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Advances in the evaluation of the stability and characteristics of the amino acid and amine derivatives obtained with the *o*-phthaldialdehyde/3-mercaptopropionic acid and *o*-phthaldialdehyde/*N*-acetyl-L-cysteine reagents High-performance liquid chromatography–mass spectrometry study

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Abstract

The composition of the amino acid and amine derivatives obtained with the *o*-phthaldialdehyde (OPA)/3-mercaptopropionic acid (MPA) and with the OPA/*N*-acetyl-L-cysteine (NAC) reagents was investigated by on-line HPLC–electrospray ionization MS. The initially formed derivatives proved to be, as expected, the corresponding isoindoles while their transformed species contained one additional OPA molecule. Based on the MS spectra of all transformed OPA derivatives a reaction pathway is suggested. This reaction mechanism was supported both by the molecular ions of the endproducts and by the presence of several selective fragment ions that served as an explanation to the structure of the believed to be less stable OPA derivatives. It has been shown that more than one OPA derivative forms in all those cases when the compound to be derivatized does contain the $NH_2–CH_2–R$ moiety. Thus, amino acids like e.g. glycine, histidine, β-alanine, γ-aminobutyric acid, ϵ-aminocaproic acid, ornithine, and also several aliphatic mono- and diamines provide more than one OPA derivative. Analytical consequences of this experience were utilized by altering the reagent's composition. Reagents containing mole ratios of [OPA]/[MPA] or [OPA]/[NAC]=1/50 resulted 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.

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

1. Introduction

In the last three years several characteristics of the *o*-phthaldialdehyde (OPA) derivatized amino acids [1–5] and OPA-derivatized amines known as having low stability, such as glycine, γ-aminobutyric acid (GABA), β-alanine, histidine, ornithine, lysine, ϵ-aminocaproic acid, as well as $C_1–C_5$ aliphatic mono, di- and polyamines, including also the relevant biogenic amines [6], revealed comparable stability as

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any other amino acids [1–5]. It was found that the above selected amino acids and amines provided more than one OPA derivative [2–6].

Depending on the composition of the derivatizing reagent (mole ratios of OPA to the SH-group containing additive) and that of the concentration of reactants, the amount of the primarily formed derivative became considerably influenced: in the cases of the above mentioned amino acids a second, a third and/or a fourth derivative appeared and increased on the chromatogram with a simultaneous decrease of the initially formed ones [2–6]. The transformation rate of the successively formed derivatives was found to be pH dependent. A decrease of the pH of the derivatization mixture, usually before elution [4,5], leads to the decrease of responses. Accordingly pioneer literature data [7–13] the existence of the mono- and dithiosubstituted isoindoles [7–11], as well as the decomposed species of the initially formed OPA derivatives (e.g. glycine, GABA, β-alanine, histidine and monoamines) [12,13] were assumed.

This work was undertaken in order to clarify the structure of the transformed OPA derivatives and the reaction mechanism they are originating from. Two approaches were followed: stoichiometric investigations were carried out with simultaneous photodiode array (DAD) and fluorescence (FL) detections, while structure elucidations were obtained by on-line LC–MS applying simultaneous HPLC–UV–MS detections [electrospray ionization (ESI) MS in the positive mode].

2. Experimental

2.1. Materials

OPA, 3-mercaptopropionic acid (MPA), *N*-acetyl-L-cysteine (NAC), amino acids and amines were obtained from Sigma (St. Louis, MO, USA) and from Serva (Heidelberg, Germany). HPLC-grade methanol and acetonitrile were from Fischer Scientific UK (Leicestershire, UK). All other reagents were of the highest purity available.

2.2. Standard solutions

Standard solutions of free amino acids were distilled water as described earlier [14], in ~$3.7\cdot10^{-4}$ *M* concentration and further diluted, one by one or together in selected grouping: final concentrations corresponded to the mole ratios of OPA/amino acid=20/1.

Stock solution of OPA contained 0.25 g OPA (weighed with analytical precision) in 50 ml methanol (further on referred to as: methanolic OPA stock solution).

2.3. Buffer solution

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

2.4. Reagent solutions

OPA/MPA reagent was prepared by mixing, in order of listing, 2.0 ml methanolic OPA stock solution (0.01 g OPA≅$7.5\cdot10^{-5}$ *M*), 6.0 ml borate buffer and 18.7–312 μl MPA solutions ($5.46\cdot10^{-3}$ –0.273 g≅$2.25\cdot10^{-5}$ *M*–$3.75\cdot10^{-3}$ *M*, corresponding to the mole ratios of OPA/MPA=1/3 –1/50). The pH of reagent was adjusted to pH 9.3±0.05 with 1.0 *M* sodium hydroxide and completed to the final 10.0 ml volume with the buffer solution.

OPA/NAC reagent was identically prepared from 2 ml methanolic OPA solution (0.01 g OPA≅$7.5\cdot10^{-5}$ *M*), 6.0 ml borate buffer containing 0.0367–1.83 g NAC (corresponding to the mole ratios of OPA/NAC=1/3 –1/50): final pH 9.3±0.05.

2.5. Derivatization

2.5.1. Characterization of the reagent solutions

Blank measurements were performed with freshly prepared (reagent's age ≥90 min [2]) reagent solutions, stored in the refrigerator, at ~4°C, for various periods of time and injected by the robotic autosampler, every day at least twice (Waters 717, thermostatted for ~4°C).

2.5.2. Reproducibility study of the isoindole derivatives

Derivatizations were performed with reagent solu-

tions prepared at least 90 min before use, and retained no longer than ≤9 days. The same amount of reagent solutions (100 μl) were mixed up with 100 μl of variously diluted solutions of free amino acids and/or amines.

3. Chromatography

3.1. Simultaneous DAD and FL detection

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

Detections have been performed simultaneously: DAD (Waters 996) and FL (Waters 274) detectors were connected in order of listing. Blank tests, concentration dependence have been recorded between 190 and 400 nm (DAD) and evaluated at 334 nm [OPA/MPA(NAC)/amino acids], as well as at the optimum fluorescence wavelengths (excitation 337 nm, emission 454 nm).

3.2. Simultaneous DAD and MS detection (performed at DSM Research, Geleen, Netherlands)

The system was a Hewlett-Packard (HP) 1100 instrument (Agilent Technologies, Waldbronn, Germany), including a DAD and atmospheric pressure ionization/electrospray ionization MS detection, a quaternary pump, an Autosampler, operating with the Chemstation Software of Agilent A.08.03. The column used was a Nucleosil 120-5C_{18} (125×4 mm).

DAD and MS detectors were applied simultaneously, connected them in order of listing. Blank tests and concentration dependence have been recorded between 190 and 600 nm (DAD), evaluated at 334 nm (OPA/MPA-amino acids), MS detections were performed with ESI in the positive mode (mass range: 50–1500 mass units; gas temperature: 340°C; capillary V: 3.50 kV.

3.3. Simultaneous UV and MS detection (carried out at Plant Health and Soil Conservation Station, Budapest, Hungary)

The apparatus was a Spectra System (Thermo-Separation Products, San Jose, CA, USA), consisted of UV 2000 (for two wavelengths) and Finnigan Aqua (ThermoQuest, Manchester, UK) MS detectors, P 2000 quaternary pump, As 2000 Autosampler, operating with the Software of Xcalibur, RevisionB 1997. The column was Hypersil ODS bonded phase, 150×4 mm, 5 μm, used with 20×4 mm guard column.

Detections have been performed simultaneously, applying the UV 2000 and MSD Finnigan Aqua detectors, connected them in order of listing. Blank tests, concentration dependence have been recorded between 190 and 900 nm (UV), evaluated at 334 nm (OPA/NAC-amino acids and/or OPA/NAC-amines), MS detections were performed with ESI in the positive mode (mass range: 50–1600 mass units; gas temperature: 200°C (flow-rate 200 μl/min) or 380°C (flow-rate 1 ml/min); capillary V: 3.5 kV.

The eluent system used for DAD(UV)–MS detections consisted of two components: (A) eluent was 0.0025 *M* ammonium acetate of pH 7.2, while (B) eluent was prepared from 0.005 *M* ammonium acetate–acetonitrile–methanol (46:44:10) (mixed in volume ratios and titrated with 1 *M* acetic acid or 1 *M* ammonium hydroxide to pH 7.2). Different gradient programs were followed as described earlier [2].

4. Results and discussion

4.1. On-line HPLC–MS studies

Composition of the OPA/MPA-amino acids was measured at DSM. Glycine, β-alanine, ε-aminocaproic acid and lysine were investigated as their OPA/MPA derivatives (Table 1, Fig. 1(1–4)). Data revealed unambiguously, that the initially formed products of these four amino acids were the well-known isoindoles while their transformed versions,

Table 1
Fragmentation patterns of the OPA/MPA and OPA/NAC derivatives of amino acids and amines [MW(OPA)=134.1; MW(MPA)=106.1; MW(NAC)=163.2)]

Amino acids, amines	Fig. No.	MW	First OPA/MPA derivative's ions				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 -$2H_2O$	$OPAH^+$			Special fragment**
Glycine	1/1	75.1	280.3	**280.0**		175.0	**414.0**	396.0	280.0		135.1			278.2
β-Alanine	1/2	89.1	294.3	**294.0**		189.1	**428.0**	410.0	294.0	338.0				
Lysine	1/3	146.2	555.6	**555.2**	511.0	405.1#	**689.0**	671.0	555.0	338.0				
ϵ-NH_2-caproic acid	1/4	131.2	336.4	**336.1**	292.0	231.1	470.1	**452.2**	336.1		135.1			
Amino acids, amines	Fig. No.	MW	First OPA/NAC derivative's ions				Transformed OPA/NAC derivative's ions							
			MH^+ calc.	MH^+ obt.	MNa^+	MH^+ – m/z=129	MH^+ + OPA	MNa^+ + OPA	MH^+ + OPA –H_2O	MNa^+ + OPA +Na	MNa^+ + OPA +2Na	MH^+ + 2OPA *	MNa^+ + 2OPA *	Special fragment **
Glycine	1/5	75.1	337.4	**337.1**	359.1	208.0	471.2	**493.1**	453.0					278.9
β-Alanine	1/6	89.1	351.4	**351.1**	373.0	222.0	485.1	**507.2**	467.2					279.1
GABA	1/7	103.1	365.4	**365.1**	387.1	236.1	528.6	**521.2**	481.2	543.1	564.8			279.1
Histidine	1/8	155.2	417.5	417.3	**461.4&**	310.3								
Ornithine	1/9	132.1	655.7	**655.3**	677.2	–	789.2	**811.2**	771.3	833.2	855.2			
Lysine	1/10	146.2	669.8	**669.3**	691.4	–	803.3	**825.3**	785.3	847.3	869.3			
ϵ-NH_2-caproic acid	1/11	131.2	393.5	**393.3**	415.3	264.3	527.6	527.4	509.4	**549.4**	571.4			
n-Propylamine	1/12	59.1	321.4	**321.3**	343.3	192.2	455.5	477.4	**437.4**	499.4				
Putrescine	1/13	88.2	611.5	**611.5**	633.5	–	745.6	**767.6**	727.6	789.6	811.5	879.5	**901.6**	600.6**
Cadaverine	1/14	102.2	625.5	**625.5**	647.5	–	759.6	**781.6**	741.6	803.6	825.6	893.7	**915.6**	614.6**
Agmatine	1/15	130.2	392.5	**392.4**	414.4	263.3	**526.5**							
Tyramine	1/16	137.2	399.5	**399.4**	421.3	270.3	533.5	555.4	**515.4**	577.5				
Spermidine	1/17	145.2	407.5	–	–	–	–	–	**505.6##**					

Indications: concentrations: [OPA]/[MPA]([NAC])/[amino acid]([amine])=20/60/1, $1=\sim 1\cdot 10^{-9}$; bold printed=abundant ions; *=Fragment ions belonging to the transformed species of putrescine3; **=Special fragment ions=MH^++OPA(–H_2O–R_1–R_2); 600.6**=m/z=879.5–279.1; 614.6**=m/z=893.7–279.1; 405.1#=MH^+–(COO+MPA); **461.4&=**MNa^++Na; 505.6##=MH^++OPA–$2H_2O$.

glycine, β-alanine, lysine and ϵ-amino caproic acid, without exception, contain an additional molecule of OPA.

In the light of these introductory data, taking into account the importance and consequences of these results, further confirmation was needed. Thus, MS measurements have been extended: (i) to several primary amino group containing compounds that provide more than one OPA derivative, as well as (ii) to OPA derivatives obtained with another SH-group containing (OPA/NAC) reagent. The structure of the OPA/NAC-amino acids and OPA/NAC-amines (Table 1, 1/5–1/17) were examined at the Plant Health and Soil Conservation Station (Budapest, Hungary). Results, in all cases investigated, repeatedly revealed that the transformed versions of the initially obtained isoindoles contain an additional OPA molecule. Spectra of the individual derivatives proved to be particularly informative (Table 1, Fig. 1/5–1/17)). They served as unambiguous evidence both to earlier results with OPA derivatives of –NH–CH_2– bonding (Table 2), and, to the suggested reaction scheme (Fig. 2). Evaluating the MS spectra the following conclusions can be drawn.

4.2. Fragments of the initially formed derivatives

Table 1, ions from the initially formed OPA/MPA and OPA/NAC derivatives, Fig. 1 1/1–1/17, compounds indicated by number1, as well as ornithine2 and lysine2. (Note: 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

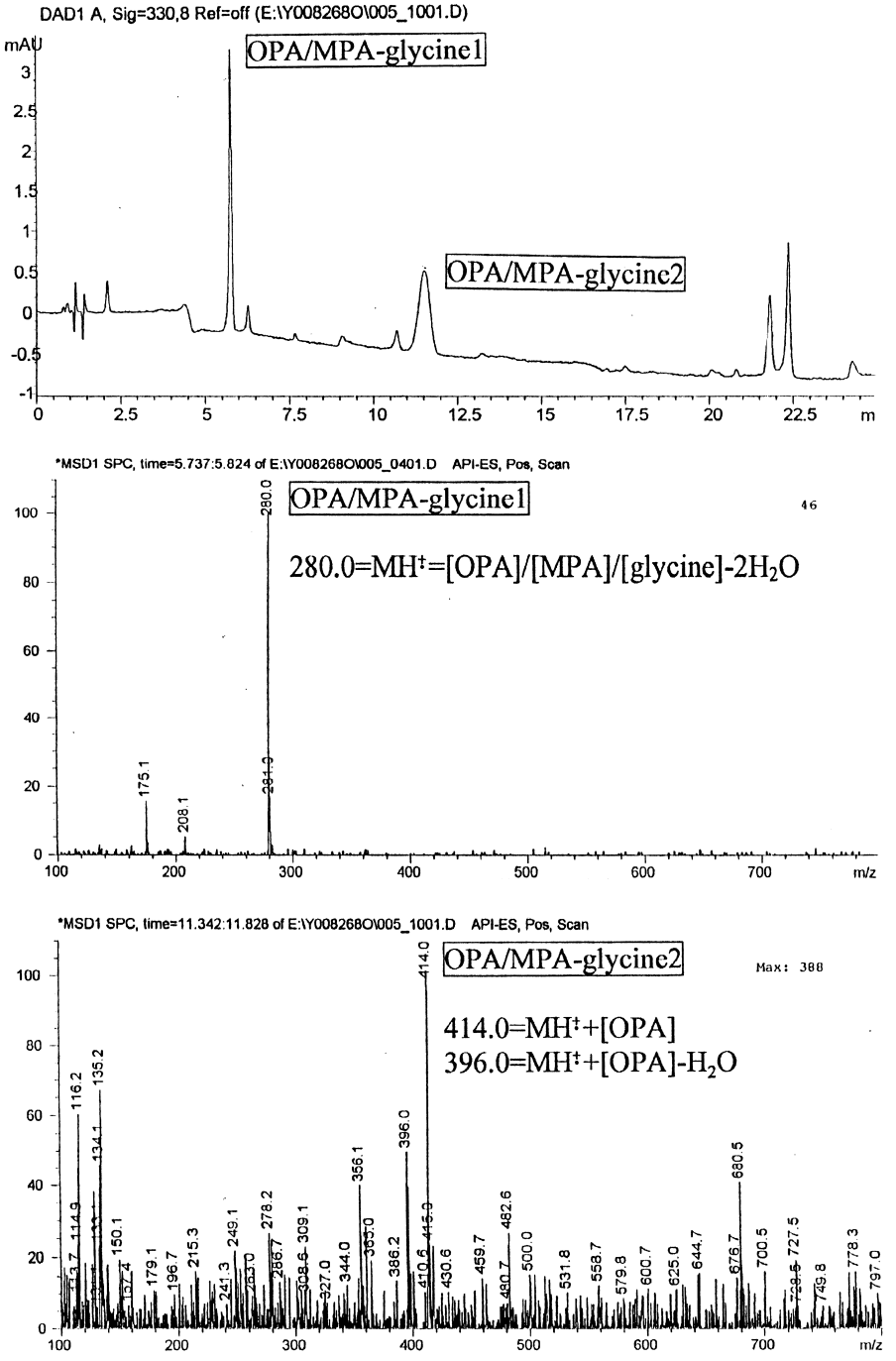


Fig. 1. DAD (UV) chromatograms (first lines), MS spectra of the initially obtained (spectra in second lines) and transformed (spectra in third and fourth lines) OPA/MPA (1–4) and OPA/NAC (5–17) derivatives. Indications: spectra in second lines, OPA derivatives indicated by number1, as well as ornithine2, lysine2, SPD2; spectra in third lines, OPA derivatives indicated by number2, as well as ornithine3, lysine3; spectra in fourth lines, cadaverine3 and putrescine3. Abbreviations: ϵ-aminocaproic acid, (ϵ-ACA); *n*-propylamine (n-PA); putrescine, (PUT); cadaverine, (CAD); agmatine, (AGM); tyramine, (TYR); spermidine, (SPD). Detailed composition of fragments in Table 1.

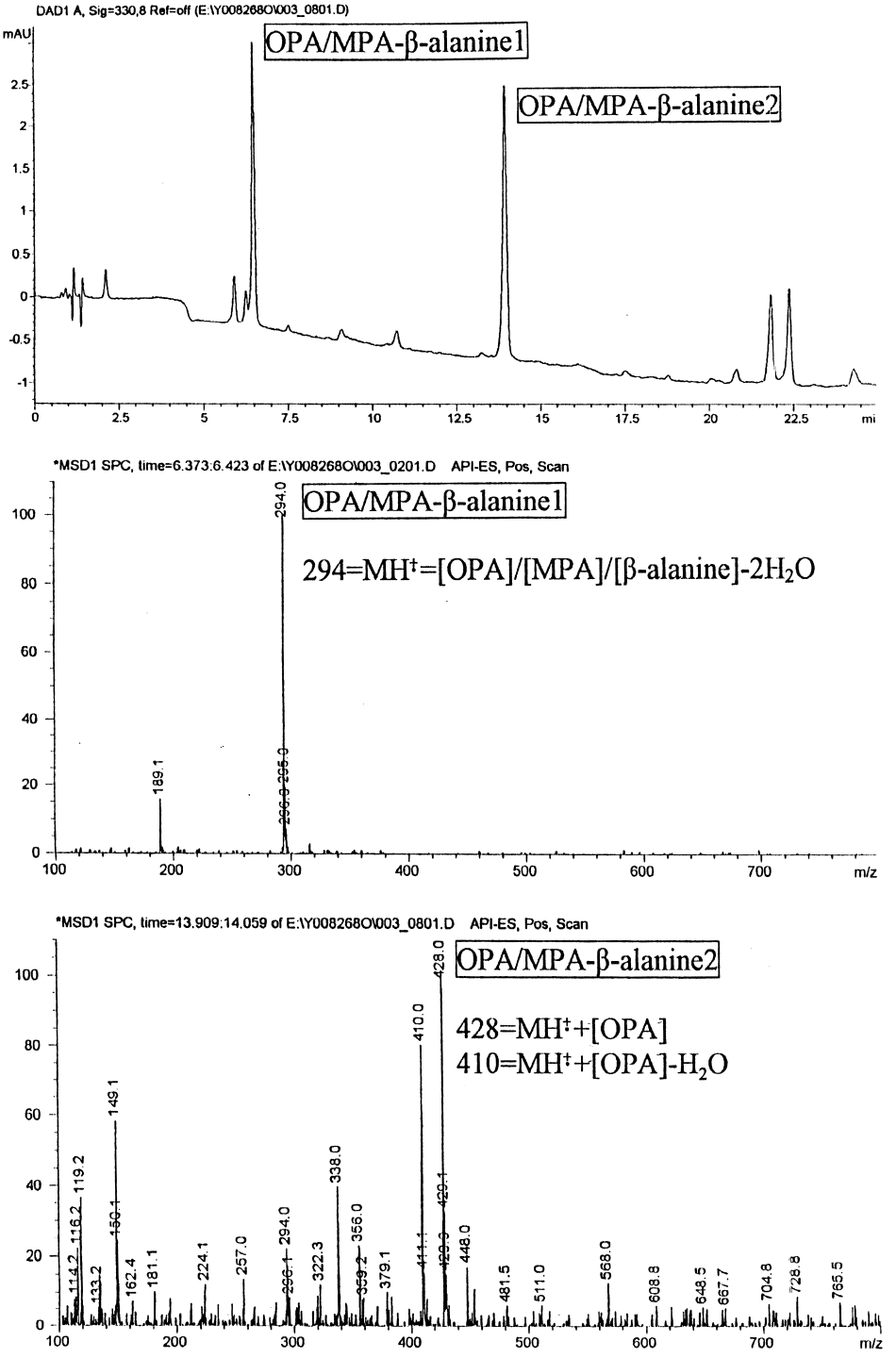
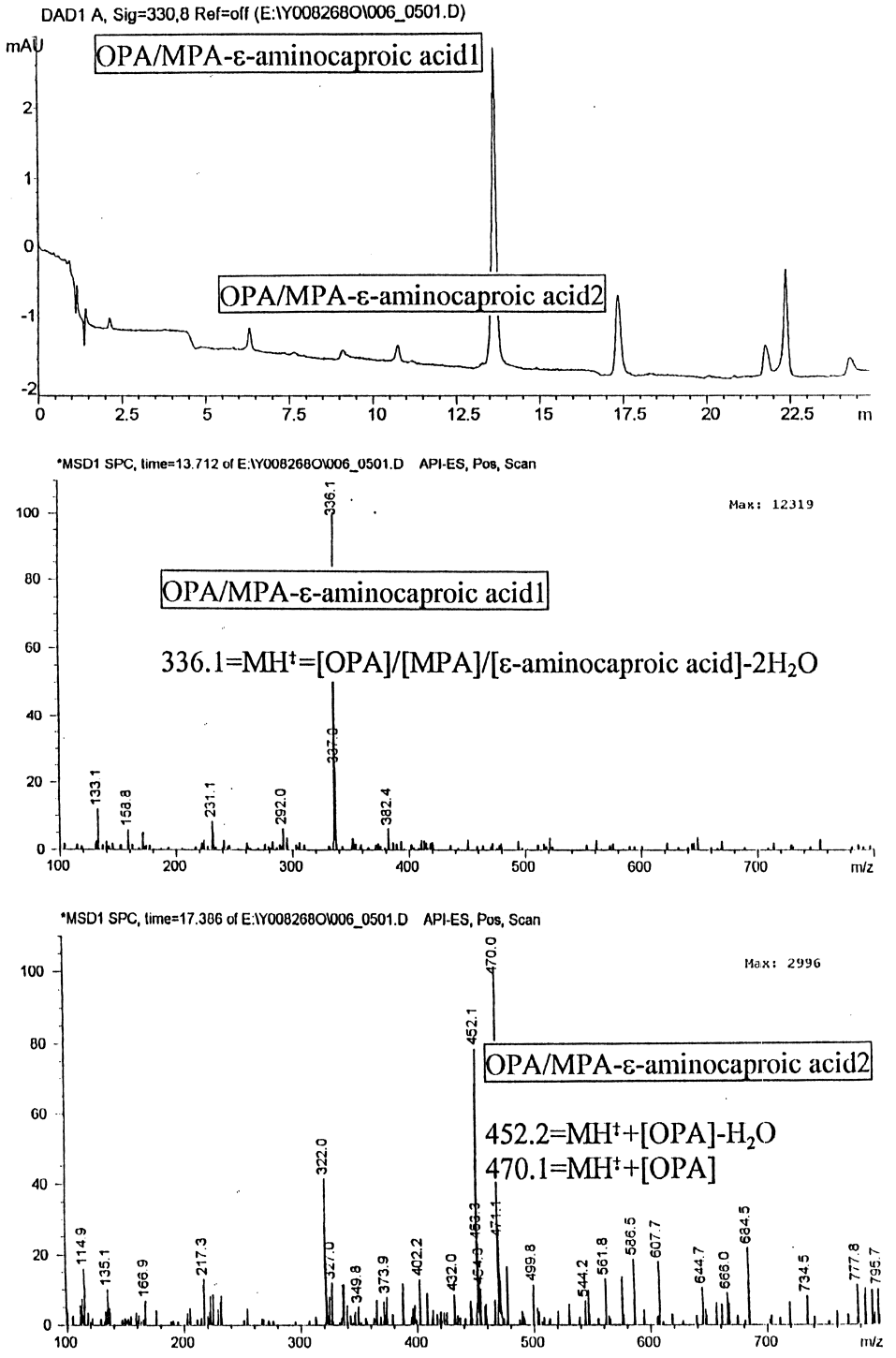


Fig. 1. (*continued*)



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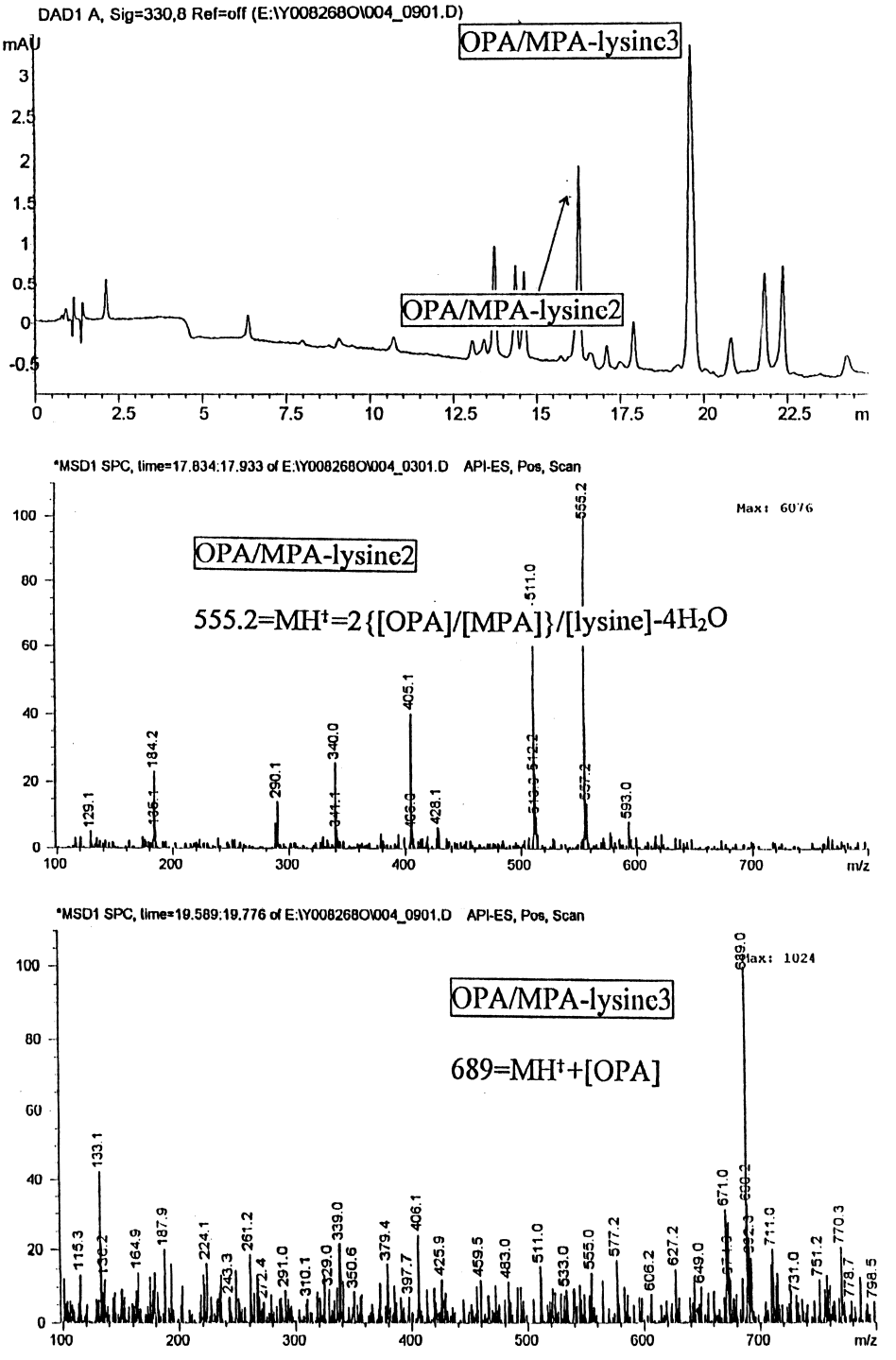

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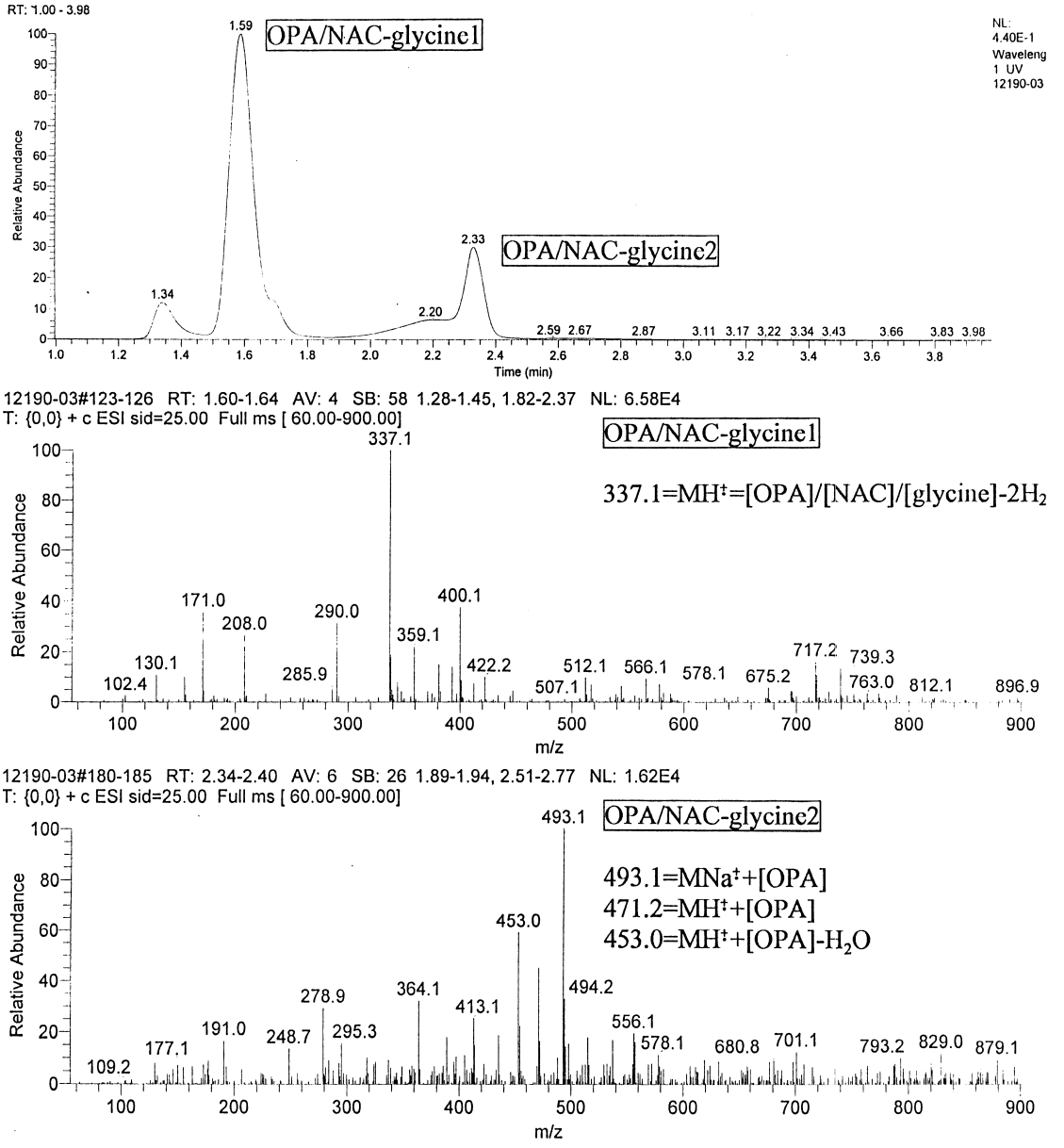


Fig. 1. (*continued*)

abundant fragments, in spectra of all initially formed derivatives, with three exceptions (OPA/NAC derivatives of histidine, tyramine (TYR) and spermidine (SPD).

(ii) Further selective fragment ions (SFIs) 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. 1/1, 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 1, 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-

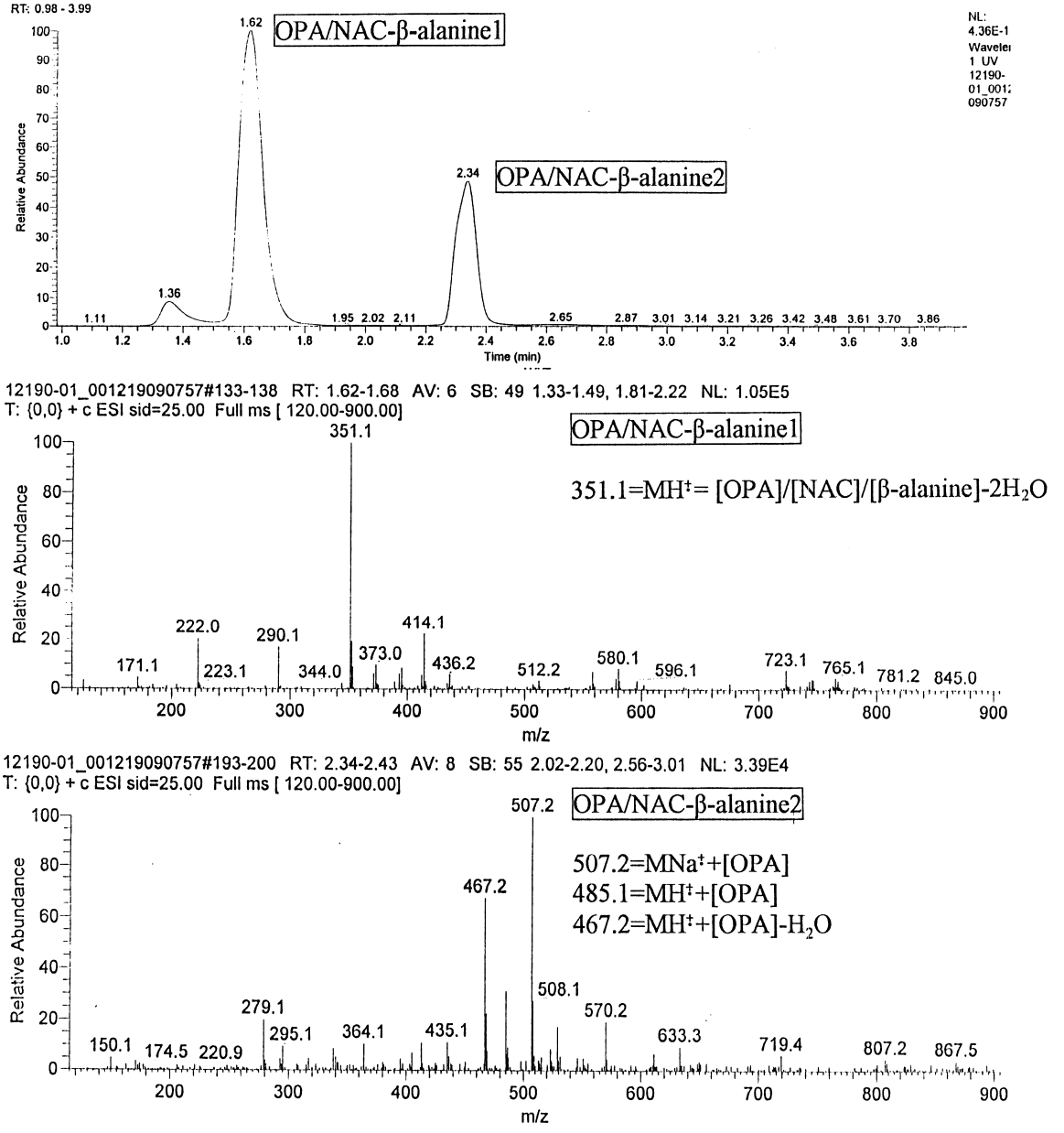


Fig. 1. (*continued*)

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. 1/1–1/4), spectra of glycine1, β-alanine1, ϵ-aminocaproic acid1 and lysine2).

4.3. Fragments of the transformed derivatives

Table 1, ions of the transformed OPA/MPA and OPA/NAC derivatives, Fig. 1/1–1/17), compounds indicated by number2, (with the exception of

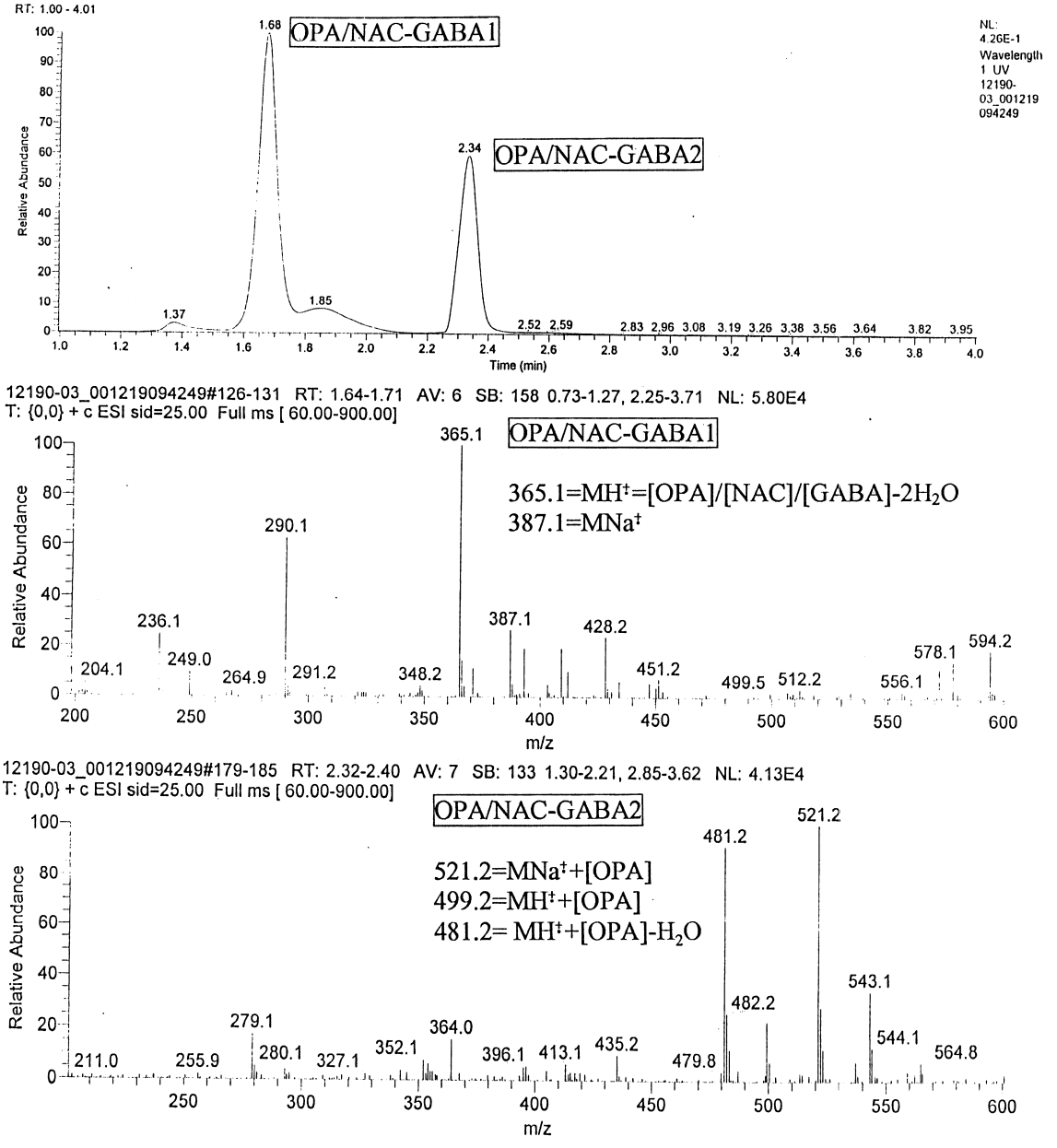


Fig. 1. (*continued*)

ornithine2 and lysine2 which are initially formed OPA species), as well as ornithine3, lysine3, cadaverine3 and putrescine3.

(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 ionized).

(ii) Dehydrated species — obtained from the transformed molecular ions by elimination of one

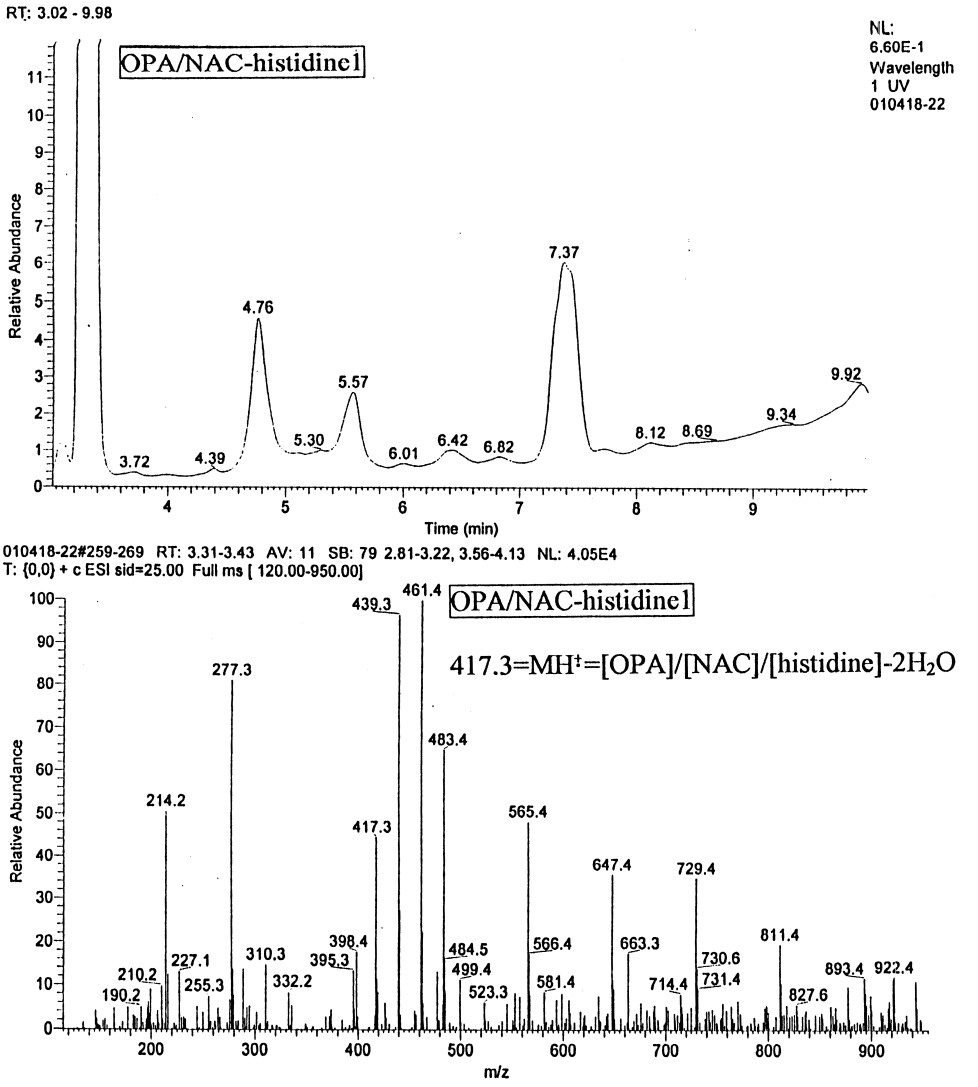


Fig. 1. (*continued*)

molecule of water ($MH^+ + OPA\text{-}H_2O$) — were masses of high intensity. The presence of these fragments is of primary importance corresponding to the stabilized endproduct of our proposed mechanism-scheme (Fig. 2: compounds VII and VIII).

(iii) Special fragments (Table 1, m/z values in the last vertical column) both with the same masses (m/z 278.2–279.1) and those originated by loss of this ominous mass (m/z 879.5–278.9 = 600.6 and m/z 893.7–279.1 = 614.6) furnished convincing evidence to the suggested reaction pathway, as shown in Fig. 2 to the origin and to the final structure of the transformed products (compound VIII of Fig. 2).

(iiia) 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

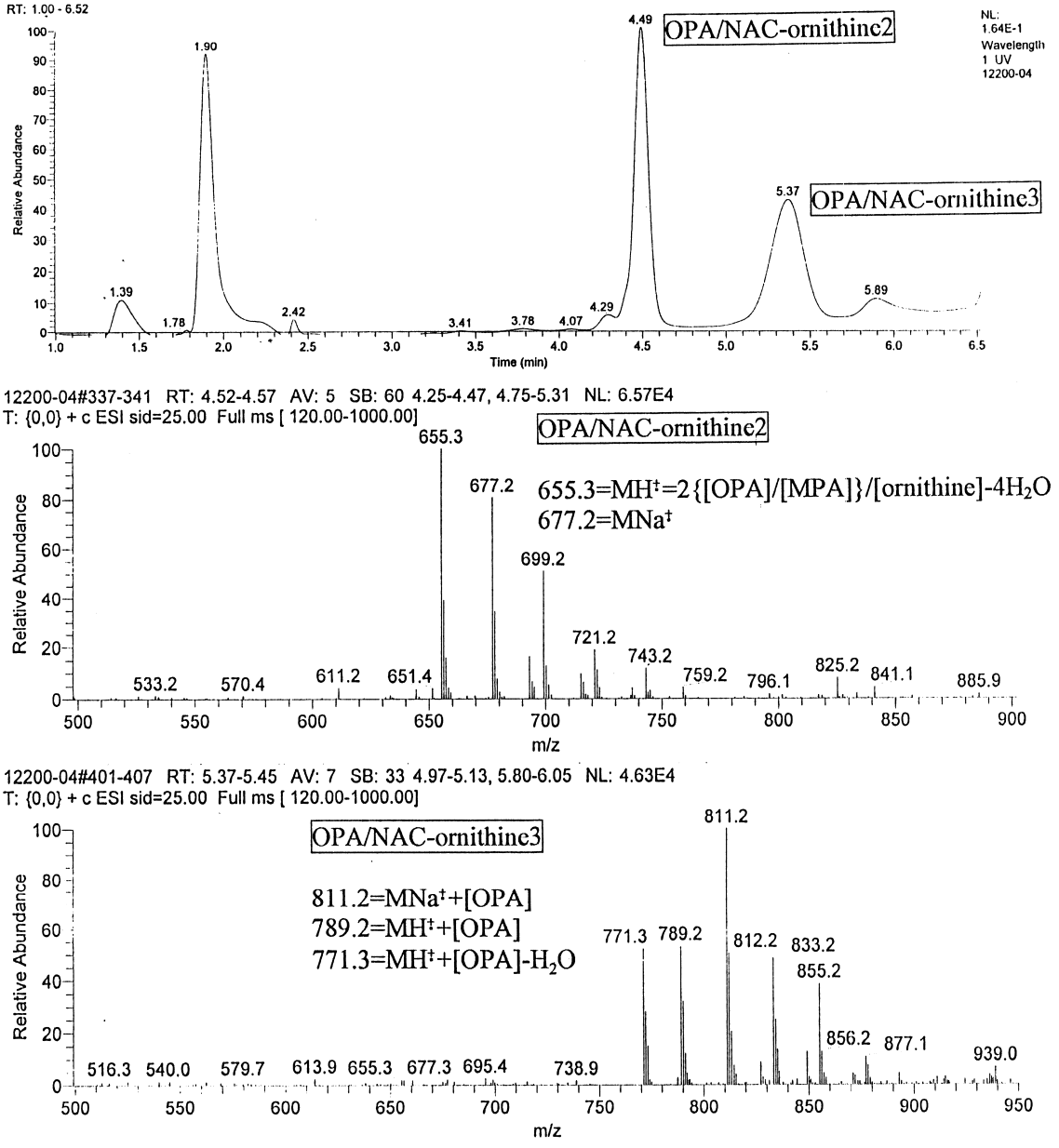


Fig. 1. (*continued*)

the transformed endproduct, belonging both to the SH group and to the NH_2 containing parts, (Fig. 2: R^1 and R^2 substituents), further on: aliphatic chain free endproduct).

(iiib) Ions with masses of m/z 600.6 and m/z 614.6 detected in the spectra of putrescine3 and cadaverine3 (Fig. 1/13 and 1/14)) proved to be a confirmation of our reaction scheme. Both originating from the corresponding, transformed, dimer molecular ions ($MH^+ + 2OPA$) by loss of the aliphatic chain free endproduct (Table 1, Fig. 1/13): 879.6–600.6 = 279.0; Fig. 1/14: 893.7–614.6 = 279.1) This observation can be explained by the reaction pathway detailed in Fig. 3.

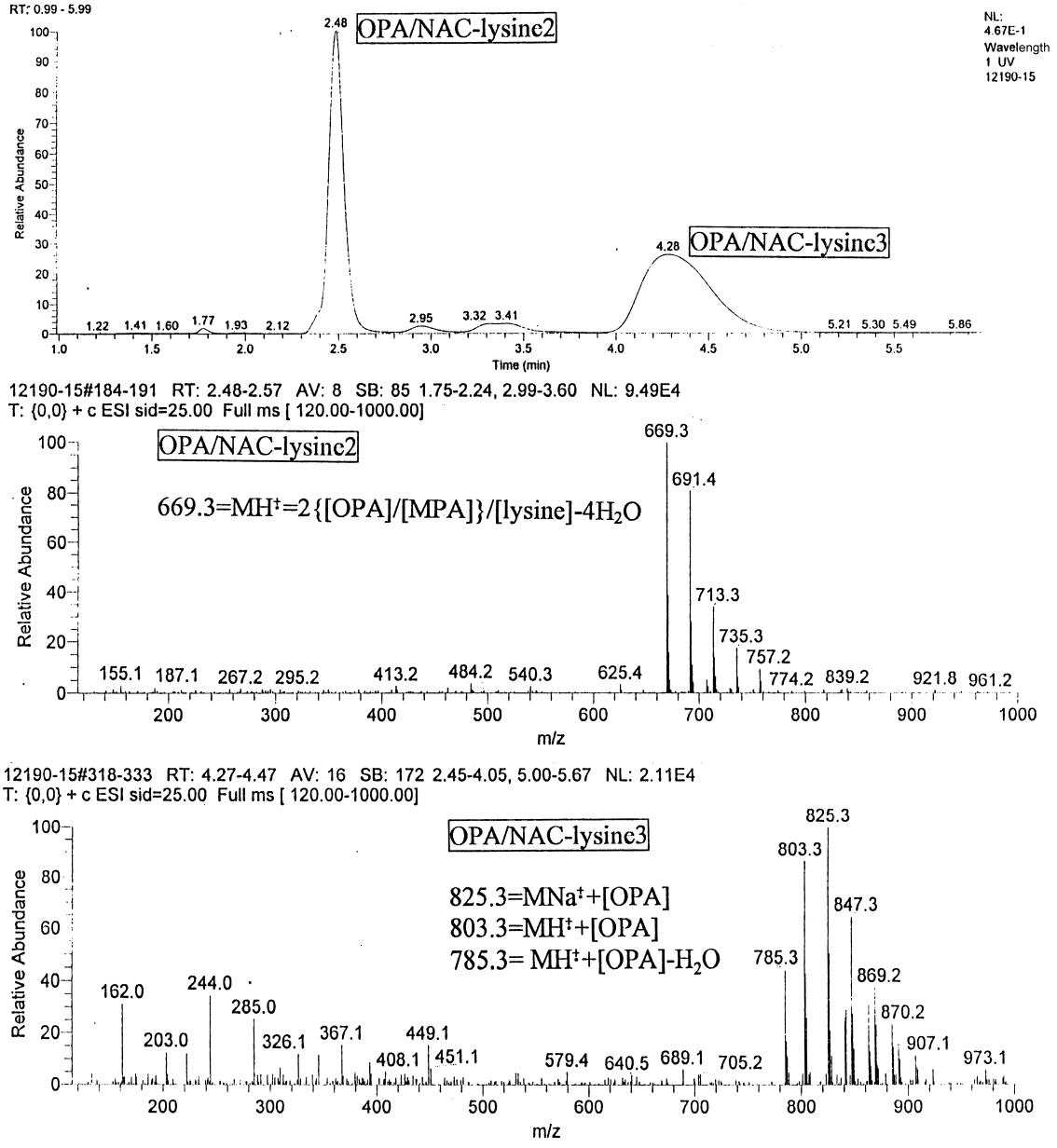


Fig. 1. (*continued*)

The formation of fragments m/z 600.6 and m/z 614.6 implies two consecutive steps. The initial step, i.e., the prerequisite condition involves the intermolecular-transhydration and subsequent aromatization of the transformed dimer resulting in species II (Fig. 3: via species I to II). Species II can be regarded as a prerequisite condition to a [3,3′]-sigmatropic rearrangement followed by cleavage of the molecule into the aliphatic chain free endproduct (Fig. 3, IIa: m/z 279) and into the stabilized remainder of the molecule (Fig. 3, IIb: m/z 600.6 and m/z 614.6).

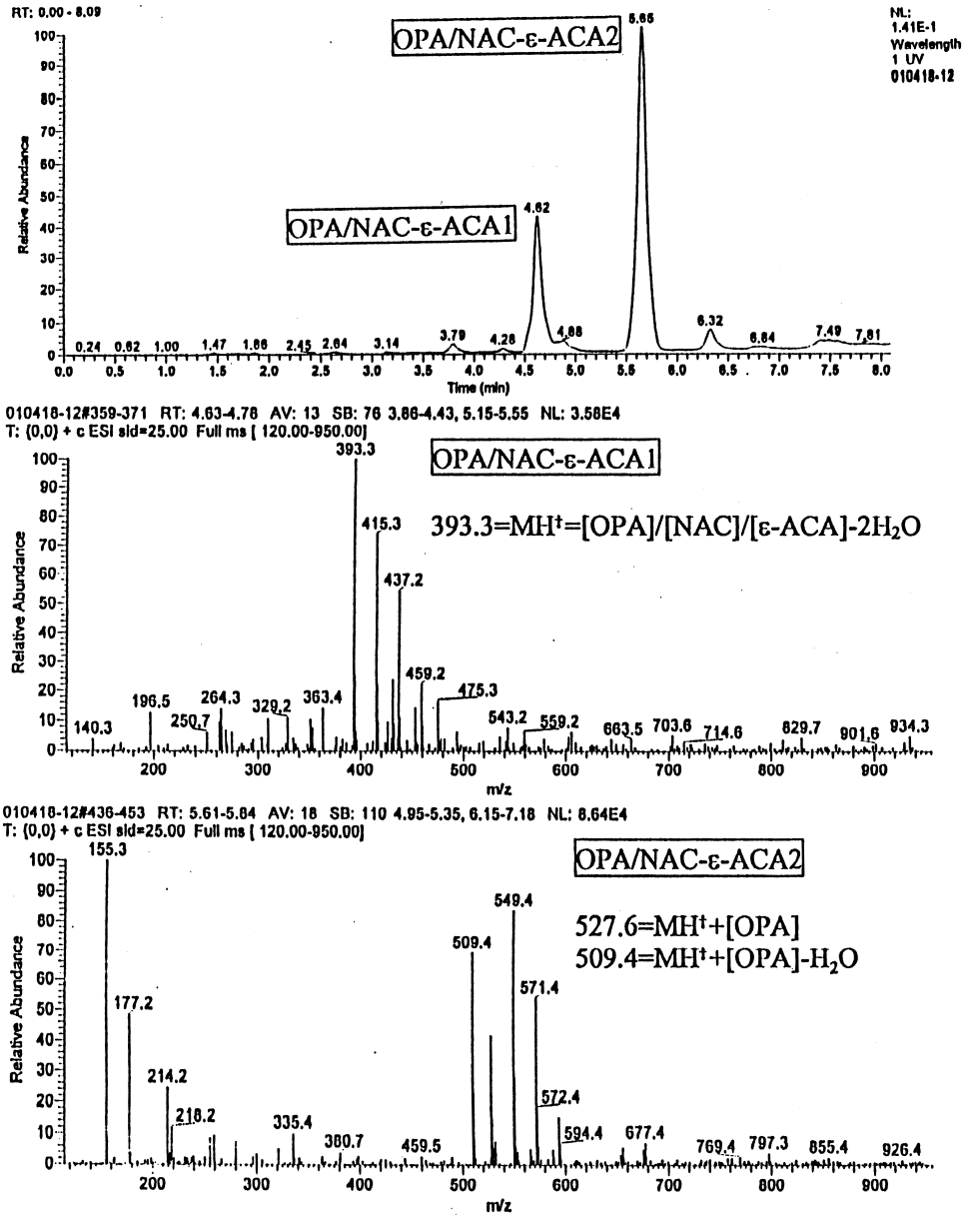


Fig. 1. (*continued*)

4.4. 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 [2–6] 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 2). 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. Refer-

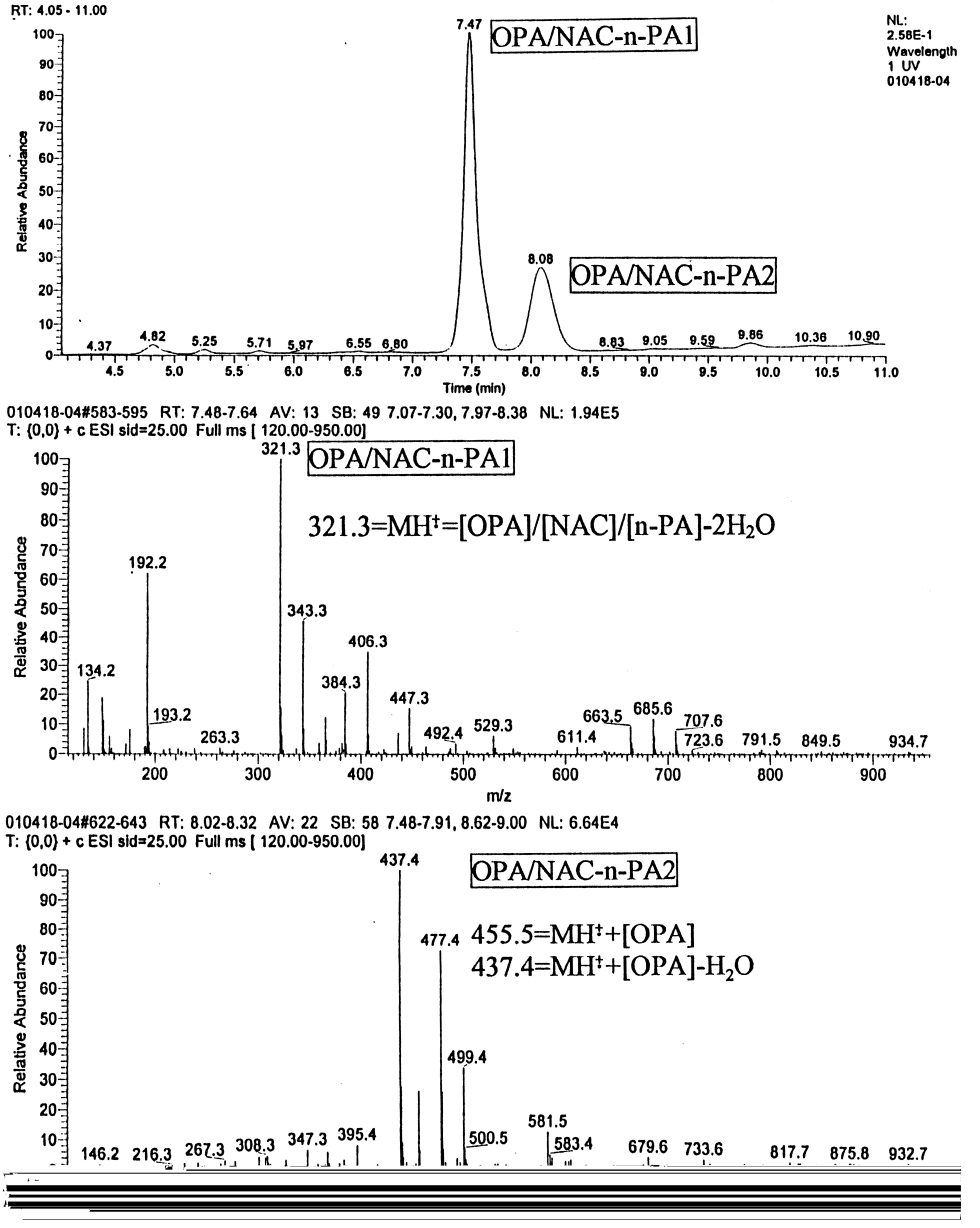
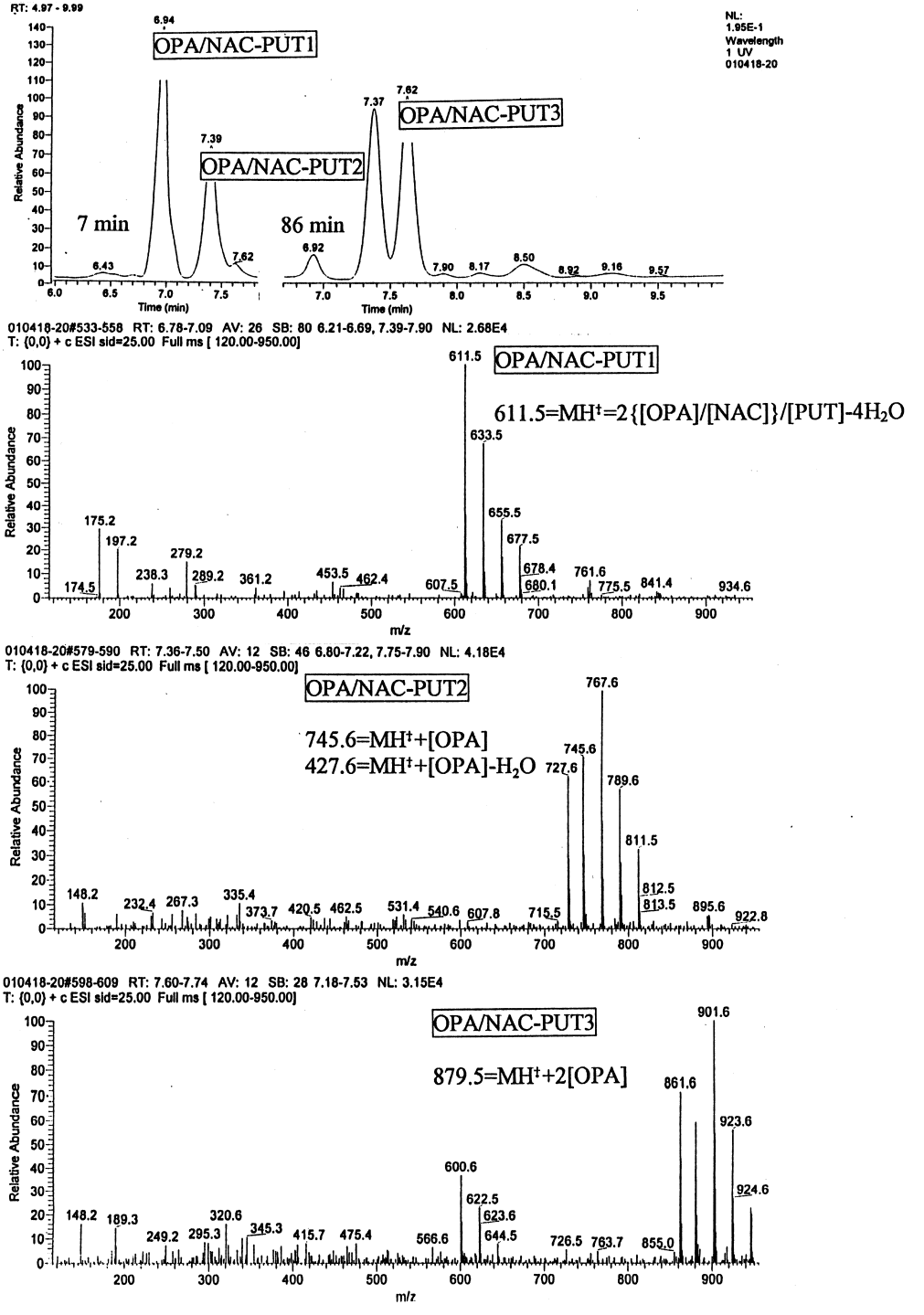


Fig. 1. (*continued*)

ring to Fig. 2 the mobilizable H atom of the CH_2 group next to the isoindole Nitrogen initializes the reaction with an additional OPA molecule (Fig. 2: 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. 2. This as-



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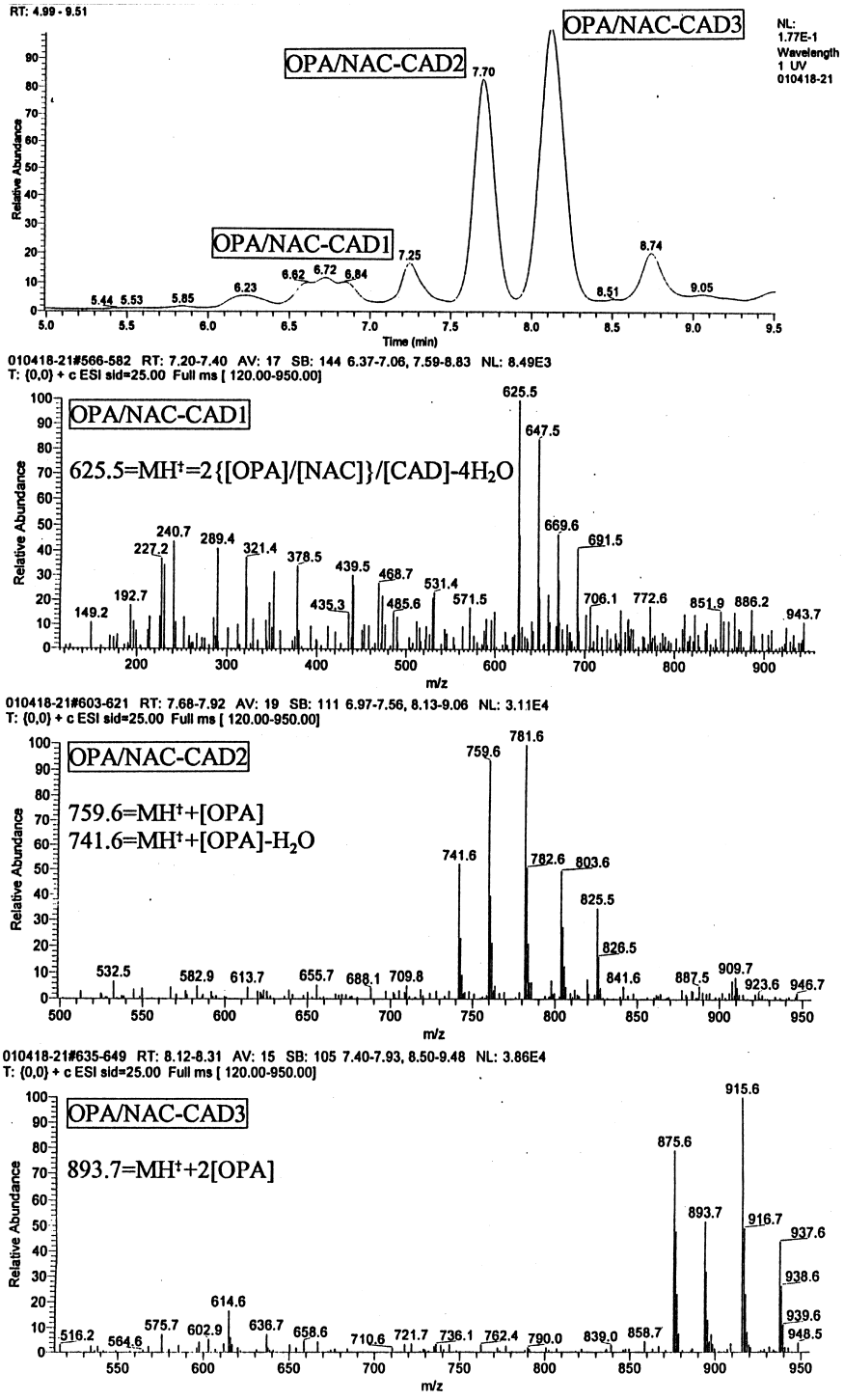

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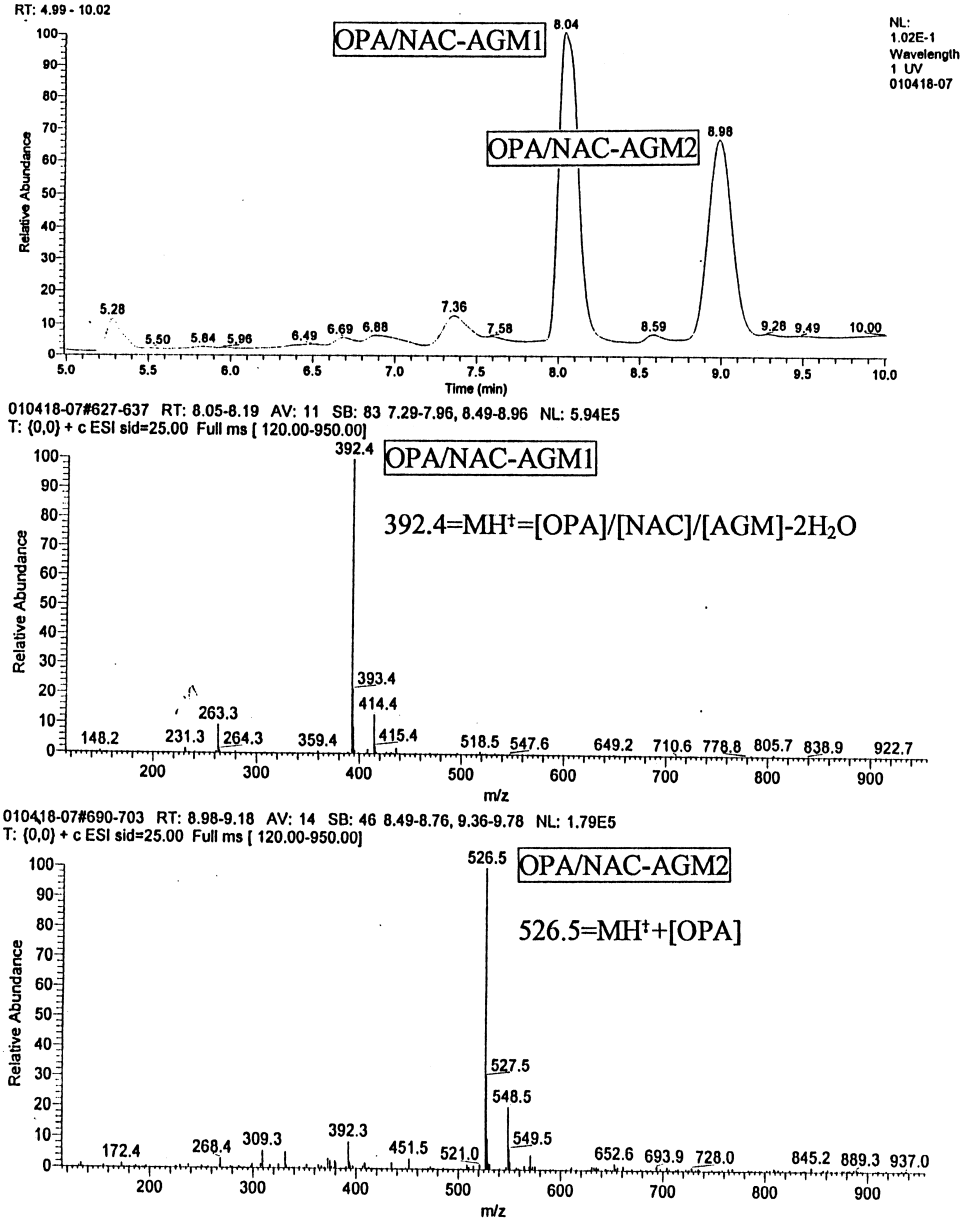

Fig. 1. (*continued*)

sumed mechanism was supported by MS data (Figs. 1/1–1/17, 2 and 3) and further completed by analytical results.

4.5. Considerations of analytical consequences

In the light of our recent stoichiometric experiences [5] and in the knowledge of the composition of the transformed derivatives — based on their MS spectra (Fig. 1/1–1/17) and on the reaction pathway they are originating from (Figs. 2 and 3) — we decided to change the composition of the reagent. The mole ratio of OPA to the SH additive was altered for OPA/MPA and to OPA/NAC = 1/50.

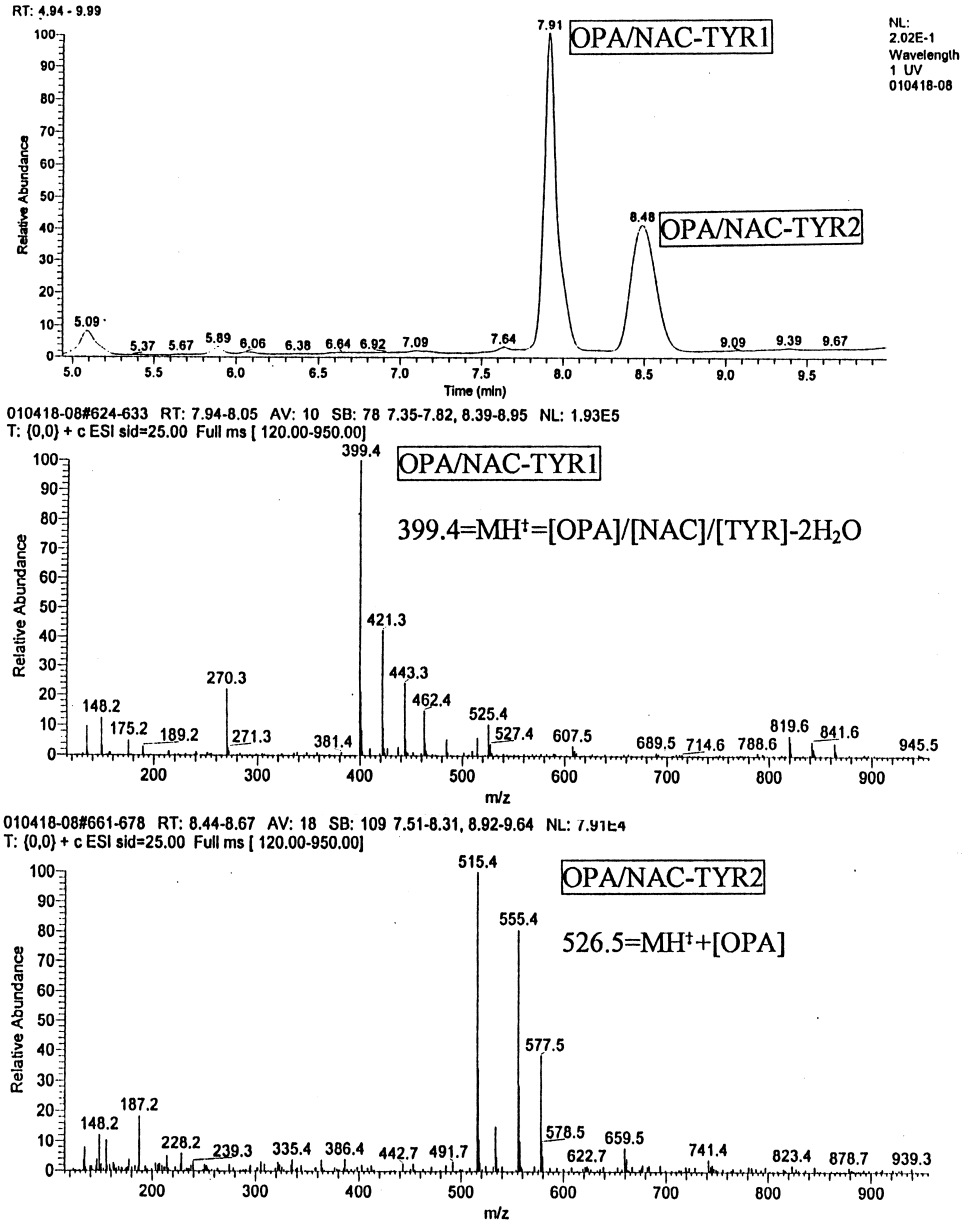

Fig. 1. (*continued*)

Since, recently [5] we found that the higher the concentration of the SH-group additive (MPA or NAC) the lower the amount of the transformed product. It means that by changing the mole ratios of OPA/MPA and OPA/NAC from 1/0.5 to 1/10 resulted averagely in a fourfold decrease of the transformed derivative: ranging from 80% (OPA/MPA and OPA/NAC = 1/0.5) to 20% (OPA/MPA

Table 2
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, iso-C_4 amines	NH_2CH_3, NH_2–$(CH_2)_{2-5}$
C_2–C_5 Diamines	NH_2CH_2–$(CH_2)_{1-4}$–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)–(CH2)$_2$–*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–*C*NH_2CH_3–CH_3

OPA/NAC 1/50 derivatized ones, could be considerably decreased as expected: unfortunately the quantitative inhibition of side reactions, in contrary to data obtained with several amines [6], could not be achieved. Moreover, in the case of the diamino group containing ornithine and lysine, new species have been detected (Table 3, Fig. 4: derivatives indicated by X1 and X2 marks) and the reaction rate of histidine and ornithine became slower (Table 4).

(ii) OPA/NAC-ornithineX1 and OPA/NAC-lysineX1 (Fig. 4) were eluted before the initially formed main products (ornithine2, lysine2), while OPA/MPA-ornithineX2 and OPA/MPA-lysineX2 (not shown) were detected after the transformed ones (ornithine3 and lysine3).

The amounts of all four species furnish substantially the same characteristics: both their fluorescence intensities and their UV responses decrease considerably with increased reaction time and became incorporated into the initially formed derivatives (Table 3). In the case of ornithine the 24.5% fluorescence intensity of OPA/NAC-ornithineX1, found after 7 min reaction time, disappeared and became incorporated into OPA/NAC-ornithine2 (its fluorescence after 7 min: 75.6%, after 3 h: 93.7%), while the 31.4% of OPA/MPA-ornithineX2, defined on its fluorescence, decreased after 3 h for 1.3%, and, became also incorporated into OPA/MPA-ornithine2 (its fluorescence after 7 min: 67.8%, after 3 h: 91.2). In lysine's cases the amount of OPA/NAC-lysineX1 is of secondary importance ($\leq$4.6%) while in parallel to the decrease of OPA/MPA-lysineX2 ($\leq$14.4%) OPA/MPA-lysine3 became increased (Table 3).

(iii) As to the decreased reaction rate of histidine and ornithine (Table 3: responses after 7 min) extended reaction times needed to be tested: to achieve maximum response values 30 min reaction time proved to be satisfactory (Table 4).

(iii) Regarding the impact of the SH-group addi-

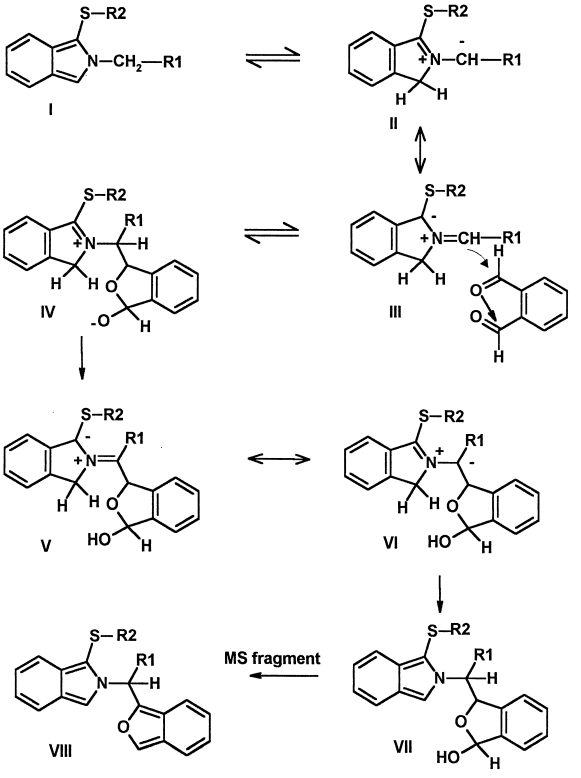

Fig. 2. Reaction pathway of the formation/transformation of the multiple derivative providing amino acids and amines (list of compounds in Table 2).

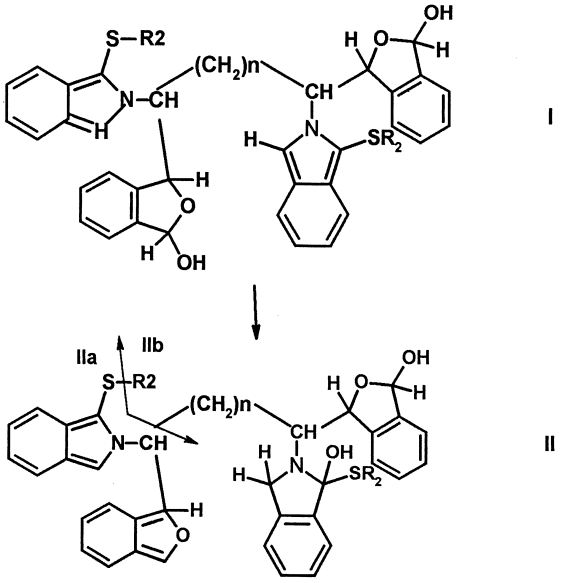

Fig. 3. Origin of ions m/z 278.2–279.1, m/z 600.6 and m/z 614.6 obtained from the transformed molecular ions (MH^+) of putrescine3 (m/z 879.6–279.0) and cadaverine3 (m/z 893.7–279.1); ($MH^+ = 2\{[OPA][NAC]\}[A] + 2[OPA] - 4H_2O$). Detailed data in Table 1.

tive it has been repeatedly proven [2–6] that the use of the OPA/NAC reagent is to be preferred: the percentage of side products obtained with the OPA/NAC reagent are lower, and their overall stability proved to be higher.

5. Conclusion

(1) Based on our MS spectra of the multiple derivatives furnishing OPA-amino acids and amines the nightmare of their instability found in the corresponding literature [12] has been solved. Since according to an earlier note [12] ‘‘. . . neither explanation appears to adequately explain the manner in which excess OPA catalyzes the degradation of Ic’’ (author’s note: Ic = isoindole)

(2) We confirmed for the first time that the common characteristic of the believed to be less stable OPA derivatives is associated with their original molecular structure: they do contain the $-NH_2-CH_2-R$ moiety and all of them furnish more than one OPA derivative.

(3) In the light of the common structure of the multiple OPA derivatives providing compounds a plausible reaction pathway has been described. According to the proposed mechanism hydrogen atoms of the $-CH_2-$ group (neighbor to the primary amino group) play the key role in the reaction with an additional molecule of OPA, resulting in consecutive OPA derivatives transformed from the initially obtained species.

(4) In the knowledge of the structure of the transformed derivatives an alternative reagent composition was tested in order to decrease the ‘‘ready to react’’ free OPA concentration. Changing the molecular ratios of OPA/MPA and/or OPA/NAC from 1/3 to 1/50 resulted both in decreased amounts of

Table 3
Stability and characteristics of the OPA/NAC and OPA/MPA-amino acids as a function of the reaction time (7 min, 3 h, 6 h), based on Fluorescence (FL) and DAD (UV) detections

Amino acid	Ret Time (min) *	UV maxi-mum, (nm)	FL detection			UV detection			FL detection			UV detection			FL detection			UV detection		
			OPA/NAC=1/3**						OPA/NAC=1/50						OPA/MPA=1/50**					
			Response(%)***			Response(%)***			Response(%)***			Response(%)***			Response(%)***			Response(%)***		
			7 min	3 h	6 h	7 min	3 h	6 h	7 min	3 h	6 h	7 min	3 h	6 h	7 min	3 h	6 h	7 min	3 h	6 h
Glycine0	5.88	334	0.1	0.1	0.1	0.6	0.8	0.8	1.0	2.3	3.3	0.2	0.8	1.4	–	–	–	–	–	–
Glycine1	6.43	334	95.7	82.3	75.4	97.0	84.4	74.9	97.7	92.7	90.7	99.3	96.2	95.0	93.5	97.1	96.4	94.0	96.5	94.9
Glycine2	7.88	339	4.2	17.6	24.5	2.4	14.8	24.3	1.3	5.0	6.1	0.5	3.0	3.6	6.5	2.9	3.6	6.0	3.5	5.1
Int unit/pM			*5.43*	*5.68*	*5.74*	*0.43*	*0.45*	*0.45*	*5.30*	*5.54*	*5.68*	*0.42*	*0.42*	*0.42*	*5.13*	*4.58*	*4.27*	*0.41*	*0.39*	*0.37*
GABA0	6.13	334	0.1	0.3	0.5	0.1	0.3	0.5	0.4	1.6	2.8	0.2	0.7	1.2	–	–	–	–	–	–
GABA1	6.93	334	96.6	80.3	68.9	96.3	80.5	68.9	98.2	92.3	90.3	99.3	96.7	95.9	83.4	79.5	77.3	87.5	78.0	70.6
GABA2	7.88	339	3.3	19.4	30.6	3.6	19.2	30.4	1.4	6.1	6.9	0.5	2.6	2.9	16.6	20.5	22.7	12.5	22.0	29.4
Int unit/pM			*5.01*	*5.19*	*5.23*	*0.41*	*0.43*	*0.43*	*4.89*	*5.14*	*5.22*	*0.40*	*0.40*	*0.40*	*5.78*	*5.34*	*4.83*	*0.40*	*0.39*	*0.38*
β-Alanine0	7.62	334	–	–	–	–	–	–	0.2	1.8	3.0	0.5	1.6	2.4	0.8	1.1	0.3	–	1.5	3.4
β-Alanine1	8.68	334	96.0	84.2	78.5	94.9	85.7	78.5	97.0	94.2	92.5	98.0	96.4	95.3	97.3	95.6	93.8	99.5	95.7	93.5
β-Alanine2	12.32	339	4.0	15.8	21.5	5.1	14.3	21.5	2.8	4.0	4.5	1.5	2.0	2.3	1.9	3.3	3.2	0.5	2.8	3.1
Int unit/pM			*4.57*	*4.69*	*4.79*	*0.40*	*0.42*	*0.42*	*4.68*	*4.77*	*4.83*	*0.41*	*0.41*	*0.41*	*5.13*	*4.49*	*3.91*	*0.39*	*0.35*	*0.31*
Histidine0	5.70	334	4.3	4.7	4.8	5.0	5.7	5.7	–	–	–	–	–	–	–	–	–	–	–	–
Histidine1	8.12	334	40.5	41.5	44.1	64.0	65.0	64.1	46.1	47.7	49.4	74.2	74.0	74.9	77.4	75.9	75.1	86.3	84.2	81.1
Histidine2	12.07	353	55.2	53.8	51.1	31.0	29.3	30.2	53.9	52.3	50.6	25.8	26.0	25.1	22.6	24.1	24.9	13.7	15.8	18.8
Int unit/pM			*3.86*	*3.65*	*3.48*	*0.35*	*0.33*	*0.33*	*1.94*	*3.33*	*3.31*	*0.18*	*0.32*	*0.32*	*2.78*	*4.75*	*4.75*	*0.19*	*0.33*	*0.34*
OrnithineX1	9.80	334	–	–	–	–	–	–	24.4	–	–	5.5	–	–	–	–	–	–	–	–
Ornithine2	13.08	334	88.5	66.1	53.9	96.2	80.1	69.3	75.6	93.7	92.6	94.5	98.1	97.6	67.8	91.2	89.5	73.1	94.8	94.2
Ornithine3	13.40	339	11.5	33.9	46.1	3.8	19.9	30.7	–	6.3	7.4	–	1.9	2.4	0.6	7.5	9.0	1.0	3.9	4.7
OrnithineX2	14.98	334	–	–	–	–	–	–	–	–	–	–	–	–	31.4	1.3	1.5	25.9	1.3	1.1
Int unit/pM			*1.06*	*1.21*	*1.35*	*0.59*	*0.59*	*0.59*	*1.10*	*1.37*	*1.31*	*0.60*	*0.67*	*0.66*	*1.45*	*1.23*	*1.07*	*0.62*	*0.56*	*0.46*
LysineX1	7.58	334	–	–	–	–	–	–	4.6	2.0	2.0	1.2	0.8	0.8	–	–	–	–	–	–
Lysine1	8.92	334	93.3	62.6	46.6	96.5	75.8	61.7	94.5	90.8	88.2	99.6	95.0	94.4	83.0	84.4	82.7	86.4	91.8	90.9
Lysine2	9.48	339	6.7	37.4	53.4	3.5	24.2	38.3	0.9	7.2	9.8	0.4	5.0	5.6	2.6	14.0	15.6	1.3	7.0	7.9
LysineX2	11.63	334	–	–	–	–	–	–	–	–	–	–	–	–	14.4	1.6	1.7	12.3	1.2	1.2
Int unit/pM			*2.03*	*2.43*	*2.66*	*0.82*	*0.82*	*0.81*	2.19	2.26	2.27	*076*	*0.78*	*0.76*	*1.90*	*1.79*	*1.44*	*0.63*	*0.59*	*0.47*

Indications: *=retention times of the OPA/NAC derivatives (the corresponding OPA/MPA derivatives elute by some seconds later); **concentrations=[OPA]/[NAC]/[amino acids] and [OPA]/[MPA]/[amino acids]=20/1000/1 ($1=1\cdot10^{-9}$); ***=%, expressed in the total of responses.

the transformed species and in an increased stability of the total of derivatives. However, the quantitative elimination of the consecutive side reactions could not be achieved. Thus, in the case of amino acids, (in contrast to amines [6]) the altered reagent composition remained of theoretical importance. In order to obtain optimum analytical condition the earlier optimized [2–5] reagent composition (mole ratio of OPA/SH-group additive=1/3) and gradient elutions (ensuring the separation and quantitation of all derivatives) are to be followed.

Acknowledgements

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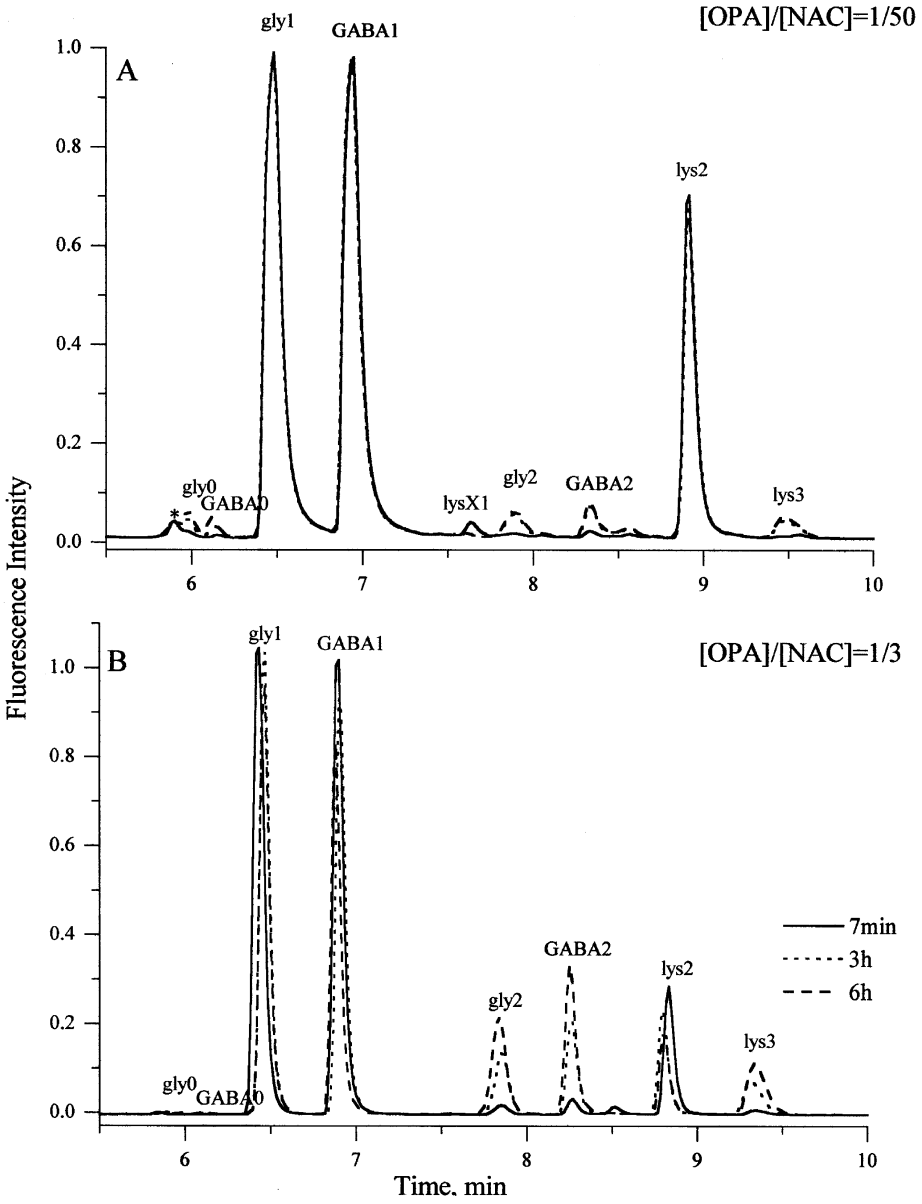

Fig. 4. Fluorescence chromatograms of glycine (gly), γ-aminobutyric acid (GABA) and lysine (lys) obtained after various reaction time (7 min, 6 h) with reagents of different mole ratios: [OPA]/[NAC]/[amino acid] = 20/1000/1 (A), 20/60/1 (B); Peaks: OPA/NAC derivatives of gly0, GABA0, gly1, GABA1, gly2, GABA2, lysX1, lys2, lys3.

Table 4
Stability and characteristics of the OPA/NAC-histidine and OPA/NAC-ornithine as a function of the reaction time (7, 15, 30 and 45 min)[a]

Amino acid	Ret time, (min) *	FL detection				UV detection			
		1/50**							
		Response(%)***				Response(%)***			
		7 min	15 m	30 min	45 min	7 min	15 min	30 min	45 min
Histidine1	8.12	41.6	40.7	40.5	40.7	74.8	73.8	73.6	73.9
Histidine2	12.07	58.4	59.3	59.5	59.3	25.2	26.2	26.4	26.1
Int unit/pM		*1.94*	*2.69*	*3.31*	*3.31*	*0.21*	*0.26*	*0.32*	*0.32*
OrnithineX1	9.80	24.4	5.7	1.1	0.5	5.5	1.8	1.0	0.8
Ornithine2	13.08	75.6	54.1	98.6	99.1	4.5	96.7	97.6	97.8
Ornithine3	13.40	–	0.2	0.3	0.4	–	1.5	1.4	1.4
Int unit/pM		*3.60*	*3.55*	*3.56*	*3.60*	*0.63*	*0.65*	*0.66*	*0.66*

Indications as in Table 3
[a] Based on fluorescence and DAD detections; [OPA]/[NAC]/[AAs]=20/1000/1 ($1=1\times10^{-9}$).

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