine3			0.30	0.20	0.40	0.30	0.50	0.40	0.50	0.60	0.60	2.10	0.90	10.80	1.20	19.00
Integrator unit/pmol			**0.39**	**0.37**	**0.39**	**0.37**	**0.39**	**0.38**	**0.39**	**0.37**	**0.39**	**0.36**	**0.38**	**0.29**	**0.38**	**0.23**
β-Alanine1			99.50	98.30	99.60	97.50	9.40	96.70	99.60	95.30	99.40	–	98.40	79.10	97.50	69.60
β-Alanine2			–	1.70	0.10	2.50	0.20	3.40	0.10	4.30	0.20	–	0.90	14.80	1.90	19.00
β-Alanine3			0.50	–	0.30	–	0.40	–	0.30	0.40	0.40	–	0.70	6.10	0.60	11.40
Integrator unit/pmol			**0.39**	**0.38**	**0.40**	**0.37**	**0.40**	**0.37**	**0.39**	**0.37**	**0.40**	–	**0.39**	**0.27**	**0.39**	**0.18**
GABA1			100	99.10	99.90	98.20	100	97.20	99.90	96.20	99.90	91.30	99.50	81.70	98.90	92.80
GABA2			0.0	0.90	0.10	1.80	0.0	2.80	0.10	3.80	0.10	8.70	0.50	18.30	1.10	7.20
Integrator unit/pmol			**0.39**	**0.37**	**0.39**	**0.37**	**0.39**	**0.37**	**0.39**	**0.37**	**0.39**	**0.35**	**0.39**	**0.25**	**0.39**	**0.20**
Ornithine1			100	99.90	100	99.80	99.90	99.70	99.90	99.60	99.90	98.80	9.60	96.40	99.40	92.50
Ornithine2			–	0.05	–	0.15	–	0.20	–	0.30	–	0.70	–	2.40	–	6.45
Ornithine3			0.0	0.05	0.00	0.05	0.10	0.05	0.10	0.10	0.10	0.50	0.40	1.20	0.60	1.05
Integrator unit/pmol			**0.66**	**0.61**	**0.70**	**0.66**	**0.71**	**0.68**	**0.73**	**0.69**	**0.74**	**0.67**	**0.75**	**0.49**	**0.75**	**0.37**
Lysine1			99.90	–	99.80	99.50	9.90	98.70	99.80	97.30	98.00	–	99.30	83.30	98.60	72.10
Lysine2			–	–	–	0.34	–	0.75	–	1.20	–	–	0.10	6.40	0.20	9.20
Lysine3			0.10	–	0.20	0.16	0.10	0.55	0.20	1.50	0.20	–	0.60	10.30	1.20	18.70
Integrator unit/pmol			**0.50**	–	**0.53**	–	**0.55**	**0.53**	**0.54**	**0.53**	**0.56**	–	**0.56**	**0.35**	**0.56**	**0.25**

Indications as in Tables 1–6 as well as: Response, % = based on the total of derivatives; GABA = γ-aminobutyric acid; – = derivative was not formed; [OPA]/[ET]/[amino acid] = 70/700/1; 1 = 1×10^{-9} mol/10 μl. Isocratic elution conditions: 50 °C, 1 ml/min; for histidine, glycine, β-alanine and GABA: 30 % eluent A + 70 % eluent B. For ornithine and lysine: 70 % eluent B + 30 % acetonitrile.

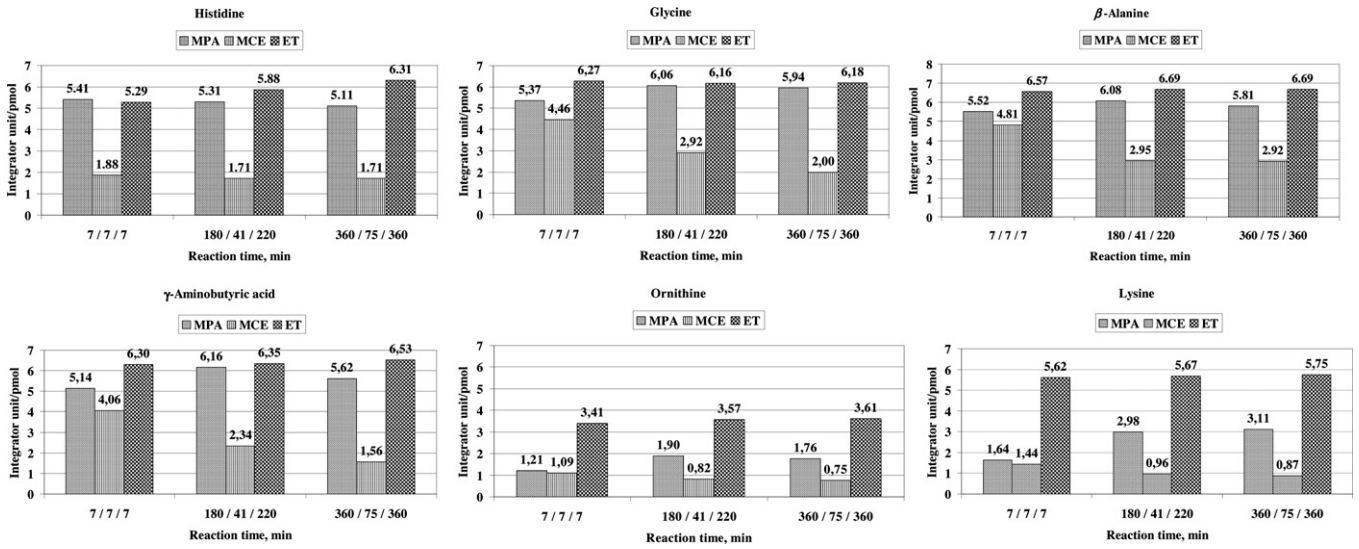

Fig. 9. Comparison of the fluorescence response values of the multiple derivative providing amino acids as a function of the *o*-phthalaldehyde reagent's SH-additive and the reaction time. With permission from Ref. [18].

analysis of amino acids and amines) to tolerate the inconvenience of ethanethiol's odour.

An additional advantage of the OPA/ET derivatization is worth emphasizing: that the responses of the OPA/ET derivatives are calculated primarily from a single product (Tables 7 and 8 and Fig. 9) while the quite comparable responses and stabilities of the OPA/MPA and OPA/NAC derivatives are obtained on the basis of two or three species [3–19].

Table 8
Comparison of responses and stabilities of alanine and lysine derivatives, as a function of the *o*-phthalaldehyde reagent's SH-additives, under the same conditions: OPA/ET = 1/3 reagent, 80% (v/v) methanol content; pH = 9.30; [OPA]/[amino acid] = 40/1, 1 = 1×10^{-9} M.

Amino acid⇓	t_R, min⇓	UV_{max}, nm⇓	Response, %					
			Fluorescence detection			UV detection		
o-phthalaldehyde/3-mercaptopropionic acid								
Reaction time, min⇒			7	47	307	7	47	307
Alanine	10.82	334	100	100	100	100	100	100
Integrator unit/pmol			**4.35**	**4.33**	**4.08**	**0.36**	**0.36**	**0.34**
Lysine1	10.95	343	2.93	3.27	13.28	0.10	1.21	5.72
Lysine2	11.43	334	88.46	84.61	54.50	93.35	89.88	76.34
Lysine3	11.55	339	8.61	12.11	32.22	6.55	8.91	17.94
Integrator unit/pmol			**1.81**	**1.82**	**2.17**	**0.72**	**0.73**	**0.71**
o-phthalaldehyde/N-acetyl-L-cysteine								
Reaction time, min→			7	47	267	7	47	267
Alanine	10.77	334	100	100	100	100	100	100
Integrator unit/pmol			**3.66**	**3.66**	**3.67**	**0.39**	**0.39**	**0.41**
Lysine1	11.22	334	97.15	95.09	87.30	99.78	99.21	95.83
Lysine2	11.33	339	2.85	4.91	12.70	0.22	0.79	4.17
Integrator unit/pmol			**1.52**	**1.54**	**1.55**	**0.77**	**0.75**	**0.75**
o-phthalaldehyde/2-mercaptoethanol								
Reaction time, min→			7	47	220	7	47	220
Alanine	11.53	334	100	100	100	100	100	100
Integrator unit/pmol			**4.60**	**4.32**	**3.31**	**0.34**	**0.32**	**0.24**
Lysine1	12.20	339	0.69	1.37	3.99	5.42	9.88	20.37
Lysine2	12.30	334	0.81	0.94	3.42	5.08	5.71	14.50
Lysine3	12.63	339	0.16	0.48	1.70	0.09	0.17	0.65
Lysine4	12.80	334	94.44	93.53	87.16	87.00	81.57	60.37
Lysine5	13.02	343	3.90	3.69	3.74	2.41	2.68	4.11
Integrator unit/pmol			**2.58**	**2.35**	**1.53**	**0.63**	**0.60**	**0.48**
o-phthalaldehyde/ethanethiol								
Reaction time, min→			7	47	180	7	47	180
Alanine	12.12	334	100	100	100	100	100	100
Integrator unit/pmol			**5.29**	**5.27**	**5.28**	**0.35**	**0.36**	**0.35**
Lysine1	14.28	334	100	100	100	100	100	100
Integrator unit/pmol			**5.98**	**5.94**	**5.72**	**0.69**	**0.70**	**0.69**
o-phthalaldehyde/2-mercaptoethanesulfonic acid								
Reaction time, min→			7	47	140	7	47	140
Alanine	10.88	334	100	100	100	100	100	–
Integrator unit/pmol			**2.77**	**2.72**	**2.71**	**0.39**	**0.40**	
Lysine1	11.43	334	100	100	100	100	100	–
Integrator unit/pmol			**1.29**	**1.29**	**1.30**	**0.78**	**0.79**	

Indications as in Tables 1–7, as well as: elution conditions, 50 °C, 1 ml/min; gradient: 0 min: 100 % eluent A; 10–11 min: 100 % acetonitrile; 12–20 min: 100 % eluent A.

1.5.3. Comparative studies: impact on the stability of the alanine and lysine derivatives depending on the composition of OPA-thiol reagents

Throughout our recent investigations with the OPA/MPA, with the OPA/NAC, with the OPA/MCE and with the OPA/ET reagents [3–17] their methanol contents were 20% (v/v) uniformly. Since the responses of the OPA/ET amino acid and biogenic amine derivatives proved to be optimum when the reagent of ~80% (v/v) methanol content was applied [17–19], the comparative studies were carried out with all reagents of 80% (v/v) methanol content.

In order to present unquestionably comparable response values, as a function of the OPA reagent's composition, alanine and lysine {as representatives of the single (alanine) and multiple (lysine) OPA derivative providing amino acids} have been selected.

(a) And, investigated strictly under the same experimental conditions, applying the OPA/MPA, the OPA/NAC, the OPA/MCE, the OPA/ET and the OPA/2-mercaptoethanesulfonic acid (MESNA) reagents, in the molar ratios of OPA/[SH-additive] = 1/3, with 80% (v/v) methanol content, at pH = 9.30, by means of the same instrument.
(b) Derivatives have been eluted in two consecutive days, continuously (Table 8). Results revealed repeatedly and unambiguously, that significant differences are associated with the OPA reagent's SH additive, also in accordance with earlier observations [2–16], as follows:
(c) Responses based on the fluorescence intensities of the OPA/ET derivatives both in cases of alanine and lysine provide maximum values expressed in integrator units/pmol (I.u./pmol); with an exceptional high fluorescence intensity for lysine (5.98 I.u./pmol compared to the corresponding lysine derivatives from 1.29 up to 2.58 I.u./pmol), as well as, with the similarly outstanding fluorescence intensity of the OPA/ET derivative of ornithine 3.41 I.u./pmol (Table 6) compared both to the OPA/MPA and to the OPA/MCE ornithines (Fig. 9: in order of listing, 1.21 and 1.09 I.u./pmol).

The generally decreasing trend of fluorescence intensities in cases of the single (alanine) and multiple (lysine) derivatives providing amino acids proved to be unambiguously confirmed.

Alanine: OPA/ET (5.29) > OPA/MCE (4.60) > OPA/MPA (4.35) > OPA/NAC (3.56) > OPA/MESNA (2.77);
Lysine: OPA–ET (5.98) > OPA/MCE (2.58) > OPA/MPA (1.81) > OPA/NAC (1.52) > OPA/MESNA (1.29).

Very likely, the above shown trend might be in correlation with the different sterical and free functional group characteristics of the isoindole's thiol component. The shorter and the 'more neutral' the SH additive, the higher the stability and the more stable is the fluorescence intensity of the derivative in question.

Response values based on the UV absorbencies of various alanine and lysine derivatives – within the experimental error of our measurements – proved to be the same, and, proportional to the number of isoindole entities [57,58].

To explain the insertion of the study of the OPA/MESNA derivatives into this paragraph, it is to be known that these derivatives have been published as five times higher responses providing ones compared to their OPA/MCE counterparts [73]: in light of our results OPA-MESNA derivatives manifest the lowest responses of all five investigated ones.

1.6. The special behavior of histidine

The irregular behavior of histidine in its reaction with the OPA reagents has been also clarified [12]. Histidine provides more than one OPA derivative. Similarly to all those primary amino group-containing compounds that do have in their initial structure the $-CH_2-NH_2$ moiety (Table 5). The ratio of histidine's initially formed and transformed OPA derivatives depends on the temperature: very likely due to the fact that elevated temperature favors the intramolecular rearrangement of histidine resulting in the formation of the $-CH_2-NH_2$ moiety containing tautomer(s). The higher the temperature the higher the amount of species transformed. The composition of the initially and transformed OPA derivatives of histidine were identified on the basis of their on-line HPLC-MS(ESI) spectra applying selective ion monitoring and computations [12]. The initially formed species has been identified as the classical isoindole, while the transformed one contains an additional OPA molecule. Stoichiometric and MS studies confirmed:

(i) that the transformation of the initially formed OPA derivatives of histidine proved to be associated with its virtual tautomerization leaded to the species containing the $-CH_2-NH_2$ moiety. that reacts with the second OPA molecule.
(ii) The ratio of its initially formed and transformed OPA derivatives depends on the temperature, but independent on the composition of the OPA reagents: likely due to the fact that elevated temperature favors the formation of the $-CH_2-NH_2$ moiety containing HIS-tautomers.
(iii) The behavior of 3-methylhistidine (MHIS) – in its initial structure unable to tautomerization – furnishing unambiguously single and stable OPA derivative, serves as completing evidence to the mechanism of the OPA derivatization of HIS.
(iv) The composition of its initially obtained and transformed derivatives was proved on the basis of their MS spectra, confirmed by the possible reaction pathway they are originating from, applying computation.
(v) From analytical point of view it proved to be unambiguous that the consequences of virtual tautomerization cannot be avoided: consequently, in order to obtain reliable and reproducible histidine values, its content must be calculated on the basis of the total of their derivatives.

1.7. Studies on derivatization of 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 [1]: the oxidation of the secondary amino group with sodium hypochlorite or chloraminT, prior to the reaction of AAs with the OPA reagents. Japanese researchers [66,67] elaborated the practical details of this derivatization technique of two steps. Later on, an other, two steps requiring derivatization method was patented [68] and introduced in the HPLC practice [69], based on the reaction of the secondary amino group with FMOC subsequently to the reaction of the primary AAs with the OPA/MPA reagent. Recently, a special OPA/clornitrobenzofurazan (NBF) reagent was suggested to derivatize proline and OH-proline for their HPLC separation [36]. Unfortunately, the OPA/NBF reagent does not react with the primary amino group, consequently two separate reactions (OPA/MCE and OPA/NBF) and elutions had to be performed [36].

1.7.1. The two-step method: oxidation of the secondary amino group by hypochlorite prior to the reaction of AAs with the OPA reagent

The two step-method has been elaborated [45], improved [46,66,67] and patented also [70] in the postcolumn mode. Involving all of disadvantages belonging to the post-column techniques, i.e., the need of extra pumps, temperature controllable reaction

Table 9
Derivatization and chromatographic conditions of the reaction of amino acids with 9-fluorenylmethoxycarbonyl chloride: literature data.

Condition of derivatization						Elimination of reagent's excess (mM)*:	Tyr	His	No/min	Reagent blank	Ref.
Buffer			Solvent: (v/v)	FMOC: mM*; [FMOC]/[AA]^T	Time: sec						
M*	Salt	pH									
0.1	b	7.7	AC:W = 1:1	**7.5**; 31.25/1	30–40	extr: $C_5H_{12;}$ ⇒ ac	m	d	20/20	–	[2]
0.1	b	7.7–8.0	AC:ACN:W = 1:1:2	**7.5**; 37.5/1	40	extr: $C_5H_{12;}$ ⇒ ac	m	–	21/92	–	[74]
0.1	b	6.0–8.5	AC:W = 1:1	**3.75–30**; 15.6–125/1	15–120	extr: C_5H_{12}	m	–	19/54	–	[75]
0.1	b	7.7	AC:W = 1:1	**7.5**; 31.25/1	40	extr: C_5H_{12}	–	–	12/50	–	[76]
0.01	b	7.7	AC:W = 1:1	**0.5**; 11.4/1	45	ADAM (20)	m+d	m+d	19 + 2/30	–	[77]
0.05	b	9.5	THF:W = 1:1	**3.9**; 1.54/1	240^f	–	–	–	9/24	–	[78]
0.05	c	8.0	AC:W = 1:1	**2**; 16/1	600	extr: $C_5H_{12;}$EtAc = 90:10^v	m+d	d	20+1/35	–	[79]
0.1	b	7.85	AC:ACN:W = 1:1:2	**2**; 20/1	600	extr: C_5H_{12}	m+d	d	18 + 1/40	–	[80]
0.05	b or c	8.0	AC:W = 1:1	**3**; 7.2/1	60	extr: C_5H_{12} or, ADAM (30)	m+d	m+d	20 + 2/45	–	[81]
0.1	b	8.5	ACN:W = 1:1	**8**; 16.7/1	90	NH_2OH + NaOH (50)	m+d	m+d	30/30	–	[82,83]
0.1	b	7.7	AC:W = 1:1	**7.5**; 2.3/1	40	extr: C_6H_{14}	m	–	13/25	–	[84]
0.1	b	8.5	ACN:W = 1:1	**8**; 16.7/1	90	NH_2OH + NaOH (50)	m	m	15/30	–	[85]
0.11	b	8.5	AC:W = 2:2,5	**1.33**; 5/1	180	HEPA (10.5)	m	d	24/75#	–	[86]
0.18	b	8.0	AC:W = 5:6	**1.36**; 97/1	120	ADAM (12.5)	m+d	d	16 + 1/45	–	[87]
0.05	b	8/11.4	AC:W = 1:1	**3**; 75/1	2–40 min	extr: C_5H_{12}	m+d	m+d	19 + 2/40	+	[88]
0.125	b	8.8	ACN:W = 1:1	**7.75**; 18/1	90	NH_2OH+NaOH (33)	m	m	17/35	–	[89]
0.166	b	8.0	AC:W = 1:2	**0.33–4**; 1; 5.5–66.6/1	45–300	ADAM (3)	m	d	16/50	+	[90]
0.16	b	9.9	ACN:W = 2:3	**4**; 4/1	60	extr. C_7H_{16}	m	m+d	25 + 1/80	–	[91]
0.08	b	10.0	ACN:W = 2:3	**6**; 24–120/1	300	ADAM (86)	m	d	16/40	+	[92]
0.035	b	9.0	ACN:W = 4.5:5.5	**1.9**; 1/1	60	cysteic acid (4.7)	m	–	18/80	–	[93]
0.325	c	10.2	AC:W = 1:1	**1.7****; 72/1	1200	cc.HCl (460)	m	m	25/32#	+	[94]
0.125	b	8.5	ACN:W = 1:1	**7.75**; 69/1	90	NH_2OH+NaOH (30)	m	m	16/43	–	[95]
0.143	c	10.2	AC:W = 1.15:1	**2.67**; 31.4/1	600&	cc.HCl (357)	m	d	27/43#	–	[96]
0.1	b	8.0/9.0	ACN:W = 1:1; AC:W = 1:1	**0.25–5**; 2.75–55:1	1–20 min	ADAM (6.2) or HEPA (7.8)	m+d	m+d	22 + 2/40	+	[20]

Indications: AA = amino acid; W = water; AC = acetone; ACN = acetonitrile; ac = acidified with 10 μL glacial acetic acid; ADAM = adamantanamine. HCl; HEPA = heptyl amine; *Reagent, buffer and quenching agents concentrations were related to the total volume of the reaction mixture; 90:10^v = (9:10, v/v); No/min = number of compounds/elution time, in min; m = 'mono-derivative' (*N*-FMOC-histidine, *N*-FMOC-tyrosine); d = 'di-derivative' (*N,NH*-FMOC-histidine, *N,O*-FMOC-tyrosine); 240^f = drop wise; b = borate; c = carbonate; # = amino acids and amines, in total; ** = N-(9-fluorenylmethoxycarbonyl-oxy)succinimide; & = at 40 °C temperature.

coils resulting in high cost, increased time consume and decreased sensitivity. Instead of OPA/MCE-oxidized proline [66,67,70], providing low fluorescence the introduction of the OPA/NAC derivative was an advancement being as stable and as sensitive as all other OPA/NAC-AAs [45,46]. Except selected cases which inevitably need simultaneous quantitation of the primary and secondary AAs, because of the above detailed disadvantages, the hypochlorite oxidation has not gained wide acceptance.

1.7.2. Double pre-column derivatization with the OPA/MPA/FMOC reagent

The first introduction of the OPA/MPA/FMOC derivatization was patented [68] and practically described [69] in the automatic version. This pioneer work [69] contained several chromatographic proposals: the quantitation of 17 primary AAs + proline within 12 min, 28 primary AAs + OH-proline and proline within 20 min, as well as 36 primary AAs + OH-proline 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 AAs of various special matrices under optimum conditions: from bovine serum albumin, wort, beer and plants with 12 min, from plasma deprotonized by acetonitrile with 20 min and by sulfosalycylic acid with 40 min run times.

The AA contents of β-lactoglobulin and peptides, including proline, have been determined by HPLC and identified by MS, in the 10–100 pM range within 12 min [70]. Further improvements have been published [51,53,71] for the quantitation AAs in plasma [51], in potato tuber (20 AAs/8 min) [71] and 42 AAs in urine [53].

1.8. Advances in the HPLC quantitation of AAs as OPA derivatives

The separation/identification possibilities of 25 AAs as their OPA/MPA and OPA/NAC derivatives have been optimized (with particular attention to those AAs which are eluting with more than one derivative (glycine, histidine, GABA, β-alanine, ornithine, lysine), and, expected to be present in natural matrices, such as apples and in free forms). Optimum separation conditions have been reported on six RP test column (T1–T6; detailed data in paper [4]).

In the knowledge of the outstanding stability of the OPA/ET derivatives, in author's practice, the double pre-column derivatization with the OPA/ET/FMOC reagent [17,18], was preferred in comparison to the OPA/MPA/FMOC one [9,10].

2. HPLC of amino acids as 9-fluorenylmethyloxycarbonyl derivatives

2.1. Literature overview

2.1.1. The state of the art in applying the FMOC reagent to the analysis of AAs

The specific phenomena of OPA derivatization have been clarified in papers [3–19] including the 70th volume of the Journal of Chromatography Library [16].

To clear up the contradictory propositions of FMOC derivatizations, described in the literature (Table 9, Refs. [74–96]) it seemed to be worth to evaluate and to criticize them, also on the basis of our self experiences (Table 9, as well as data in the last horizontal line and in Sections 2.1.1–2.1.6 and 2.2.1–2.1.7 of this paper).

Being authors of paper [20,21] committed to the quantitative aspects of chromatographic analyses, therefore, the suggested conditions [74–96] to be comparable, have been calculated on a unified basis (relying on data, given in the papers): the reagent, the buffer and the quenching agent's concentrations were related to the total volume of the derivatization media (Table 9, values indicated by asterisk*). Described conditions [74–96] have been evaluated, one by one, in order of listing, in Sections 2.1.1–2.1.6.

Table 10
Apparent (pH of the buffer used) and effective pH values (pH of the reaction mixtures) as a function of the pH of the buffer and the organic solvent of the reagent, and the reaction time.

pH of buffer	Solvent of the FMOC reagent	pH of the reaction mixture*	Increment of pH	%, RSD*
8.0	Acetone	10.1	2.1	0.46
	Acetonitrile	9.8	1.8	0.43
9.0	Acetone	10.8	1.8	0.54
	Acetonitrile	10.6	1.6	0.43

* Indications: averages of the pH values measured in the mixtures of at least three reagent blanks and three derivatization mixtures of amino acids, immediately after mixing and after 40 min reaction times, it means the averages of 12 pH measurements, performed on the same day.

2.1.2. Buffer and pH

In the overwhelming part of reports borate buffer was used, in a wide concentration range: from 0.01 M [77] to 0.325 M [94] and from pH 6 [75] to pH 11.4 [88]. The optimum pH capacity of borate buffer can be expected at pH 10.2. However, performing derivatizations in reaction media of pH ≥ 10 would be an unfavorable selection, being associated with several disadvantages, thoroughly correlated with each others, such as:

(1) increased hydrolysis rate of the reagent,
(2) the need of high FMOC concentration to ensure its excess,
(3) the appearance of a huge FMOC-OH peak, consequently,
(4) raising difficulties in the evaluation of the neighboring amino acid derivatives (the number and identity of the coeluting species are depending on the gradient applied). As to the selected favorite buffer-pH values, found in the literature, they proved to be between pH 6 [75] and pH 11.4 [88], with a calculated average of pH 8.5.

As to the actual pH of the reaction mixture, where the derivatization reactions are effectively going on, for the time being, has not been discussed: however, concerning the special composition and the transformation processes of the reaction mixtures, the pH changes might be ≥2 pH units (Section 2.2.2, Table 10).

2.1.3. The solvent of the FMOC reagent

As to the preparation of the FMOC stock solution, according to the descriptions, in order of listing, acetone and acetonitrile (ACN) were preferred with one exception [78], using as solvent tetrahydrofuran (THF). This means, that out of 24 proposals [2,74–96] in 13 acetone [2,75–77,79,81,84,86–88,90,94,96], in 7 ACN [82,85,89,91–93,95], in 2 acetone:ACN = (1:1, v/v) [74,80] were applied to dissolve FMOC. The advantage of acetone over ACN can be attributed to its higher polarity resulting in the increased reaction rate of the amine groups with the reagent: in particular in the cases of aspartic and glutamic acids. However, the drawback, working with the acetone containing reaction media, proved to be a huge impurity product, which emerges between the FMOC-OH and the excess of FMOC and/or its derivatives. This huge impurity, together with smaller ones, has been indicated also earlier [88], however as a tentative observation only. The huge impurity product, in the understanding of the authors of this paper renders acetone less suitable for this purpose (additional disadvantages of the use of acetone are shown in Sections 2.2.3, 2.2.5 and 2.3.8).

2.1.4. The issue of the blank measurement

After an exhaustive literature overview it is hard to accept the low limit of quantitation (LOQ) values, even at the low pmol level, without reporting anything about the reagent blank runs, i.e., how impurities have been taken into account (if at all). The existence of

impurities was observed only in few papers [88,90,92,94], without their numerical characterization. Remarks were limited:

(1) to the range they are eluting [88],
(2) to their presence in general, influencing the detection limit of the procedure [90],
(3) to the presentation of a blank run showing three impurities, without commenting them [92], and,
(4) by stating that "...many unknown peaks appeared in the area of polyamines..." [94], it means (present authors' remark) after the FMOC-OH species. Thus, the importance of the quantitation of impurities was simply neglected.

In our practice several impurity peaks were found coeluting with amino acids throughout the run: certainly in spite of the fact that HPLC purity reagents and ultra clean glass wares {cleaned by cc. H_2SO_4/30% H_2O_2 = (2:1, v/v)} were used. We are convinced that the knowledge of the amounts of impurities is of primary importance, in particular in those cases, when trace amounts of amino acids are to be determined in a reliable and reproducible manner.

2.1.5. Evaluation of the quantitative aspect of the derivatization protocols

To comment whether the cited derivatization reactions, – reflecting extremely different and contradictory conditions, – might lead to quantitative reactions, yes or not, will be estimated partly on the basis of the unified literature data (Section 2.1, Table 9), partly on present author's self experiences ([20,21]; Section 2.2).

Evaluating literature proposals, in terms of their quantitative manner, it turns out, that

(1) FMOC concentrations, expressed in mM, related to the total volume of the reaction media proved to be satisfactory in all cases cited.
(2) The molar ratios of reactants, might lead all, depending primarily on the reaction time applied, to quantitative derivatization; presumably with a single exception, where no FMOC excess was applied ($[FMOC]/[AAs]^T = 1/1$ [93]). *Note*: the superscript 'T' means the total of amino acids.
(3) As to the inadequacy of the selected reaction times, in particular regarding the quantitative derivatizations of aspartic and glutamic acids, asparagine, glutamine, serine, and threonine, to evaluate them from paper to paper, unambiguously, is quite critical. However, it can be stated, it would need at least 5 min, even applying acetone containing medium, with an FMOC concentration of $\geq$0.5 mM, and with the molar ratios of the reactants like ($[FMOC]/[AAs]^T \geq 5.5/1$.
(4) Elimination of the disturbing reagent peaks, according to the pioneer works [2,74], was carried out by extracting the reaction mixture with C_5H_{12}, this procedure was applied also later on [75,76,79–81,88].

Because of the considerable loss (25–50%) of the derivatized amino acids (histidine, ornithine, lysine being partly extracted into the organic phase), instead of C_5H_{12}, as extracting agents, also the mixture of C_5H_{12}/ethyl acetate = (9:1, v/v) [79], or C_6H_{14} [84], or C_7H_{16} [91] have been suggested. Although, quantitative separation of the amino acid derivatives, by extractions, could not be achieved.

The reaction of the unused FMOC with excess adamantanamine (ADAM) [77,81,87,90,92], or by heptylamine [86], also in accordance with our self experiences, proved to be an excellent alternative to remove reagent's surplus, resulting in the spectacular decrease of the reagent peak, without any loss of the FMOC amino acids.

2.1.6. The issue of histidine and tyrosine

The registration of the presence of the one FMOC group (*N*-FMOC-histidine, *N*-FMOC-tyrosine, further on: His-1, Tyr-1) and those of the two FMOC groups containing (*N,NH*-FMOC-histidine, *N,O*-FMOC-tyrosine, further on: His-2, Tyr-2) species, was treated randomly and not consistently. The reasons for that might be attributed primarily to the shortage of a gradient program suitable for the acceptable separation of these four species.

This means, it was not preferred to separate His-1, His-2, Tyr-1, and Tyr-2 from their corresponding neighbors, with four exceptions [77,81,82,88]. In two papers [77,81] their separation was shown only without any comment on them.

Excellent separations of 21 FMOC derivatives {19 + 2 (His-2 + Tyr-2)}, 10 pmol of each [77] and 22 FMOC derivatives {20 + 2 (His-2 + Tyr-2)}, 1 nmol of each [81] were demonstrated, without further details on their distribution [77,81].

The examination on the formation and transformation of His-1, His-2, Tyr-1 and Tyr-2 were discussed, in a qualitative manner, only [82,88] and with different conceptions. Namely,

(1) in reactions carried out at pH between 10 and 12 (prepared by hydroxylamine and sodium hydroxide), applying 1 min reaction times [82], His-1 and Tyr-1 proved to be the major products. Under these conditions His-1 provides an approximately 2.5 times higher peak, in comparison to His-2, while Tyr-2 furnished 2.3 times higher product than Tyr-1.
(2) Derivatizations performed at pH 8 and at pH 11 [88], the behavior of histidines and tyrosines were characterized at two time points, after 2 min and 40 min reaction times, providing the following conclusions:

(a) Histidine
At pH 8, after 2 min, His-2 predominates, His-1 might be the twentieth part of His-2. After 40 min, His-1 disappears and also the area of His-2 decreases.
At pH 11, after 2 min and 40 min, the total of peak areas of His-1 + His-2 seem to be the same, while their area ratios changed by increased reaction time: from His-1/His-2 $\approx$ 1/1 (2 min) for His-1/His-2 $\approx$ 8/1 (40 min).
(b) Tyrosine
At pH 8, after 2 min, the area ratio of Tyr-1/Tyr-2 was $\approx$2/1, while after 40 min it became changed for Tyr-1/Tyr-2 $\approx$ 1/4.

At pH 11, after 2 min and 40 min, Tyr-2 was present, only, after both reaction times with identical areas.

Despite the authors' presumption [82,88], the formation of His-2 and Tyr-2 could not be completely formed back into His-1 and Tyr-1 by increasing the pH to 10–12 [82] and 11 [88], respectively.

On the basis of all uncertainties and contradictions reported in the literature [74–96], including the pioneer work [2], the following tasks had to be solved:

(1) to examine and to explain the reasons and consequences of contradictory literature proposals,
(2) to demonstrate the importance and the extent of the reagent blank runs,
(3) to call attention to the apparent and to the effective pH of reaction mixtures where derivatizations are really going on,
(4) to clarify the behavior and characteristics of the FMOC derivatives of AAs under strictly the same, well defined conditions, to be comparable, varying the pH, the organic solvent content, the FMOC concentration and the molar ratios of the reactants, one by one, as well as,
(5) to define the optimum derivatization conditions which ensure the unambiguous quantification even of the less reactive amino

acids, such as aspartic and glutamic acids, and the more than one derivative providing histidine and tyrosine, in a reliable and reproducible manner.

2.2. Recent experiences in author's laboratory

2.2.1. The elution shape of the FMOC amino acids and the reagent blank

The introductory task was to develop an optimized gradient elution suitable for the separation of 22 FMOC amino acids, focusing on the baseline resolution of His-2, Tyr-1, and Tyr-2 from their corresponding neighbors; it is to be noted, that under our optimum derivatization conditions His-1 is quantitatively transformed into His-2, thus, its elution time is only indicated (Fig. 10). FMOC amino acids, denoted by asterisks) do co elute with impurities; the dashed line represents the reagent impurities (blank optimization studies in Section 2.2.3).

2.2.2. The pH phenomena

Before going into details, it seems to be worth to note that no comment could be found in the literature on the apparent and effective pH of reaction mixtures.

According to our introductory experiences carried out with buffers of pH 8 and pH 9, we realized that all those pH values declared in proposals on the basis of the buffer pH applied, without exception, are to be regarded as apparent pH values. What does it mean? It means that the effective pH of reactions should be distinguished from the apparent ones. In our experiences, working under strictly the same experimental conditions, both at pH 8 and at pH 9, after mixing the reactants, the pH of the reaction mixture increased considerably (Table 10).

These pH changes are independent whether they have been measured in the mixtures of the reagent blank or in those ones containing the derivatized amino acids (Table 10), and proved to be reproducible, characterized with their relative standard deviation percentages ($\leq$0.54%, RSD).

These pH changes occur immediately after mixing and proved to be constant, thereafter at any time (shown after 40 min), before the addition of the quenching solution of ADAM or heptyl amine (HEPA).

In the light of our experiences it can be concluded that:

(1) the lower the apparent pH the larger the increment of pH changes occur in the reaction mixtures, as well as,
(2) in acetone containing media the increments proved to be by 0.2–0.3 pH units higher (at pH 8: 2.1, at pH 9: 1.8 pH units) compared to the ACN containing ones (at pH 8: 1.8, at pH 9: 1.6 units).

2.2.3. Blank value depending on the composition of the reagents as a function of time

Concerning literature data, the issue of the reagent's impurities, have been in the overwhelming part of proposals neglected, with few exceptions [88,90,92,94], without detailed comments (Section 2.1.3).

According to our working strategy we are convinced, – that in particular in those cases when a constant reagent concentration is applied, and the analyte contains also amino acids to be measured in the low pmol level, – the knowledge of the impurities in the reagent blank is obligatory. Consequently, in course of our derivatization studies, with all conditions tested blank runs have been carried out, in parallel. Impurity values, – determined as a function of the pH (pH 8, pH 9) and that of the organic solvent content of reagents (acetone, ACN), – were expressed in pmol values and indicated by the name of the coeluting amino acids (Table 11).

Evaluating data compiled in Table 11, it turns out that:

(1) the total amount of impurities (except FMOC-OH), found in a single run, does not vary considerably, in spite of the fact that the UV absorbency and the fluorescence intensity of the different impurities are considerably different (Table 11, UV, FL data in the last horizontal line).
(2) The major difference can be attributed to the huge amount of impurity, detected in the acetone containing reagents, only (t_R, 30.03 min, 96.7–110 pmol/injections), calculated with the response of the neighbor ornithine {Fig. 11 and Table 12, 185 integrator units (I.u.)/1 pmol FMOC-ornithine}. The amounts of this impurity were independent on the reaction time, on the pH of the reagent, on the detection applied, and on the producer of the HPLC grade acetone. Consequently, the total amounts of impurities proved to be about three times higher with acetone containing reagents compared to those obtained with the ACN containing ones. The additional disadvantage of this impurity can be attributed to the fact that it proved to be inseparable from Tyr-2.
(3) As to the impact of the FMOC concentration and the time passed after reagent's preparation (ACN-containing reagent, FMOC mM: 0.5–5, reaction time: 1–20 min, fluorescence detected data), it is unambiguous that both the increased FMOC concentrations and the extended times of the reactions, being in expected compromise with each others, and are resulting in increased amounts of impurities.

Concerning optimum derivatization conditions, in its selection, the width of the FMOC-OH peaks (emerging in the middle of the chromatogram: t_R = 21.03 min), served as a key argument. It means the wider the FMOC-OH peak the worse the resolution possibilities of their neighbor FMOC amino acids. As to the extreme width of the FMOC-OH peak, its amounts have been calculated by the average of the UV responses of FMOC amino acids, expressed in pmol (1.41 I.u./1 pmol). These huge FMOC-OH values, might serve as information of its disturbing presence, applying FL detection: being with several order of magnitude larger that the amounts of amino acids to be determined (pmol/injections, or less). Thus, it is obvious that the smallest FMOC-OH peak, associated with quantitative derivatization should be the starting point to find the best compromise of optimum derivatization conditions (Table 11). Regarding the peak shape and the width of the FMOC-OH peak, in accordance with the literature [90], it could be considerably improved and reduced, by adding the quenching solutions. Out of ADAM and HEPA, in our experience both proved to be identically beneficial. However, we preferred the use of ADAM: because its quantitative reaction with the excess FMOC needed 1 min [77] in comparison to HEPA which required 3 min [86]. *Note*: since all of our test derivatizations have been followed with simultaneous UV(DAD)-FL detection, the responses obtained with UV detection, proved to be well utilizable: in the estimation of the amounts of the FMOC-OH species (detailed above) and on the comparison of the response ratios obtained with FL and UV detections (Section 2.2.4 and Fig. 11).

2.2.4. Comparison of the ultraviolet (UV) and FL responses of the FMOC amino acids

The outstanding intensity of the FL detection in comparison to the ultraviolet UV one still might be of importance. Namely, out of the cited proposals, in 15 cases fluorescence detection, in 7 cases UV detection were used, and, the simultaneous UV/FL detections detection were followed in 4 descriptions [2,77,82,87]. The only conclusion in terms of the response ratios (FL response/UV response = RR) was discussed in paper [82] indicating the RR value = 25.

Table 11
Response values of impurities, calculated with the responses of the coeluting amino acids, expressed in pmol, depending on the pH, on the composition of the reaction medium (acetone, ACN) and on the reaction time (the selected conditions correspond to quantitative derivatizations).

Impurities, coeluting with amino acids	t_R, min	FMOC: mM*										
		0.5								1.5	3	5
		pH 8				pH 9				pH 9, ACN		
		AC, 10 min		ACN, 30 min		AC, 5 min		ACN, 20 min		5 min	3 min	1 min
		UV	FL	UV	FL	UV	FL	UV	FL	FL		
		pmol, expressed in the responses of the coeluting amino acids										
Asp	4.67	0	1.58	0	0.93	0	0.91	0	0.66	1.17	1.37	4.04
Glu	5.58	0	0.67	0	0.59	0	0.32	0	0.22	0.61	0.64	1.40
Hyp	8.98	1.93	0	1.89	0	2.23	0	2.66	0	0	0	0
Asn	9.67	0	0.45	0	0.45	0.50	0.25	0	0.17	0.15	0.32	0.94
Gln	10.17	0	0	0	0	0	0	0	0	0	0	1.10
Ser	10.73	11.29	11.14	11.79	11.14	6.72	6.89	5.99	5.88	11.99	10.84	11.03
Gly	11.72	10.00	8.39	11.20	8.51	5.88	5.66	4.71	4.72	8.97	7.14	9.84
Arg	12.48	1.08	1.93	2.24	2.12	0.66	0.74	0.94	0.95	1.11	1.33	3.67
Ala	13.18	2.70	2.98	2.96	2.82	1.60	1.84	1.57	1.57	3.00	2.56	3.20
Pro	13.62	1.37	2.56	2.21	2.09	1.15	1.70	1.58	2.01	3.30	3.36	8.77
Tyr-1	14.52	1.40	2.01	2.46	3.64	0.28	2.00	5.91	8.78	2.91	6.14	3.60
Val	16.83	2.82	1.75	3.17	1.70	2.42	1.37	2.87	1.57	2.63	3.38	2.36
Ile	18.93	2.97	0.65	3.16	0.72	2.71	0.49	3.50	0.58	0.63	0.52	0.79
Leu	19.33	0.60	0.81	1.33	1.07	0.76	0.61	1.35	0.87	0.84	0.99	1.69
Trp	19.92	3.30	0	5.25	0	0.69	0	0.50	0	0	0	0
Phe	20.23	0.33	0.27	0.14	0.16	0	0	0	0	0	–	–
FMOC-OH^{UV}	21.03	3.4×10^3		2.5×10^3		4.3×10^3		2.8×10^3		5.4×10^3	9.5×10^3	26×10^3
$(Cys)_2$	22.70	0.11	0	0.20	0	0.37	0	0.74	0	0	0	–
Orn	27.22	1.78	2.15	2.13	2.33	1.42	1.63	0.95	1.31	2.34	1.97	3.55
Lys	27.65	1.48	1.27	2.43	3.53	1.05	1.33	3.69	3.45	8.59	17.11	3.86
Impurity**	30.03	96.7	109.3	0	0	110.7	99.1	0	0	0	0	0
Impurities⇒***	140	148	52.6	40.7	139	123	37.0	32.5	44.9	57.7	59.9	

*Indications: FMOC concentration related to the total volume (600 μL) of the reaction medium; AC = acetone:water = (1:1, v/v); ACN = acetonitrile:water = (1:1, v/v); FMOC-OH^{UV} = the amount of reagents' hydrolysis product evaluated on the basis of its UV absorbency, at 262 nm, calculated with the response of phenylalanine (the huge peak obtained by fluorescence detection was not suitable for quantification, being out of the range of attenuation).
**Impurity calculated with the response of ornithine. ***Impurities in total, except, the amounts of FMOC-OH^{UV}.

Table 12
Derivatization yield (expressed as integrator units/pmol values), obtained in solutions of ACN:water = (1:1, v/v), at pH 9, as a function of the FMOC concentrations (0.25–5 mM)* and the time of interactions (1–60 min), with constant amount of amino acids ([amino acids]^T = 5.4 × 10^{-8} M).

Amino acids	t_R, min	FMOC: mM*; in braces:molar ratios of[FMOC]/[amino acids]^T									
		0.25; {2.75:1}			0.5; {5.5:1}			1.5; {16.5:1}			
		Reaction time, min									
		1	30	60	15	20	30	1	3	5	10
		Integrator units (I.u.)/1 pmol amino acids									
Asp	4.67	*14.48*	*63.94*	*63.89*	*106.6*	113.2	111.9	71.20	*100.3*	109.9	109.6
Glu	5.58	*23.00*	*84.76*	*85.42*	117.8	115.1	117.4	*94.95*	*114.4*	118.2	117.7
Hyp	8.98	104.6	104.5	104.9	101.8	102.4	101.9	101.7	102.6	101.7	103.9
Asn	9.67	*17.14*	*61.55*	*63.56*	82.50	82.87	83.60	*72.73*	84.53	85.05	86.03
Gln	10.17	*28.02*	*77.15*	*76.80*	85.30	85.93	86.80	*80.47*	85.87	86.22	86.38
Ser	10.73	*33.19*	92.58	92.23	91.95	92.43	92.20	91.27	91.51	91.59	92.20
Gly	11.72	*88.11*	93.34	93.15	93.63	93.34	92.98	91.36	89.75	90.10	91.44
Thr	12.12	*67.64*	106.1	106.7	109.3	106.4	106.2	107.8	109.6	108.3	108.4
Arg	12.48	*101.7*	119.7	120.1	114.3	120.0	113.8	121.7	119.7	120.3	121.3
Ala	13.18	*83.88*	132.4	128.9	133.6	134.1	131.1	132.7	134.1	134.4	134.4
Pro	13.62	*33.97*	75.67	72.03	76.38	76.70	76.67	77.50	74.66	75.12	76.15
Val	16.83	*58.24*	89.45	89.75	86.95	90.61	87.67	89.10	89.22	88.45	88.36
Met	17.22	*47.01*	81.05	81.94	79.7	83.26	79.00	80.99	80.56	79.79	79.61
Ile	18.93	*91.27*	126.3	126.6	128.8	129.2	124.7	128.9	124.8	124.3	124.4
Leu	19.33	*75.69*	118.3	120.5	115.9	121.6	114.7	119.4	120.8	118.2	114.6
Trp	19.92	1.16	1.10	1.16	1.11	1.14	1.13	1.14	1.17	1.12	1.18
Phe	20.23	*97.67*	127.2	128.6	127.4	126.0	126.8	122.4	-	127.7	125.7
$(Cys)_2$	22.70	–	–	–	*13.80*	13.12	13.00	12.60	13.18	12.40	12.79
Orn	27.22	*144.8*	184.9	185.2	188.5	184.0	182.9	187.6	183.4	185.1	184.0
Lys	27.65	*96.03*	*158.4*	160.2	159.9	162.5	162.3	165.0	161.4	161.7	165.0
FMOC–OH^{UV}	21.03	*0.87 × lO^3*	*1.8 × 10^3*	*2.1 × 10^3*	*2.4 × 10^3*	*2.8 × 10^3*	*3.1 × 10^3*	*2.8 × 10^3*	*4.2 × 10^3*	*5.3 × 10^3*	*7.9 × 10^3*
His-1#	11.30	*1.09*	6.72	6.69	6.72	0	0	7.30	6.86	0	0
His-2#	25.60	0	22.90	22.91	22.70	22.67	22.82	22.91	22.91	22.24	23.27
Tyr-1#	14.52	*24.8*	48.9	48.9	*67.0*	52.2	50.0	52.2	56.2	58.8	*65.5*
Tyr-2#	30.96	160.3	160.5	160.5	160.7	160.6	160.8	160.6	160.8	160.5	160.5

Amino acids	FMOC: mM*; in braces: molar ratios of[FMOC]/[amino acids]^T						
	3;{33:1}				5; {55:1}		Averages**(%, RSD)
	Reaction time, min						
Reaction time	1	3	5	10	1	10	
Asp	*94.75*	115.2	116.1	114.6	113.8	115.6	113.3 (2.11)
Glu	111.1	118.1	115.4	117.3	114.0	115.4	116.1 (1.89)
Hyp	103.6	101.7	103.5	104.9	104.1	102.4	103.0 (1.18)
Asn	83.04	83.38	81.84	85.96	85.67	84.64	84.1 (1.65)
Gln	84.44	85.76	84.25	85.39	85.47	83.71	85.5 (1.07)
Ser	92.26	92.55	90.87	90.79	90.69	90.56	91.7 (0.78)
Gly	92.59	95.92	92.72	95.33	91.99	92.07	92.7 (1.80)
Thr	104.9	108.0	106.9	108.5	107.0	107.1	107.4 (1.20)
Arg	118.0	113.6	118.7	114.2	117.6	118.7	118.1 (2.37)
Ala	132.8	131.2	132.9	135.6	136.0	131.5	133.1 (1.40)
Pro	74.75	72.33	73.94	77.34	75.14	76.85	75.4 (2.22)
Val	88.03	87.43	85.87	86.47	88.93	85.56	88.1 (1.66)
Met	79.70	79.51	80.10	79.14	80.75	80.83	80.4 (1.41)
Ile	122.1	123.5	119.7	124.6	127.5	122.3	125.2 (2.18)
Leu	116.6	114.2	113.4	116.1	116.7	118.6	117.3 (2.15)
Trp	1.15	1.16	1.09	1.09	1.17	1.11	1.14 (2.65)
Phe	–	–	–	–	–	–	126.5 (1.49)
$(Cys)_2$	12.08	12.34	12.28	*11.82*	–	–	12.64 (3.14)
Orn	181.0	181.8	187.0	183.9	185.5	183.1	184.5 (1.12)
Lys	163.6	161.0	161.2	165.0	161.1	165.6	162.5 (1.20)
FMOC–OH^{UV}	*5.5 × 10^3*	*9.4 × 10^3*	*12xl(f*	*18 × 10^3*	*9.4 × 10^3*	*26 × 10^3*	–
His-1#	0	0	0	0	0	0	6.86 (3.73)
His-2#	22.74	23.13	22.71	22.98	23.48	22.88	22.9 (1.23)
Tyr-1#	48.7	*42.9*	51.9	51.9	51.8	0.00	51.9 (6.0)
Tyr-2#	160.5	160.5	160.4	160.5	161.0	160.5	160.6 (0.10)

Indications: as in Table 11, as well as: ** = averages of quantitative responses, corrected by the blank values, shown in Table 11, normalized for 1 mL/min flow rate; italic printed values have been omitted from the mean; () = in parentheses RSD percentages; – = no data available; His-1#, His-2#, Tyr-1# and Tyr-2# = calculated values, based on responses while His-2 and Tyr-2 were present exclusively, His-1 and Tyr-1 are indicated by 0, being quantitatively transformed to His-2 and Tyr-2; (see also Figs. 14a and b–21a and b).

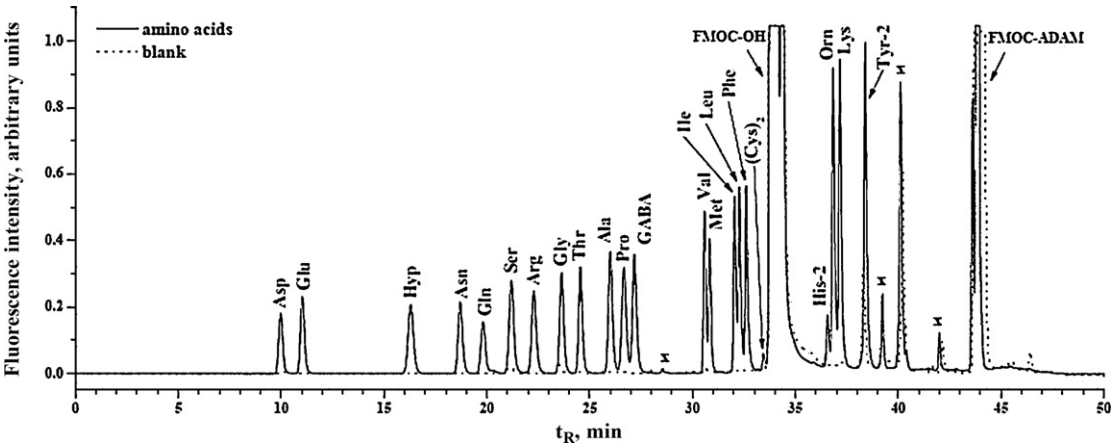

Fig. 10. Elution shape of the FMOC derivatives of amino acids (continuous line), obtained with fluorescence detection, together with the impurities of reagent blank (dashed line); derivatized under optimized conditions (pH 9, ACN containing reagent; FMOC 0.5 mM) [20]; *Note:* derivatives indicated by asterisk do contain impurities from the reagent blank.

The results of our studies obtained with the 22 FMOC amino acids, derivatized under optimum conditions (Section 2.2.5) are shown by Fig. 11.

Comparing the numerical responses, expressed as I.u./1 pmol amino acids the particular advantage of FL detection has been repeatedly confirmed.

According to our results obtained with the selected 22 FMOC amino acids:

(1) FL detected responses varied between 1.14 and 184.5 I.u./1 pmol values with an average response of 99.9 I.u./1 pmol (Table 12 and Fig. 11), while
(2) UV detected responses proved to be between 0.80 and 2.49 I.u./1 pmol values with an average response of 1.41 I.u./1 pmol (Fig. 11).

The response ratio (RR) values calculated from the FL and UV responses (Fig. 11), reveal that with the exceptions of Trp (RR = 0.69), $(Cys)_2$ (RR = 5.1) and His-2 (RR = 28.6), the RR values varied between methionine (Met, RR = 69) and glycine (Gly, RR = 105), manifesting an average of RR = 70.9, which proved to be 2.8 times higher (70.9/25 = 2.8) as published earlier [82].

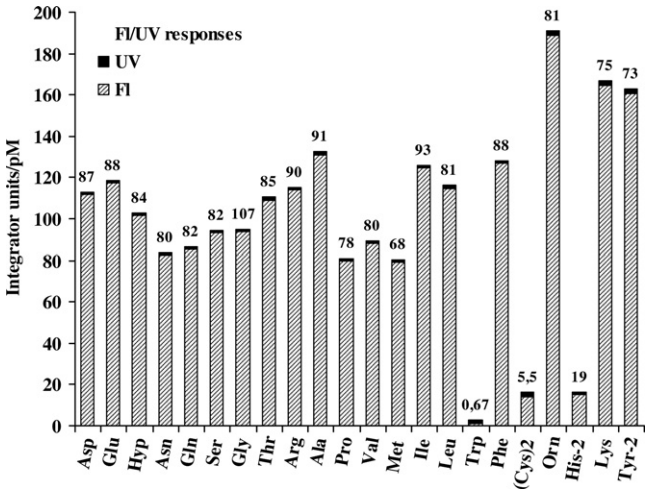

Fig. 11. Comparison of the responses of the FMOC amino acids obtained by UV and FL detections shown by their response ratios (FL/UV responses, expressed in integrator units (I.u.)/1 pmol (pM) amino acid derivative.

2.2.5. Derivatization optimization study

As the first step, in the frame of these investigations, the FMOC concentration and the derivatization time were varied, at pH 9 and at constant amino acid concentrations. Consequently, the molar ratios of reactants became changed accordingly (Table 6: $([FMOC]/[AAs]^T = 2.75:1–55:1$).

The efficiency of derivatizations was shown by the responses of the FMOC derivatives, expressed in arbitrary units (I.u./1 pmol amino acid).

Derivatizations have been regarded as quantitative ones, providing reproducible responses, within the experimental error of our analyses, characterized with their relative standard deviation percentages (≤6.0%, RSD with an average of 1.5%, RSD).

On the basis of our most important expectations, i.e., to obtain

(1) quantitative derivatization within the shortest reaction time,
(2) with the smallest FMOC-OH peak as possible, as well as,
(3) with the quantitative evaluation possibility of both derivatives of histidine (His-1, His-2) and tyrosine (Tyr-1, Tyr-2), the following conclusions could be drown (Table 12):
   (a) The use of 0.25 mM FMOC concentration, – in spite of the smallest FMOC-OH peak obtained, – proved to be unsatisfactory, even after 60 min reaction time (Table 12 italic printed response values in the first three vertical columns); thus, these condition had to be omitted.
   (b) Increasing the FMOC concentrations (0.5–5 mM) associated with increased molar ratio conditions of reactants ($[FMOC]/[AAs]^T = 5.5:1–55:1$) resulted in several quantitative conditions. From 1 min (last group of vertical columns FMOC, 5 mM, 1 min) up to 20 min (second vertical group of columns, FMOC 0.5 mM, 20 min), in between with additional conditions suitable for quantitation purposes {FMOC concentrations: 1.5 mM (5 min) and 3 mM (3 min)}.
   (c) On the basis of the above detailed results (data in Tables 11 and 12), as the best compromise (remaining on the safe side both with the width of the FMOC-OH peak and with the time of derivatization), as optimum derivatization condition the FMOC concentration of 0.5 mM and 20 min reaction time was selected.
   (d) As a result of our exhaustive and consistent optimization studies this is the first time that the consequent follow-up of the formation and transformation of the FMOC-histidine (His-1, His-2) and the FMOC-tyrosine derivatives (Tyr-1, Tyr-2) resulted in reproducible responses of all four species (detailed study in Section 2.2.7).

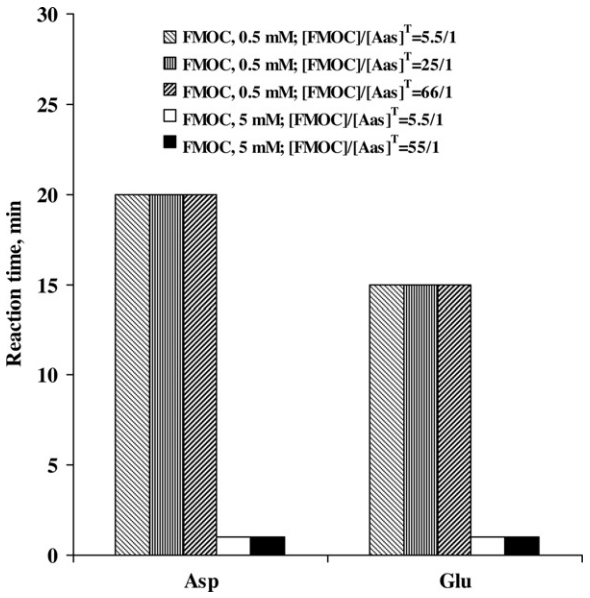

Fig. 12. Comparison of the reaction times needed for the quantitative derivatizations of aspartic and glutamic acids, depending on the mole concentration of the FMOC in the reagent and the molar ratios of the reactants ([FMOC]/[AAs]^T).

As the second step of our derivatization studies we wanted to know whether the rate limiting step of quantitative results are associated with the FMOC concentration of the reaction mixture, or with the molar ratios of the reactants?

The answer to this question was given by applying constant FMOC concentrations, varying the amounts of amino acids. Studies were approached from two directions, confirmed as quantitative derivatization conditions (Table 12, FMOC 0.5 mM, 20 min and 5 mM, 1 min), like:

(a) at 0.5 mM FMOC concentrations, performing 20 min reaction time, the amount of amino acids were decreased resulting in the increased molar ratios of [FMOC]/[AAs]^T from 5.5 (Table 12) through 25/1 to 66/1 (Fig. 12), while, in parallel,
(b) at 5 mM FMOC concentrations, performing 1 min reaction time, the amount of amino acids were increased resulting in the decreased molar ratios of [FMOC]/[AAs]^T from 55/1 (Table 12) to 5.5/1 (Fig. 12).

Results obtained were shown by the spectacularly same responses of the less reactive amino acids, like aspartic and glutamic acids (certainly, in accordance with the responses of the rest of the other 20 species), confirming the evidence, that:

(a) the higher the FMOC concentration the faster the derivatization reaction. With other words,
(b) by increasing the molar ratios at 0.5 mM FMOC concentrations do not result in decreased reaction time, 20 min was needed at all three conditions, correspondingly,
(c) by decreasing the molar ratios at 5 mM FMOC concentrations, 1 min reaction time was satisfactory in both cases.

In conclusion, based on the above detailed studies, carried out:

(i) under constant AA concentration (1. step of our optimized derivatizations, data in Table 12), varying the amounts of the FMOC, and,
(ii) under constant FMOC concentration (2. step of our optimized derivatizations, selected data in Fig. 12), varying the amounts of AAs, it has been confirmed that the rate limiting step of derivatization are limited by the actual concentration of the FMOC.

Note: The strange scheme of Fig. 12 serves to emphasize the primary importance of the FMOC concentration. The selected values are obtained as a result of detailed derivatization study carried out for 1, 10, 15, 20, 30 and 60 min reaction times (not shown), under the indicated molar ratios of reactants. It means that the selected reaction times, needed for quantitative derivatization, are strongly confirmed with derivatizations of shorter and longer reaction times, respectively.

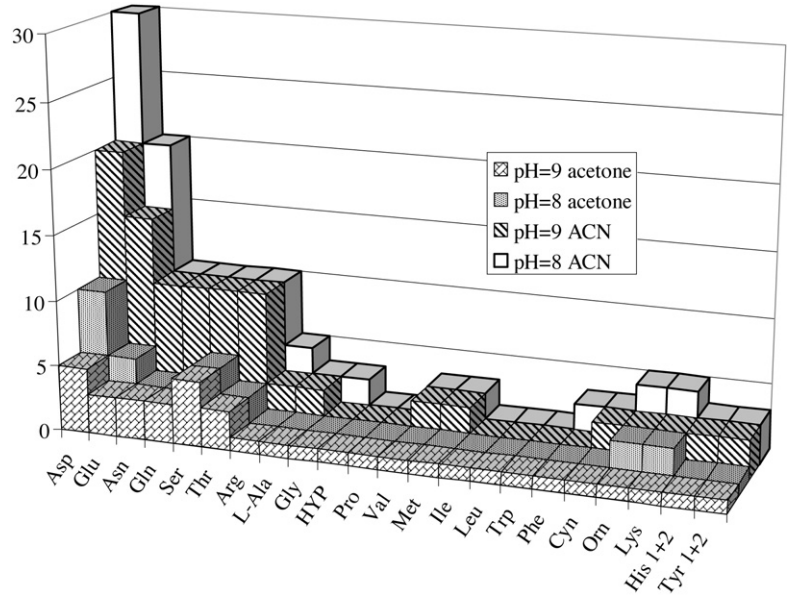

Fig. 13. Comparison of the times of quantitative derivatizations of the selected 22 amino acids, under optimum conditions (FMOC 0.5 mM, [FMOC]/[AAs]^T = 25/1) as a function of the pH and the organic solvent of the FMOC reagents Figs. 14–21. The formation and transformation of His-1, His-2, Tyr-1 and Tyr-2, obtained with fluorescence detection, as a function of the pH and the reaction time. Formation and transformation conditions (Figs. 14–21) were expressed in pmol values; results in Figs. 14–17 were obtained with ACN and in Figs. 18–21 with acetone containing reagents.

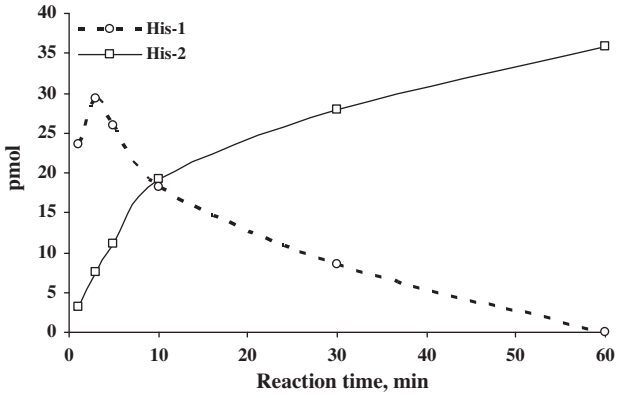

Fig. 14. The formation and transformation of His-1, His-2 in ACN containing media (pH 8).

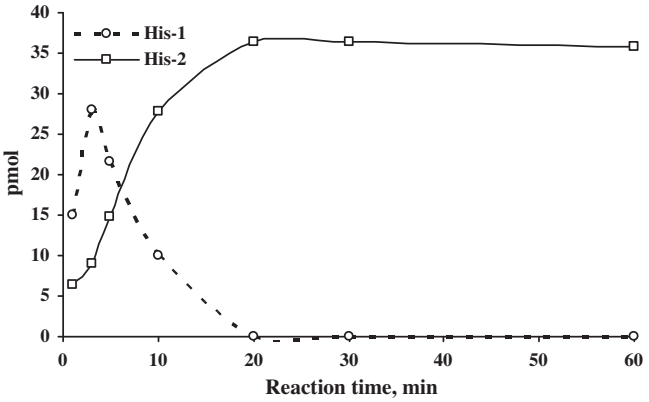

Fig. 15. The formation and transformation of His-1, His-2 in ACN containing media (pH 9).

2.2.6. The advantage of acetone versus ACN as organic solvent of derivatizations

As a completion of our optimization studies, the presentation of the advantage of the use of acetone as solvent was performed for practical conditions.

In spite of the fact that in our understanding ACN is the best choice for FMOC derivatization (detailed arguments in Sections 2.2.3 and 2.2.7), for selected practical tasks, not involved in the separation of the FMOC derivatives between the FMOC-OH and the FMOC-adamantan peaks, the use of acetone instead of ACN might provide useful information.

In the course of our derivatization studies, performed under strictly the same experimental conditions (except the organic solvent was varied), the reaction times needed for quantitative reactions were compared (Fig. 13).

Data obtained confirmed that acetone, based on its considerable higher polarity, favors the faster derivatizations, both at pH 8 and at pH 9, equally. Thus, in acetone containing media, at pH 9, quantitative derivatization can be achieved within 5 min reaction time, including all amino acids, while for quantitative results in ACN containing media, 20 min reaction time was needed.

2.2.7. The formation and transformation studies of the FMOC derivatives of histidine and tyrosine

In order to characterize all four derivatives {*N*-FMOC-histidine (His-1), *N*-FMOC-tyrosine (Tyr-1), *N,NH*-FMOC-histidine (His-2), *N,O*-FMOC-tyrosine (Tyr-2)} with reproducible, comparable responses (Table 12, measured and calculated responses), conditions performed had to be met the following requirements:

(1) a special gradient which enables the complete baseline resolution of all four species [20],
(2) to obtain at least one derivatization condition which provides the transformed species (His-2, Tyr-2) exclusively, to be able to calculate separate responses for all four species, and,
(3) to confirm, that His-2 and Tyr-2, in the time range of analytical interest (20–60 min), are stable and suitable for quantitation purposes.

All these requirements {(1), (2), (3)} have been achieved in the frame of our derivatization optimization studies (Section 2.2.5, Table 12), completed by the detailed formation and transformation of histidine and tyrosine. These detailed studies have been carried out at 0.5 mM FMOC concentration, at pH 8 and at pH 9, in parallel, both in ACN (Figs. 14–17) and in acetone (Figs. 18–21) containing media.

Due to the different reaction rates of the second FMOC groups with the deprotonated N of the histidine's imidazole- and with the deprotonated O of the tyrosine's phenol group, thus, the overall formation and transformation rate both for histidine and for tyrosine were slower in the ACN (Figs. 14–17) compared to the acetone (Figs. 18–21) containing solutions.

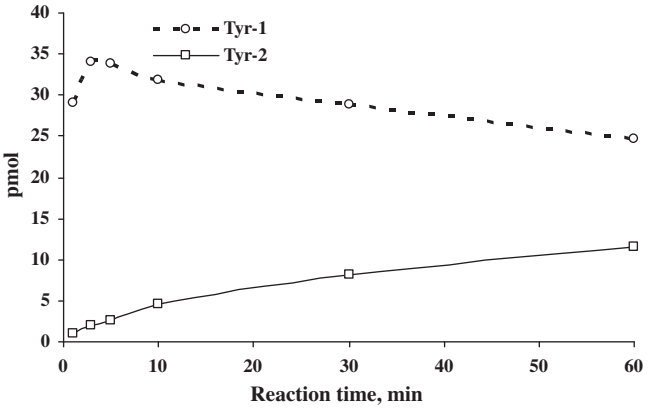

Fig. 16. The formation and transformation of Tyr-1, Tyr-2 in ACN containing media (pH 8).

In accordance with our derivatization optimization studies (Section 2.2.5, Table 6) the evaluation of the formation and transformation processes are calculated in details, in ACN containing solutions (Table 12 and Figs. 14–17).

(a) The exclusive presence of His-2 was determined, out of sixteen, in ten conditions, presented in Table 12 and Figs. 14 and 15.

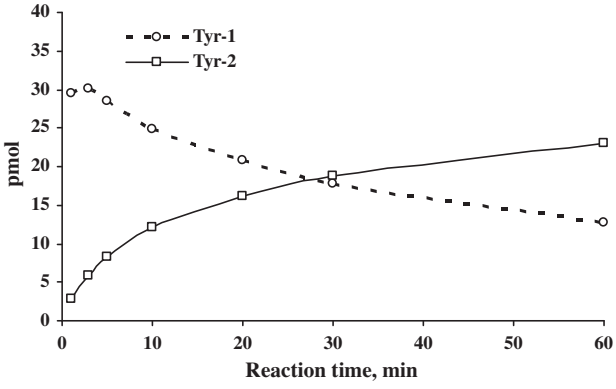

Fig. 17. The formation and transformation of Tyr-1, Tyr-2 in ACN containing media (pH 9).

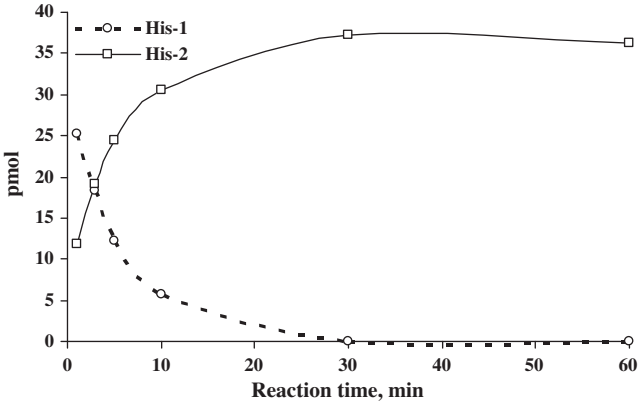

Fig. 18. The formation and transformation of His-1, His-2 in acetone containing media (pH 8).

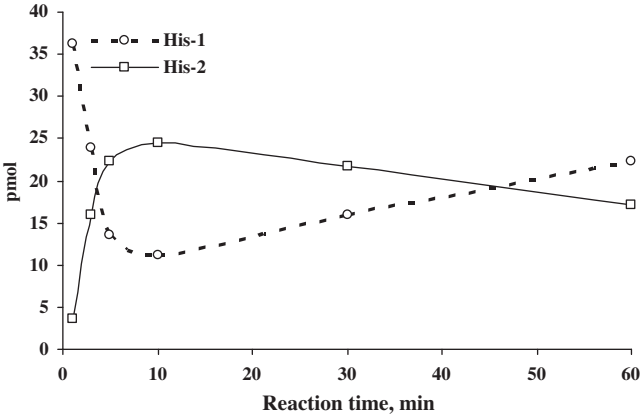

Fig. 19. The formation and transformation of His-1, His-2 in acetone containing media (pH 9).

This means, that the quantitative transformation of His-1 was indicated by 0 (Table 6), and the responses of His-2 proved to be 22.9 I.u./1 pmol histidine, confirming its excellent reproducibility (≤1.23%, RSD) and its stability (Fig. 15).

(b) While, the exclusive presence of Tyr-2, out of 16, could be achieved in a single condition, only (Table 6). This means, that the quantitative transformation of Tyr-1, indicated by 0 (Table 6: 5 mM FMOC, 10 min reaction time), needs extremely high FMOC concentration and even suitable compromise of reaction time and pH conditions. In spite of the fact being in possession of this single 'measured response' only, the reality of all those further response values calculated from this only one (Table 12 Tyr-1: 0, Tyr-2: 160.5 I.u./1 pmol), resulted in acceptable reproducibility and in acceptable stability, characterized by the total of Tyr-1 + Tyr-2 (Table 12 and Fig. 17).

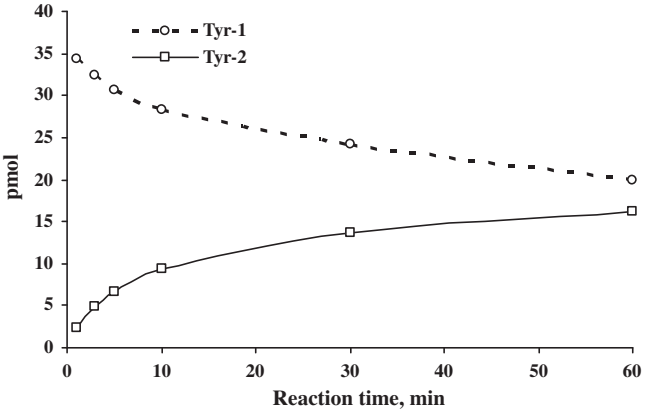

Fig. 20. The formation and transformation of Tyr-1, Tyr-2 in acetone containing media (pH 8).

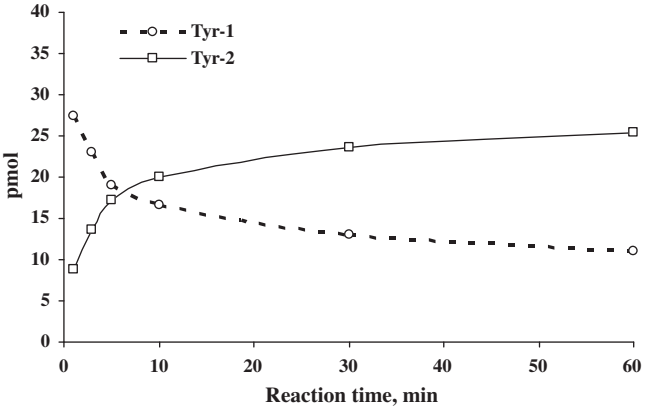

Fig. 21. The formation and transformation of Tyr-1, Tyr-2 in acetone containing media (pH 9). *Note:* Figs. 10–21, without exception, have been reproduced with permission from Ref. [20].

(4) As to the numerical responses of the initially formed and transformed species, values revealed a considerable higher intensity of the transformed ones (His-1/His-2 = 1/3.3 and Tyr-1/Tyr-2 = 1/3.2), due to the increased intensity of the second FMOC groups.

(5) Regarding the pH dependence of the transformation rate of His-1, His-2, Tyr-1 and Tyr-2, shown on the basis of their peak shapes (Figs. 14–17) and the corresponding curves consisting from the calculated pmol values of species (Figs. 14–17) provided the expected correlations. Transformations obtained under strictly the same conditions, except the apparent pH of derivatizations (pH 8, pH 9), revealed that the higher the pH the faster the transformation rate. This experience proved to be in accordance with the deprotonation phenomena of the species and can be explained with the different reaction rate of the second FMOC group which became favored by increasing the pH of the reaction media.

(6) As to the impact of the acetone containing media on the formation and transformation of histidines and tyrosines (Figs. 18–21) the trend of processes are comparable, however faster as in ACN containing media, consequently, less suitable for quantitation purposes.

(a) Comparing the transformation of His-1 to His-2, or vice versa (reaction times at Figs. 14, 15, 18 and 19) it turns out that in order of listing, the quantitative transformation in ACN containing media needed 60 min (Fig. 14, pH 8) and 20 min (Fig. 15, pH 9) and in acetone containing media 10 min (Fig. 18, pH 8,). However, in acetone at pH 9 at 10 min (Fig. 19a), instead of the only presence of His-2 its transformation back to His-1 starts up.

(b) The distribution of Tyr-1 and Tyr-2 (Figs. 16, 17, 20, 21) reflects the presence of both derivatives. At pH 8 the amounts of Tyr-2 are increasing at the expense of Tyr-1, in ACN containing medium slower (Fig. 16) compared to the acetone containing one (Fig. 20). The transformation of Tyr-1 to Tyr-2 at pH 9, in acetone containing media proved to be faster (Fig. 21: Tyr-1 ≅ Tyr-2, at 7–8 min), compared to its transformation in ACN (Fig. 17: Tyr-1 ≅ Tyr-2, at 27–28 min).

3. Conclusion

(1) A detailed literature overview of papers dealing with the OPA (in the presence of various SH-group containing additives) and with the FMOC derivatizations were documented, summarized, critically evaluated: uniformly from quantitative analytical point of view.
(2) Primarily the background of uncertainties, contradictory literature data were cleared up on the basis of stoichiometric studies, confirmed by on-line PDA and fluorescence detections, in selected cases also by on line LC–PDA–(ESI)MS(MS) support.
(3) Particular attention was paid on the more than one product providing amino acids.
(4) In the cases of OPA derivatization it was proved, that the believed to be less stable amino group containing compounds (glycine, β-alanine, γ-aminobutyric acid, ornithine, lysine, aliphatic mono and diamines, without exception, do contain the '$-CH_2-NH_2$' moiety.
   - Due to the peculiarity of the '$-CH_2-NH_2$' moiety containing amino acids/amines, their expected transformed products, – containing one additional OPA molecule (UV_{max} 339 nm), and/or one additional SH-compound (UV_{max} 343 nm), – are formed also with all SH group containing regents.
   - In comparison to all corresponding investigated species (OPA–MCE, OPA–MPA, OPA–NAC, OPA–ET), stability and molar response data confirm the doubtless requirement of the come-back to the OPA–ET derivatization. This consideration needs the scientific conviction of the community of analytical chemists (involved in the analysis of amino acids and amines) to bear the horror of ethanethiol's odor (One of the most important advantages of the OPA–ET derivatization can be regarded its benefit for the well known, lowest stability furnishing methylamine. In the frame of our study it has been confirmed that, as OPA–ET-derivative, also methylamine provides high and stable responses up to 3 h reaction time).
   - Comparing fluorescence responses of the single derivative providing, the '$=CH-NH_2$' moiety containing amino acids, as a function of the OPA reagents' SH-additive they were derivatized with, it has been shown that,
     (a) stabilities are independent on their composition and
     (b) the lowest response values were obtained by the OPA–MCE derivatives.
(5) In the case of FMOC derivatizations the key points of the contradictions were thoroughly associated with the extremely different derivatization conditions proposed in the literature.
(6) On the basis of our stoichiometric derivatization studies various conditions resulting in the quantitative yield of the FMOC derivatives of 22 amino acids, including the less reactive aspartic and glutamic acids and the two species providing histidine and tyrosine derivatives have been summarized. It was confirmed that:
   – to perform the elutions of blank measurements and to deduct the responses of impurities from the corresponding amino acid derivatives is inevitable necessary, in particular when the constituents of the analyte are present in the low pmol range, as well as,
   – to select the conditions for optimum derivatization proved to be a matter of compromise: such as the pH and the organic solvent of the reaction medium, as well as, the FMOC concentration and the time of derivatization. The most important points of our selection were, to obtain
     (a) quantitative derivatization for the shortest reaction time,
     (b) with the smallest FMOC-OH peak as possible, as well as,
     (c) with the quantitative evaluation possibility of both derivatives of histidine (His-1, His-2),
     (d) and tyrosine (Tyr-1, Tyr-2). In conclusion, - based on the above detailed aims, performed,
     (e) under constant amino acid concentration varying the amounts of the FMOC, and,
     (f) under constant FMOC concentration varying the amounts of AAs, – it has been confirmed that the rate limiting step of derivatizations is determined by the concentration of the FMOC.

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