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Behavior and characteristics of biogenic amines, ornithine and lysine derivatized with the *o*-phthalaldehyde–ethanethiol–fluorenylmethyl chloroformate reagent

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Abstract

The stability and characteristics of the ornithine (Orn), lysine (Lys), putrescine (Put), cadaverine (Cad), 1,7-diaminoheptane (Diah), spermidine (Spd) and spermine (Spm) derivatives obtained with the *o*-phthalaldehyde (OPA)–ethanethiol (ET)–fluorenylmethyl chloroformate (FMOC) reagent has been investigated. The stoichiometry of the introduced, two-step derivatization process has been followed by photodiode array (DAD) and fluorescence (FL) detections, simultaneously, while the composition of derivatives was confirmed by on-line HPLC–electrospray ionization (ESI) MS measurements. Depending on the composition of the OPA reagents, in addition to the secondary amino group-containing Spd and Spm, under common aqueous conditions also Orn and Lys do react with FMOC resulting in derivatives of various compositions. Applying the OPA–ET reagent of increasing methanol (Met) content (38–80%, v/v) the formation of the FMOC group containing Orn and Lys derivatives could be considerably decreased. Optimum elution condition (18 min, including equilibration) was developed for the simultaneous quantitation of Orn, Lys, Put, Cad, Diah, Spd and Spm, in the presence of the rest of protein amino acids. The practical utility of the method was demonstrated by the analysis of mouse tissues. Average reproducibility of quantitations, characterized with the relative standard deviation percentages of fluorescence intensities and UV responses, in order of listing, proved to be 2.1% and 2.1%, respectively.

Keywords: Biogenic amines; Ornithine; Lysine; Derivatization; LC

1. Introduction

In the recent years, several characteristics of the reaction between the primary amino group-containing compounds and the OPA reagents, relating to the behavior of amino acids [1–10,12] and amines [6–11,13], have been clarified in our laboratory [1–13]. Due

(i) to the fast transformation of the initially formed OPA–3-mercaptopropionc acid (MPA) and OPA–*N*-acetyl-L-cysteine (NAC) derivatives of biogenic amines (BAs) to further products [7], as well as
(ii) to the limited, therefore, unfeasible possibility providing literature proposal [14] (HPLC of the OPA–MPA derivatives of Put, Cad, Spd and Spm at 10 °C temperature),

we have been looking for new approaches [6–11,13]. This paper describes a basically improved possibility for the quantitation of BAs together with Orn and Lys: applying the OPA–ET–FMOC reagent, a new combination of the two-step derivatization technique. The unexpected behavior of Orn and Lys, the stability, transformation properties and composition of derivatives as well as the analytical applicability of the protocol will be given in details.

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2. Experimental

2.1. Materials

OPA, MPA, ET, FMOC, Orn·HCL, Lys, Put·2HCl, Cad·2HCl, Diah (as internal standard, IS), Spd·3HCl and Spm·4HCl were obtained from Sigma (St. Louis, MO, USA) and from Serva (Heidelberg, Germany). HPLC-grade methanol (MET) and acetonitrile (ACN) were products of Riedel-de Haën purchased from Sigma-Aldrich (D-30926 Seelze, Germany). All other reagents were of the highest purity available.

2.2. Standard solutions

Standard solutions of free amino acids and amines have been prepared with distilled water in the concentrations of $\sim 1–2 \times 10^{-2}$ M (with analytical precision) and further diluted before use. Stock solution of OPA contained 0.75 g OPA (weighed with analytical precision) in 50 mL MET (further on: methanolic OPA solution).

2.3. Buffer solution

Borate buffer (BB) stock solution (ST) was mixed from 0.8 M boric acid, containing 0.8 M potassium chloride and completed with different volume ratios of 0.8 M sodium hydroxide (SH), and water (W) to obtain BB of various pH values, such as, ST–SH–W (1:1:0) (BB: pH ≅ 10.5); ST–SH–W (2.4:1.4:1) (BB: pH ≅ 9.3); ST–SH–W (1.5:0.5:1) (BB: pH ≅ 8.8); ST–SH–W (1.2:0.2:1) (BB: pH ≅ 8.3).

2.4. Reagent solutions

[OPA]–[ET] (1:10) reagents—used under optimum analytical conditions—were obtained by mixing, in order of listing, 2.5 mL methanolic OPA, 2.0 mL borate buffer (BB: pH 9.3) and 200 μL ET and completed with MET up to 10.0 mL.

The final pH of the OPA/ET reagents, due to their high MET concentration, proved to be higher than the original BB. It means the pH values of the OPA–ET reagents—prepared with BB of pH values of 10.5, 9.3, 8.8 and 8.3—in order of listing, were 10.7, 9.8, 9.6 and 9.3, i.e., higher by 0.2, 0.5, 0.8 and 1.0 pH units, respectively.

The molar ratios of OPA to the SH-additive were varied from the OPA–ET (1:1) to the OPA–ET (1:50). These reagents were prepared as above with the only exception that the amounts of ET were varied from 20 to 1000 μL.

FMOC reagent was prepared from 0.17 g FMOC (weighed with analytical precision and made up to 5.00 mL with acetonitrile. It means that the molar ratios of the reagent's constituents were [OPA]–[ET]–[FMOC] ≈ (1:10:0.4), if not otherwise stated.

2.5. Derivatization

2.5.1. Characterization of the reagent solutions

Blank elutions were performed with freshly prepared reagent solutions (reagent's age ≥ 90 min [2]), saved in the refrigerator (∼4 °C) and injected by the robotic Autosampler, every day at least two times.

2.5.2. Studies with the OPA/ET reagents

Derivatizations were performed with reagents prepared at least 90 min earlier before use and saved no longer than 3 days. In contrary to our earlier experiences, obtained with the OPA–MPA [2], with the OPA–NAC [2], as well as with the OPA–2-mercaptoethanol (MCE) [10] reagents, the OPA–ET reagent should be freshly prepared, every third day. The calculated amounts of reagent solutions were mixed with the selected amounts of amino acids and amines and were reacted for 60 s before injection (if not otherwise stated).

2.5.3. Derivatization procedure (optimum condition)

Two-step reactions have been carried out as follows:

(i) in the first step, the OPA–ET (1:10) reagent was added with the molar ratios of [OPA]–[amino acids + amines] ≥20:1, followed after 60 s;
(ii) in the second step by the FMOC reagent, with the molar ratios of [OPA]–[amino acids + amines]–[FMOC] ≥20:1:5, for an additional 60 s. Total derivatization time, 2 min.

2.6. Chromatography

2.6.1. Stability and stoichiometric studies: simultaneous DAD and FL detection

The system was a Waters HPLC instrument (Waters Pharmaceutical Division, Milford, MA, USA), consisted of a Waters 996 DAD and a Waters 474 FL detectors, a Waters 600 Controller quaternary pump with a thermostable column area and a Waters 717 Autosampler, operating with the Millennium Software (version 2010, 1992–95, validated by ISO 9002). The columns were Hypersil ODS bonded phase (5 μm), 200 mm × 4.6 mm + 30 mm × 4.6 mm guard column (column1), or 150 mm × 4.6 mm + 20 mm × 4.6 mm guard column (column2).

Detections have been performed simultaneously: DAD and FL detectors were connected in order of listing. Blank tests, stoichiometric investigations have been recorded between 190 and 400 nm (DAD) and evaluated at 334/262 nm, as well, as at the optimum fluorescence wavelengths of isoindoles (Ex/Em = 337/454 nm).

On-line HPLC–ESI-MS studies simultaneous UV and MS detection was carried with a Thermo Finnigan TSQ Quantum AH apparatus (Thermo Finnigan, LC–MS Division, San Jose, CA, USA), consisted of a Surveyor DAD detector a TSQ Quantum AH detector, a Surveyor Autosampler, operating with the Xcalibur software 1.4 SRI.

Detections have been performed simultaneously, applying the Surveyor DAD and the TSQ Quantum AH detectors, connected in order of listing. Blank tests, concentration dependence have been recorded between 190 and 900 nm (UV), evaluated at 334 nm (OPA–ET-amino acids and 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); $V_{capillary}$: 3.5 kV).

2.7. Elution programs

According to the requirement of the various derivatives containing systems different elution programs have been followed. Applying three eluents: eluent A (prepared from 0.1 M sodium acetate–ACN–MET (46:44:10), mixed in volume ratios and titrated with CH_3COOH or NaOH to pH 7.2 ± 0.05), as well as, MET and ACN.

Final analytical and fragmentation studies were performed on column1 and column2, respectively, at 50 °C (flow rate, 1.8 mL/min), the following gradient protocol was applied: 60% A and 40% MET for 4 min; at 4.1 min changed for 30% A and 70% ACN, then increased ACN until 100%, linearly, up to 9 min, and, hold 100% ACN for additional 3 min. At 12.1 min, the initial 60% A and 40% Met were used (total elution time, 18 min). (Note: data in tables, obtained with various flow rate of eluents, without exception, in order to be comparable, have been calculated to 1 mL/min flow rate.)

3. Results and discussion

3.1. Derivatization of BAs with the OPA–ET reagents

Introductory investigations of BAs—based on our recent promising experiences with OPA–ET derivatization of the C_6–C_8 aliphatic amines [11]—were carried out with the OPA–ET reagent. Results revealed that, however, the peak profiles and responses of Put, Cad and the single secondary amino group-containing Spd could be accepted, in favor of the two secondary amino group-containing Spm, derivatization protocol should be altered/improved (Table 1, Fig. 1). The tiny OPA–ET-Spm's response (∼0.2 integrator unit/pM Spm) does not allow its reproducible/sensitive quantitation.

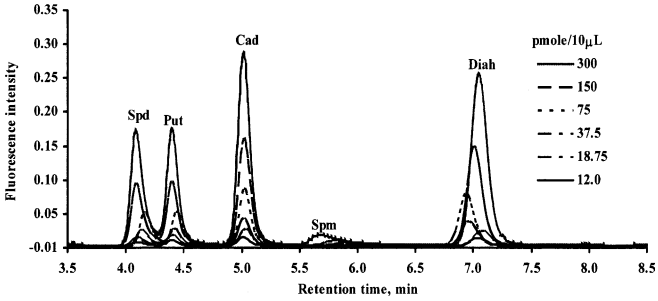

Fig. 1. Fluorescence detected chromatograms of various amounts of the OPA–ET derivatives of Spd, Put, Cad, Spm and Diah (internal standard) obtained with the [OPA]–[ET] (1:10) reagent; reaction time, 90 s (detailed results in Table 1).

3.2. Derivatization of BAs with the OPA–ET–FMOC reagents

To increase OPA–ET-Spm's response our attention was drawn to the two-step derivatization process, based on the pioneer experience with proline and hydroxyproline [15]. This principle [15], related exclusively to the two secondary amino group-containing amino acids, gained vide acceptance [16]: applying in the first step, the OPA–MPA; in the second step, the FMOC reagents [15,16].

In the present work, BAs were transformed into their OPA–ET derivatives in the first step, followed derivatization with FMOC in the second step. As a result of FMOC labeling Spd and Spm provided spectacularly excellent peak profiles, increased fluorescent responses and good reproducibility (Table 2, Fig. 2).

3.3. Extension of the OPA–ET–FMOC derivatization protocol to Orn and Lys

This special derivatization and chromatographic elution method was developed in the presence of the rest of free protein amino acids, in order to quantitate the free Orn and Lys contents of biological tissues, together with BAs, being primarily their precursors (Orn ⇒ Put, Lys ⇒ Cad).

(i) Introductory data, obtained with the OPA–ET–FMOC reagent, revealed that both the OPA–ET-Orn and the OPA–ET-Lys derivatives do transform into various products. These unexpected derivatives, in addition to their isoindole structure (UV maximum at 334 nm), do

Table 1
Quantitation of different amounts of the OPA–ET derivatives of biogenic amines (BAs) in model solutions on the basis of their fluorescence intensities (Ex/Em = 337/454); OPA–ET (1:10) reagent's methanol content 20% (v/v) (Fig. 1)

Amines	Retention time (min)	Integrator units/1 pmol biogenic amine							
		300	150	75	37.5	18.75	12.0	Average*	RSD (%)
Spermidine (Spd)	3.97–4.15	4.10	4.40	4.55	4.44	4.61	4.46	4.43	4.0
Putrescine (Put)	4.23–4.43	3.89	4.27	4.38	4.31	5.49	5.23	4.60	13.5
Cadaverine (Cad)	4.98–5.02	6.54	7.18	7.36	7.18	8.92	8.40	7.60	11.6
Spermine (Spm)	5.88–5.90	0.37	0.27	0.29	0.23	0.24	0.20	0.27	22.3
1,7-Diaminoheptane (Diah)	6.88–7.02	5.49	5.95	6.22	6.02	7.48	7.69	6.48	13.8

Indications: *Average(s), obtained from three separate tests have been calculated on the basis of integration units/1 pmol values.

Table 2
Quantitation of different amounts of the OPA–ET–FMOC derivatives of biogenic amines (BAs) in model solutions on the basis of their fluorescence intensities (Ex/Em = 337/454) and on their UV absorbance at 334 nm; OPA–ET reagent's methanol content 20% (v/v) (Fig. 2)

Amines	Retention time (min)	Integrator units/1 pmol biogenic amine							
		300	150	75	37.5	18.75	12.0	Average*	RSD (%)
On the basis of fluorescence detection									
Putrescine (Put)	6.00–6.06	4.55	4.62	4.67	4.77	4.52	4.70	4.64	2.0
Cadaverine (Cad)	7.08–7.16	8.64	8.61	8.62	8.35	8.21	8.26	8.45	2.3
1,7-Diaminoheptane (Diah)	10.30–10.36	7.91	8.12	8.28	8.13	7.95	7.97	8.06	1.8
Spermidine (Spd)	10.83–10.91	7.15	6.90	7.00	*6.06*	*5.70*	*5.56*	7.02	1.8
Spermine (Spm)	12.90–13.01	*4.93*	*4.61*	*4.28*	*3.81*	*3.66*	*2.80*	–	–
On the basis of UV detection									
Putrescine (Put)	5.97–6.03	0.69	0.70	0.70	0.66	0.72	0.68	0.69	3.0
Cadaverine (Cad)	7.05–7.13	0.66	0.67	0.68	0.66	0.69	0.64	0.67	2.7
1,7-Diaminoheptane (Diah)	10.26–10.32	0.63	0.62	0.61	0.61	0.60	0.60	0.61	1.9
Spermidine (Spd)	10.80–10.88	0.56	0.50	0.54	0.53	0.52	0.52	0.53	3.9
Spermine (Spm)	12.87–13.07	0.40	0.39	0.41	*0.33*	*0.30*	*0.28*	0.40	2.5

Indications: as in Table 1; italic printed values: have been omitted from the average values, they are read and used from the calibration curves; (–) no data available.

contain also the characteristic UV maximum values of the FMOC derivatives at 262 nm. On the basis of this experience, in order to inhibit/decrease this undesired process to the possible minimum extent, detailed stoichiometric investigations were needed.

(ii) Varying the molar ratios of the OPA–ET from 1:1 to 1:50, it turned out, that on the impact of the OPA–ET–FMOC reagent, the initially formed OPA–ET derivatives (Fig. 3: Orn1, Lys1) transform into various species (Fig. 3: Orn2–Orn5, Lys2–Lys5). The amounts of different products could be considerably influenced by the OPA–ET molar ratios, though not quantitatively eliminated: the higher the SH-additive concentration in the reagent, the lower the amount of transformed species.

Evaluating these data from analytical point of view, it turned out that minimum transformation takes place applying the reagent of OPA–ET (1:50) molar ratio: unfortunately, also this slowed down reaction provided transformation products (Fig. 3: Orn2–Orn5, Lys2–Lys5). Completing these data with the behavior of BA, on impact of OPA–ET ratios in the OPA–ET–FMOC reagent, as compromise for further studies the OPA–ET (1:10) molar ratio was selected (fast reaction, maximum response values for Spm). However, to optimize the two-step protocol, further approach seemed to be necessary.

(iii) The next change in the derivatization conditions related to the MET content of the medium: this alteration proved to be of primary importance: the higher the alcohol content, the lower the number and the amounts of the transformation species (Fig. 4). Increasing the reagent's MET content (38–80%, v/v) resulted in an additional advantage: the medium remains clear also after addition of the FMOC reagent.

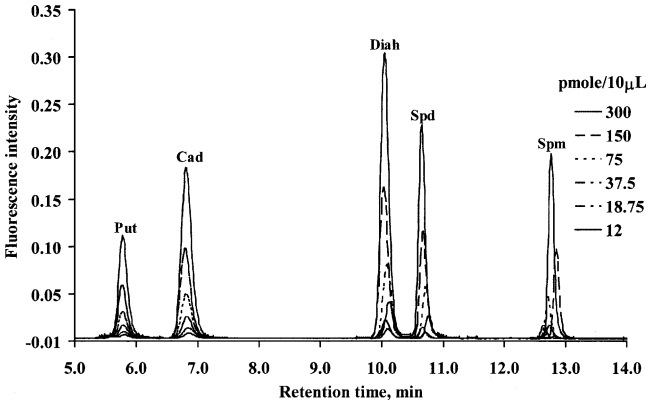

Fig. 2. Fluorescence detected chromatograms of various amounts of the OPA–ET–FMOC derivatives of Spd, Put, Cad, Spm and Diah (internal standard) obtained with the [OPA]–[ET]–[FMOC] (1:10:0.13) reagent; reaction time, 90 s + 90 s (detailed results in Table 2).

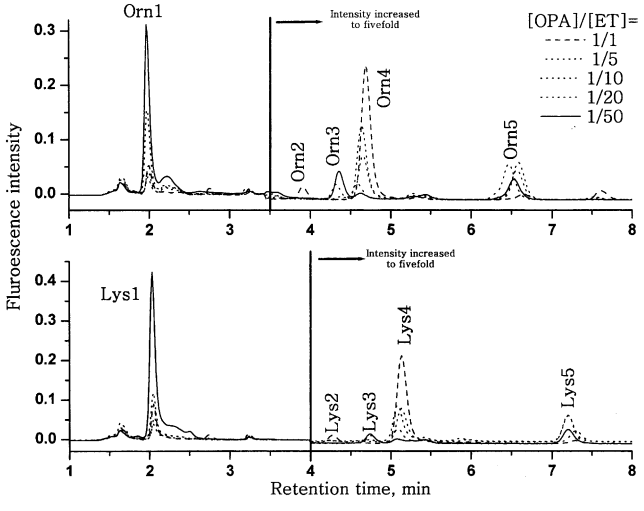

Fig. 3. Fluorescence detected chromatograms of the OPA–ET–FMOC derivatives of Orn and Lys obtained with various ET containing reagents [OPA]–[ET]–[FMOC] (1:1:0.3–1:50:0.3); chromatographic conditions: column2; elution: 0 min: MET 70%, A eluent 30%, 4 min: MET 75%, A eluent 25%, 4.1–10 min: 100% methanol, 10.1–18 min: MET 70%, A eluent 30%; flow rate, 1.5 mL·10^{-1}; temperature, 50 °C.

Table 3
Stability and characteristics of the OPA–ET derivatives of ornithine (Orn), lysine (Lys), putrescine (Put), cadaverine (Cad) and diaminoheptane (Diah) as a function of the reaction time based on fluorescence and UV detections

Amino acid/BA	Retention time (min)	UV_{max} (nm)	Response (%)															
			Fluorescence, reaction time (min)								UV, reaction time (min)							
			1	3	7	16	32	64	Average*	RSD (%)	1	3	7	16	32	64	Average*	RSD (%)
Orn1	3.13	334	99.03	99.14	99.93	98.87	98.83	98.61			100	99.76	99.80	99.60	99.12	99.29		
Orn2	4.98	334	0.50	0.52	0.66	0.55	0.57	0.75			0.00	0.16	0.12	0.28	0.30	0.33		
Orn3	5.75	349	0.45	0.34	0.41	0.57	0.61	0.65			0.00	0.08	0.08	0.12	0.18	0.37		
Int.u./1 pM			3.07	3.00	3.02	3.04	3.03	3.06	3.04	0.85	0.69	0.68	0.69	0.69	0.69	0.70	0.69	0.92
Lys1	3.54	334	99.95	99.98	99.95	99.87	99.81	99.27			99.95	99.97	99.89	99.91	99.83	99.25		
Lys2	5.82	334	0.02	0.00	0.00	0.04	0.06	0.15			0.00	0.00	0.02	0.00	0.00	0.03		
Lys3	6.73	339	0.03	0.02	0.05	0.09	0.13	0.59			0.05	0.03	0.10	0.09	0.17	0.72		
Int.u./1 pM			7.78	7.74	7.58	7.84	7.93	7.74	7.77	1.51	0.89	0.89	0.87	0.90	0.90	0.89	0.89	1.23
Put1	9.15	339	0.90	0.89	0.99	1.34	1.66	2.44			0.84	0.92	0.90	0.90	1.08	1.65		
Put2	9.57	334	99.10	99.11	99.01	98.66	98.34	97.56			99.16	99.08	99.10	99.10	98.82	98.35		
Int.u./1 pM			4.97	4.90	4.94	4.97	4.98	4.96	4.95	0.59	0.77	0.77	0.77	0.77	0.77	0.77	0.77	0.0
Cad1	9.45	339	0.10	0.l6	0.55	0.57	1.34	2.78				0.00	0.02	0.39	2.66	2.66		
Cad2	9.96	334	99.90	99.84	99.45	99.43	98.66	97.22			100.0	100.0	99.98	99.62	97.34	97.34		
Int.u./1 pM			11.87	11.85	11.81	11.99	12.10	12.04	11.94	0.98	1.17	1.16	1.14	1.18	1.15	1.15	1.16	1.27
Diah1	10.13	339	0.07	0.11	0.21	0.39	0.73	1.47			0.11	0.07	0.17	0.37	1.35	1.35		
Diah2	10.88	334	99.93	99.89	99.79	99.61	99.27	98.53			99.89	99.93	99.83	99.63	98.65	98.65		
Int.u./1 pM			10.28	9.65	9.67	9.82	9.93	9.63	9.83	2.54	0.79	0.78	0.79	0.80	0.78	0.78	0.79	1.04

Indications as in Tables 1 and 2; AAs, amino acids; response (%), calculated on the total of derivatives; Int.u., integrator units.

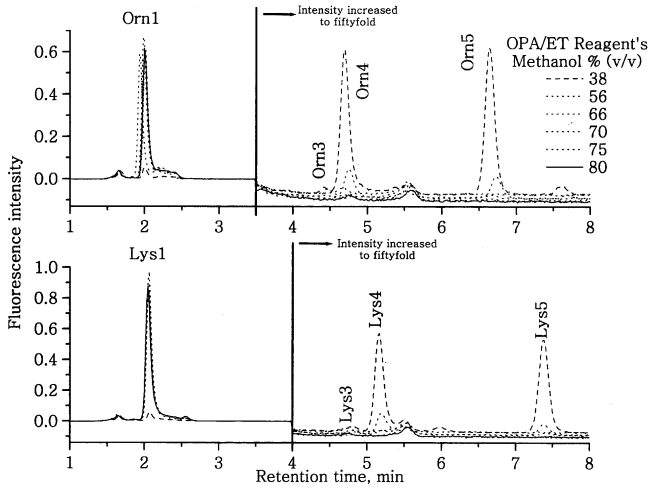

Fig. 4. Fluorescence detected chromatograms of the OPA–ET–FMOC derivatives of Orn and Lys obtained with various MET (38–80%, v/v) containing reagents [OPA]–[ET]–[FMOC] (1:10:0.3); chromatographic conditions as in Fig. 3.

3.4. Stability studies on Put, Cad, Diah, Spd, Spm, Orn and Lys derivatives obtained with the OPA–ET–FMOC reagents

Based on derivatization studies summarized in Sections 3.1–3.3 (Tables 1 and 2, Figs. 1–4), it seemed to be necessary to characterize stability of derivatives under the most promising, 80% (v/v) MET containing conditions: depending on the reaction time in thorough connection with the OPA–ET–FMOC reagent's composition and with the optimum pH values of the two-step derivatization process.

(i) In the first step, the stability of the OPA–ET derivatives of Orn, Lys, Put, Cad and Diah have been separately tested in 80% (v/v) MET containing solution: in order to be able to follow also the tiny amounts of their transformed species (Table 3). Results revealed that less than 1% transformation of the initially formed products can be expected after 1–3 min reaction times, only.

(ii) In the second approach, the optimum conditions of the second step process was to be defined (reagent's FMOC content and its pH value).

(iii) The reagent's FMOC concentration at pH 9.3 were varied in the range of the molar ratios as follows: [OPA]–[ET]–[FMOC] (1:10:0.06–1:10:0.6). It turned out that for sper-mine's maximum responses the molar ratios of FMOC–OPA ≥0.3:1 is to be followed.

(iv) As to the optimum pH values of the simultaneous derivatizations of the Put, Cad, Spd and Spm derivatives—carried out with the OPA–ET reagents of pH 8.6, 9.2, 9.6 and 10.6—it has been proved that the isoindole formation is fast and quantitative in the pH range tested (pH 8.6–10.6) compared to the FMOC labeling step (pH 9.2–9.6). Thus, the pH 9.3–9.4 has been chosen as optimum reaction medium.

Table 4
Stability and characteristics of the OPA–ET–FMOC derivatives of spermidine (Spd) and spermine (Spm) as a function of the reaction time based on fluorescence and UV detections; reagent: OPA–ET–FMOC (1:10:0.4), 80% (v/v) methanol content, pH 9.3

BA	Retention time (min)	UV_{max} (nm)	Response (%)										UV, reaction time (min)								
			Fluorescence, reaction time (min)																		
			1 + 1	3 + 1	1 + 3	1 + 7	1 + 16	1 + 32	1 + 64	Average	RSD (%)		1 + 1	3 + 1	1 + 3	1 + 7	1 + 16	1 + 32	1 + 64	Average	RSD (%)
Spd1	11.34	262/334	99.25	99.23	99.10	98.28	98.77	98.36	98.61				99.39	99.37	99.19	98.41	99.01	99.12	99.29		
Spd2	12.27	262/339	0.75	0.77	0.90	1.72	1.23	1.61	0.65				0.61	0.81	0.81	1.59	0.99	0.18	0.37		
	Int.u./1 pM⇒		8.04	7.93	8.13	8.44	8.21	8.00	*7.58*	8.13	2.3		0.78	0.77	0.77	0.79	0.77	0.76	*0.71*	0.77	1.3
Spm1	12.56	262/334	99.05	98.54	99.05	99.0	98.4	97.8	95.8				99.28	98.77	99.32	99.25	98.68	98.24	96.56		
Spm2	13.70	262/339	0.95	1.46	0.95	1.0	1.6	2.2	4.2				0.72	1.23	0.68	0.75	1.32	1.76	3.44		
	Int.u./1 pM⇒		6.69	*5.61*	6.80	6.46	6.05	6.20	*5.24*	6.44	4.9		0.61	0.61	0.65	0.62	0.60	0.60	*0.51*	0.62	3.0

Indications as in Tables 1–3.

Table 5
UV maximum values of the OPA–ET–FMOC-Spd and the OPA–ET–FMOC-Spm derivatives at 262 nm and at 334 nm; reagent: OPA–ET–FMOC (1:10:0.5)

Methanol, % (v/v)	Spermidine			Spermine		
	$A_{262\,nm}$	$A_{334\,nm}$	A_{262}/A_{334}	$A_{262\,nm}$	$A_{334\,nm}$	A_{262}/A_{334}
38	519	251	2.06	672	207	3.25
56	570	275	2.07	782	241	3.25
66	564	275	2.05	758	244	3.11
70	664	317	2.09	920	289	3.18
75	554	266	2.08	789	244	3.23
80	637	305	2.09	971	299	3.25

Indications: A, absorbency.

(v) Prior to characterize overall stability properties of the [OPA]–[ET]–[FMOC] derivatives of Spd and Spm, reactions were carried out after 1 + 1 min and 3 + 1 min (OPA + FMOC) derivatization steps. Since differences between the 1 and 3 min lasting OPA/ET reactions have not been found, the stability of the OPA–ET–FMOC derivatives have been followed and compared after 1 + 1, 1 + 3, 1 + 7, 1 + 16, 1 + 32 and 1 + 64 min reaction times of the mixed species (Table 4). Results proved that from analytical point of view the stability of derivatives are excellent. Transformation of the initially formed species do not exceed 1%.

3.5. Studies on the composition of the Put, Cad, DiaH, Spd, Spm, Orn and Lys derivatives obtained with the OPA–ET–FMOC reagents

3.5.1. UV characteristics of the OPA–ET–FMOC derivatives of Spd and Spm

As a result of DAD, following the UV maximum values of the OPA–ET–FMOC-Spd and -Spm derivatives in the 190–400 nm range, it turned out that they are providing two maximum values: at 262 and at 334 nm. Evaluating the extent of these UV absorbency values in various methanol-containing media (38–80%, v/v) they proved to be unambiguously consistent (Table 5). At 262 nm (characteristic to FMOC labeling), the OPA–ET–FMOC-Spd furnishes two times higher, the OPA–ET–FMOC-Spm derivatives three times higher absorbencies compared to their at 334 nm evaluated ones (characteristic to isoindole derivatives). This finding indicates that the molar absorbency of the two isoindole-functions seems to be approximately identical with the molar absorbency of one FMOC labeled function.

3.5.2. On-line HPLC–DAD–ESI-MS studies on the composition of the OPA–ET–FMOC derivatives of Put, Cad, Diah, Spd, Spm, Orn and Lys

These investigations have been carried out separately with BAs, including Diah as internal standard (Fig. 5A–F), as well as with Orn (Fig. 6A–G), and Lys (Fig. 7A–G): in order to have reliable possibility to evaluate all transformed species obtained from the reactions of Orn and Lys with the OPA–ET–FMOC reagent.

3.5.2.1. HPLC–DAD–ESI-MS study on the composition of the Put, Cad, Diah, Spd and, Spm derivatives. In the cases of BAs and Diah, HPLC–DAD–ESI-MS measurements confirmed their assumed, theoretical composition (Fig. 5A–F). As seen, the first line (Fig. 5A) shows the DAD profile of Put, Cad, Diah, Spd and Spm., while the forthcoming ones (Fig. 5B–F) represent their MS spectra, i.e., in order of their retention times.

The protonated molecular ions and their (by potassium) cationized versions {Fig. 5B, Put: MH^+ = 409.3 = [Put]([OPA]–[ET])$_2$, MK^+ = 447.2; Fig. 5C, Cad: MH^+ = 423.3, MK^+ = 461.2; Fig. 5D, Diah: MH^+ = 451.3, MK^+ = 489.3; Fig. 5E, Spd: MH^+ = 688.3, MK^+ = 726.3; Fig. 5F, Spm: MH^+ = 967.5, MK^+ = 1005.5}.

3.5.2.2. HPLC–DAD–ESI-MS study on the composition of Orn and Lys. As to derivatives obtained from the interaction of the OPA–ET–FMOC reagent with Orn (Fig. 6A–G) and Lys (Fig. 7A–G), they proved to be originated from identical reactions: without exceptions masses of the characteristic Orn and Lys derivatives are different in 14 mass units only (m/z = –CH_2–). (Note: due to the different HPLC systems, to the prolonged reaction times and to the 10 times diluted ammonium ion containing eluent, retention times and ratios of species—compared to those shown in Figs. 3 and 4—became modified; however, the number of species (Orn1–5, Lys1–5) and their retention orders proved to be the same.

Evaluating the DAD profiles of the various Orn and Lys derivatives (Figs. 6A, C and 7A, C) and the corresponding MS spectra of species (Figs. 6B, D–G and 7B, D–G), it turns out that:

Figs. 6A, B and 7A, B depict the initially formed classical isoindoles, i.e., the DAD profile of the OPA–ET-Orn and OPA–ET-Lys derivatives (Fig. 6A and 7A), their spectra containing the corresponding protonated molecular ion, their (by sodium) cationized versions and additional selective fragment ions {Fig. 6B, Orn1: MH^+ = 453.2 = [Orn]([OPA][ET])$_2$, MNa^+ = 475.2, $[MH–COO]^+$ = 409.3; Fig. 7B, Lys1: MH^+ = 467.3 = [Lys]([OPA][ET])$_2$, MNa^+ = 489.3, $[MH–COO]^+$ = 423.3;}.

The HPLC/MS(ESI) profile of the transformed Orn and Lys species (Figs. 6C–G and 7C–G) represent their DAD chromatograms (Fig. 6C: Orn2–Orn5, Fig. 7C: Lys2–Lys5) and their corresponding spectra (Figs. 6D–G and 7D–G).

Before going into further details, it is to be noted that all species are mixed derivatives labeled partly by the OPA–ET, partly by the FMOC reagent. In order of their formation they are the followings:

(i) Orn5 and Lys5 (Figs. 6G and 7G), the main components, are representing the protonated, dehydrated molecular ions and their (by potassium) cationized versions, the simplest, mixed derivatives {Orn5: MH^+ =

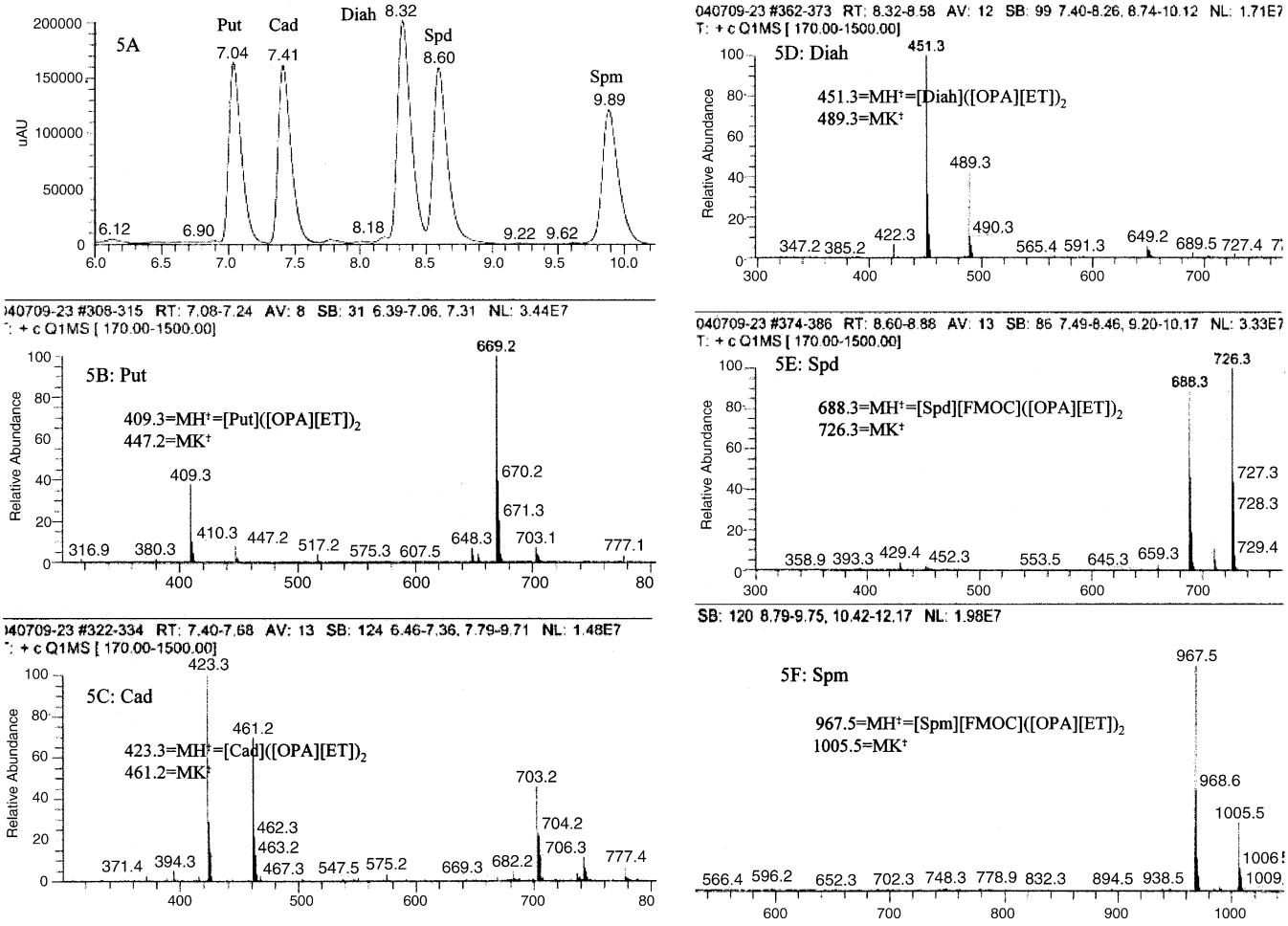

Fig. 5. DAD chromatogram of the OPA–ET–FMOC derivatives shown at 334 nm (A) and MS spectra (B–F) of Put, Cad, Diah, Spd and Spm. (Detailed composition of fragments in the text.)

497.2 = [OPA][ET][FMOC][Orn])–H_2O, MK^+ = m/z = 535.2); Lys5: MH^+ = 511.2 = [OPA][ET][FMOC][Lys]–H_2O, MK^+ = 549.2).

(ii) Orn4 and Lys4 (Figs. 6F and 7F) contain one transformed isoindole (the CH_2NH_2 moieties of these amino acids do react with an additional OPA molecule [6]), and one by FMOC derivatized amino group, in their dehydrated forms {Fig. 6F, Orn4: MH^+ = 631.2 = ([OPA][ET])[OPA][FMOC][Orn]–H_2O, MK^+ = 669.2; Fig. 7F, Lys4: MH^+ = m/z = 645.3 = ([OPA][ET])[OPA][FMOC][Lys]–H_2O, MK^+ = m/z = 683.2}.

(iii) Orn3 and Lys3 (Figs. 6E and 7E), not fully understood species, might be thoroughly correlated with Orn4 and Lys4 containing the same abundant protonated and cationized masses (Fig. 6E, Orn3: MH^+ = m/z = 467.3, MK^+ = m/z = 505.2); (Fig. 7E, Lys3: MH^+ = m/z = 481.3, MK^+ = m/z = 519.2).

(iv) Orn2 and Lys2 (Figs. 6D and 7D) meet the masses formed from Orn5 and Lys5 by the loss of one COOH group {Fig. 6D, Orn2: MH^+ = 452.2 = [OPA][ET][FMOC][Orn]–([COOH + H_2O]), MK^+ = 490.2); Fig. 7D, Lys2: MH^+ = 466.3 = [OPA][ET][FMOC][Lys]–([COOH + H_2O]), MK^+ = 504.2}.

3.5.3. *Behavior of the α,ω-amino group containing carboxylic acids in the two-step derivatization procedures*

The reaction between the FMOC reagent and the OPA–ET derivatives of Orn and Lys called attention to the possibility of reactions also with other SH-group containing OPA reagents and with additional α,ω-amino group containing carboxylic acids. Thus, remaining on the safe side all protein amino acids have been tested, one by one, in the two-step process. FMOC labeling has been carried out not only subsequently to the OPA–ET derivatization but also after reactions with the OPA–MPA and with the OPA–MCE reagents. In all cases investigated, with the only exceptions of the OPA–ET derivatized Orn and Lys, they remained intact. On this basis, we assumed that in this special process, the crucial role is associated with the two amino groups containing amino acids and, with the neutral end-group of the OPA reagent's SH-additive.

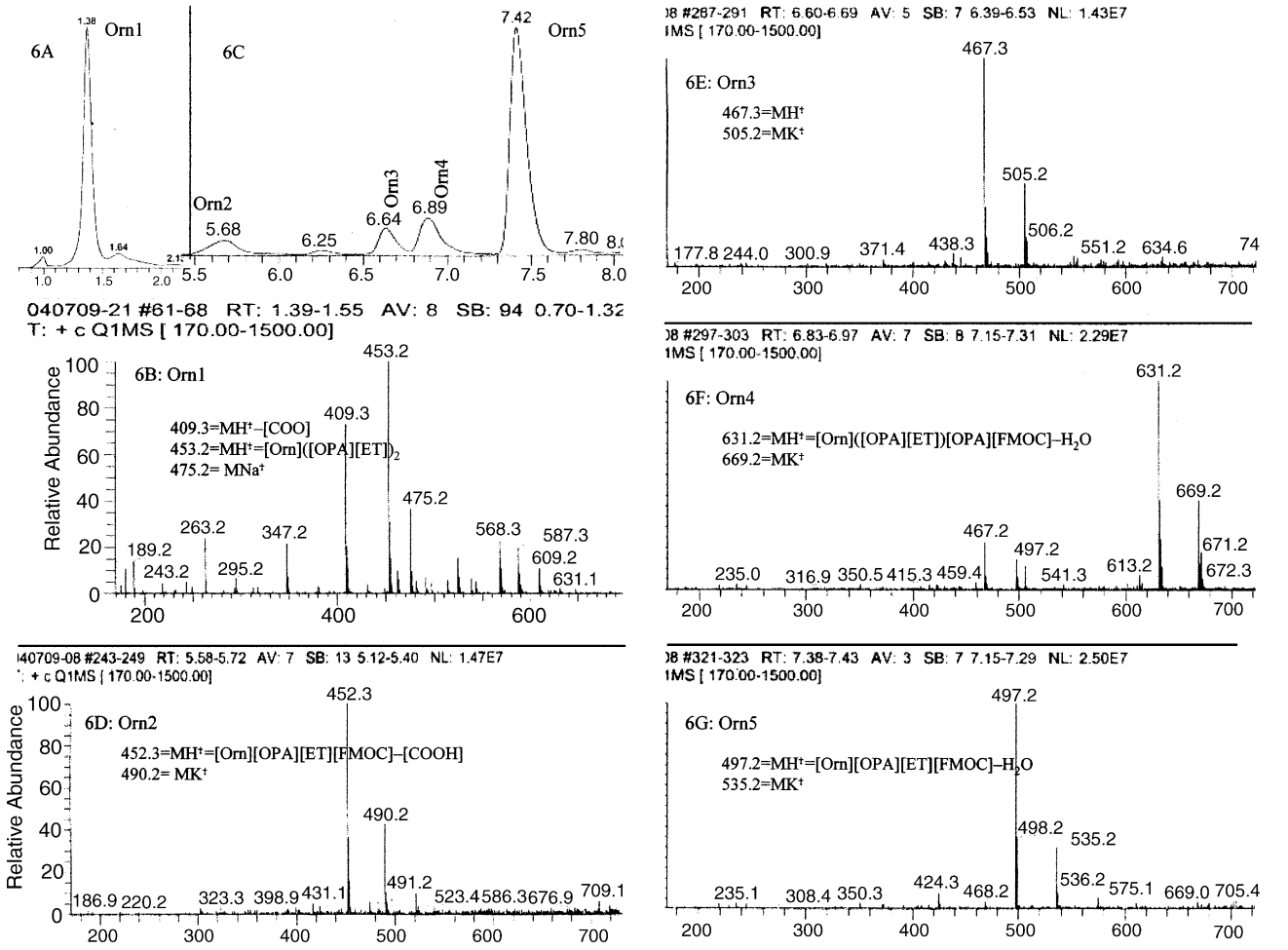

Fig. 6. DAD chromatogram of the OPA–ET–FMOC derivatives of Orn1–Orn5 shown at 334 nm (A: Orn1, 6C: Orn2–Orn5) and their corresponding MS spectra (B: Orn1, D–G: Orn2–Orn5). (Detailed composition of fragments in the text.)

To confirm this assumption, we reacted other α,ω-diaminocarboxylic acids, such as the 1,3-diaminopropionic acid (Dapa), the 2,4-diaminobutyric acid (Daba) and the 2,6-diaminopimelic acid (Dpia), one by one, in the first step with the OPA–ET and in the second one with the OPA–ET–FMOC reagent.

Data obtained both with the simultaneous DAD–FL and with the HPLC–DAD–ESI-MS detected derivatizations proved that, in addition to the classical double isoindole, in all three above detailed reactions, mixed derivatives are formed. In cases of Dapa and Daba several derivatives, in the case of Dpia three, mixed species were obtained, characteristic to its diamino/dicarboxylic functions (Fig. 8A–E).

Due to the presence of the two carboxylic groups, characteristic derivatives with characteristic retention order have been detected (Fig. 8A–C). Its classical double isoindole (Dpia1) elutes before the reagent peaks (Fig. 8A), followed, in order of listing, at Fig. 8C, with the simple mixed species (Dpia2), with the decomposition product of the transformed isoindole (Dpia3) and with the decarboxylated product of the simple, mixed species (Dpia4). Mass spectra of species Dpia1–4 (Fig. 8B, D–F) are represented by their corresponding molecular ions and by their cationized versions with potassium.

Fig. 8B represents the spectra of Dpia1: $\{MH^{+} = 511.2 = ([OPA][ET])_2[Dpia]\}$. Fig. 8D shows the dehydrated version of the simple, mixed derivative, Dpia2: ($MH^{+} = 554.2 = [OPA][ET][FMOC][Dpia]–[H_2O]$, $MK^{+} = m/z = 592.1$).

Fig. 8E reveals the spectra of Dpia3 that meets the composition of the transformed isoindole (containing one additional OPA molecule [6]), obtained by the lost of the mass $m/z = 76 = [CH_2–S–CH_2–CH_3]^{+}$, i.e., $\{MH^{+} = m/z = 645–76 = 569.2 = ([OPA][ET])_2[OPA][Dpia]–[CH_2–S–CH_2–CH_3]^{+}$, $MK^{+} = 607.2\}$.

Fig. 8F furnishes the spectra of Dpia4 that proved to be the decarboxylated molecule of the simple, mixed species, containing the transformed isoindole [6] $\{MH^{+} = 599.2 = ([OPA][ET])[OPA][FMOC]–[COOH + COO]$, $MK^{+} = 637.1\}$.

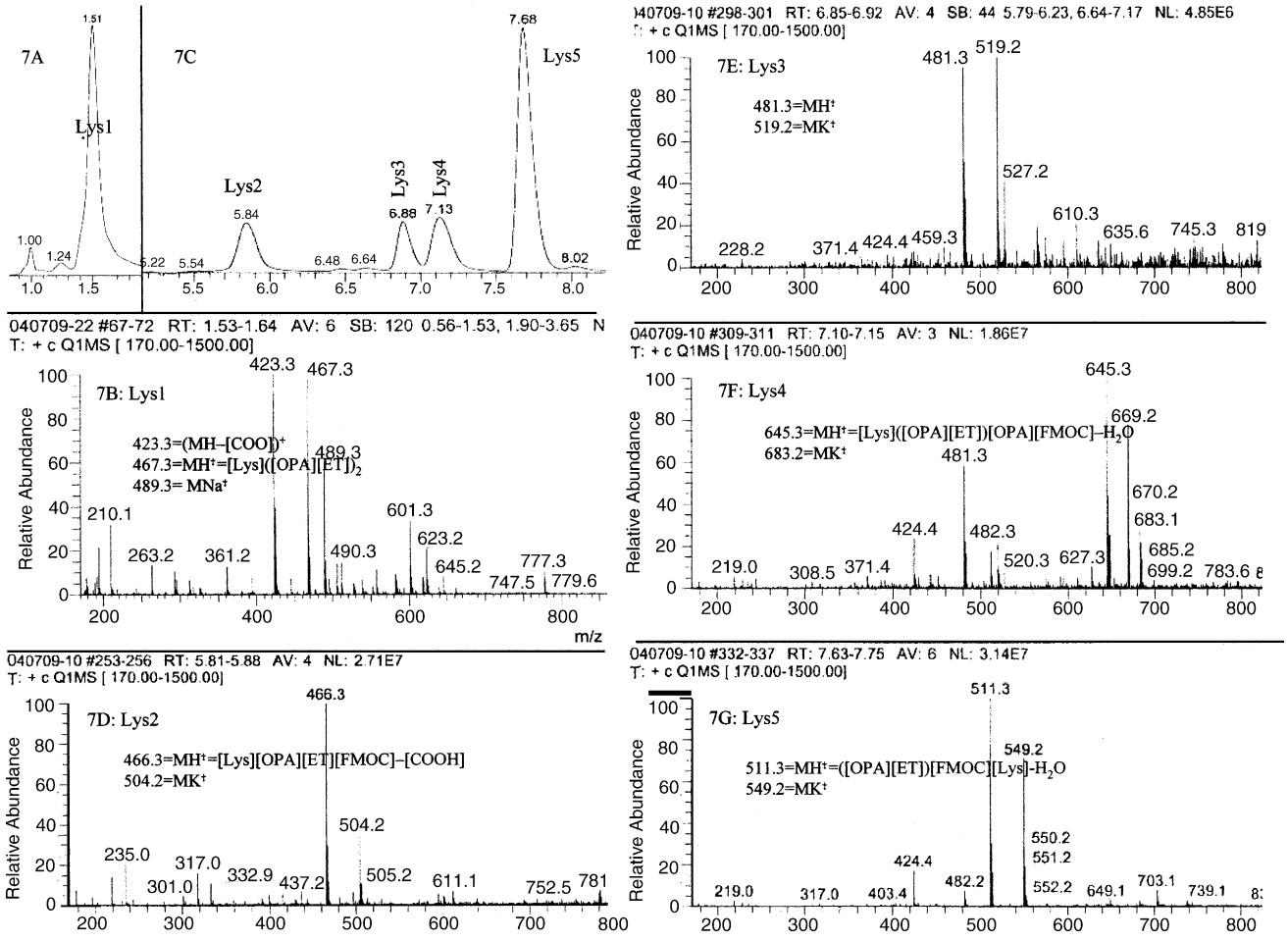

Fig. 7. DAD chromatogram of the OPA–ET–FMOC derivatives of Lys1-Lys5 shown at 334 nm (A: Lys1; 7C: Lys2–5) and their corresponding MS spectra (B: Lys1, 7D–G: Lys2–Lys5). (Detailed composition of fragments in the text.)

3.5.4. Approaches to identify the amine function of Orn and Lys ready to react with the FMOC reagent: MS–MS fragmentation study

Two possibilities had to be taken into consideration: FMOC reacts either with the amino group neighbor to the secondary C2, or with the amino group neighbor to the primary C5.

(i) Based on our earlier results carried out with the *N*-α-acetyl-L-lysine and with the *N*-ε-acetyl-L-lysine [4], it was confirmed [6] that in case of the free ε-amino group (part of the $-CH_2-NH_2$ moiety), the initially formed isoindole does transform, incorporating one additional OPA molecule. Starting from this point, and from the fact that among the mixed derivatives also the transformed, the two OPA molecule-containing species (Orn4 and Lys4) were identified, ([OPA][ET])[OPA][FMOC][Orn]/[Lys], consequently, FMOC reacts with amino group neighbor to the C2 atom.

To confirm the above assumption, the simple mixed derivative of Orn (Orn5 = m/z = 497.2 = [OPA][ET][FMOC][Orn]) was tested in the frame of a MS–MS fragmentation study. We assumed that on the basis of its selective fragment ions the FMOC reactive amino group could be unambiguously selected. Preliminary calculations revealed that the simple mixed derivative of Orn, indicated as Orn5 ([OPA][ET][FMOC][Orn]–H_2O = m/z = 497.5) is a better target compound comparing to the corresponding Lys, providing under fragmentation considerably different masses. Assuming that the cleavage takes place either between the $=CH-CH_2$, or between any of the $-CH_2-$ groups the expected fragments have been listed in Table 6.

Performing the MS–MS fragmentation of the simple mixed compound of Orn as a function of the applied voltage (Fig. 9A–D), it is clear that

(i) fragmentation takes place between the C2 and C3 carbon atom, as well as
(ii) the FMOC reactive amino group is the neighboring to C2 atom. Increasing the fragmentation voltage from 10 eV (Fig. 9A) through 20 eV (Fig. 9B) and 30 eV (Fig. 9C) up to 40 eV (Fig. 9D) the molecular ion of the simple mixed compound (m/z = 497.0–497.3) decreases with the simultaneous increase of the amount of the protonated

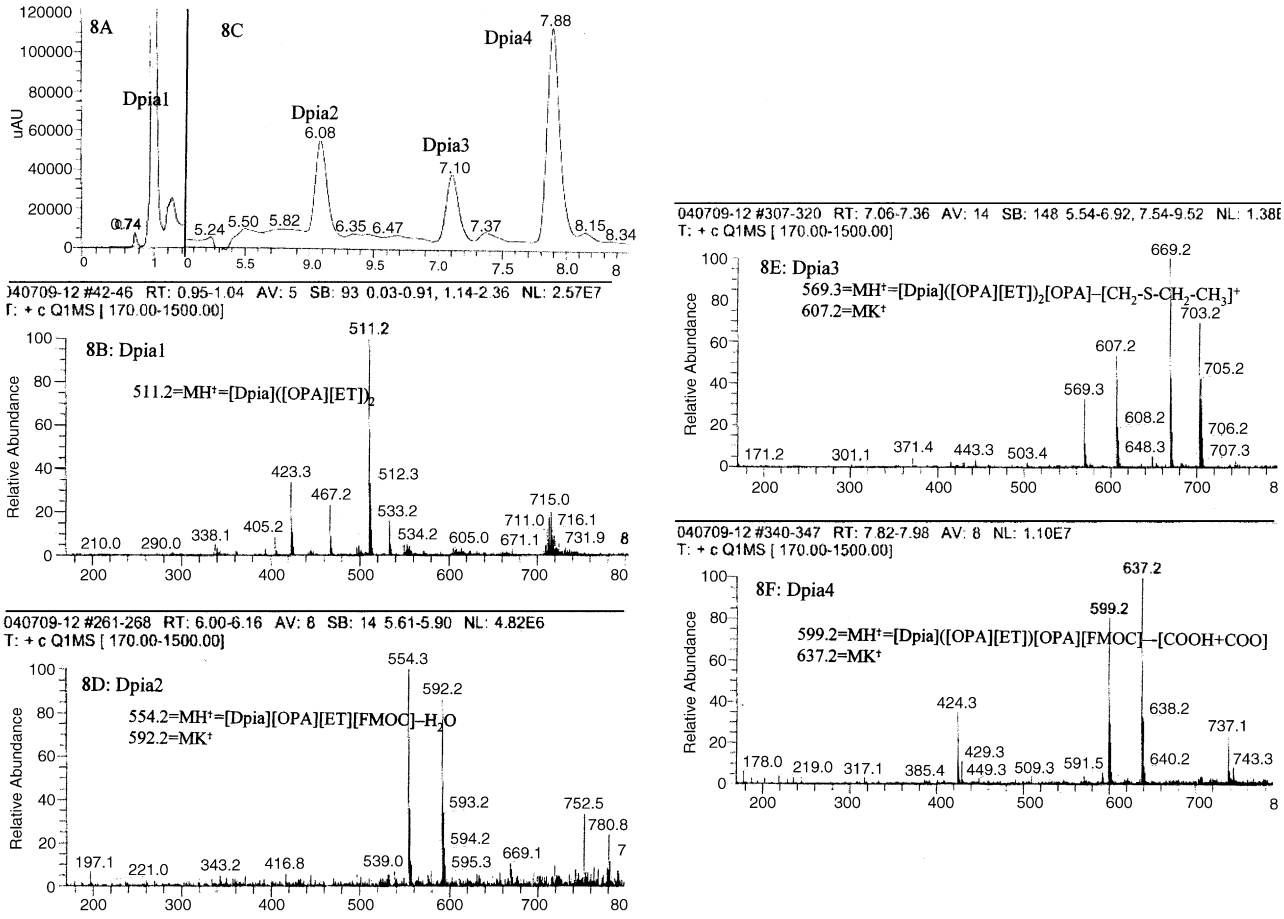

Fig. 8. DAD chromatogram of the OPA–ET–FMOC derivatives of 2,6-diaminopimelic acid (Dpia) shown at 334 nm (A and C: Dpia1–4) and their corresponding MS spectra (B, D–F: Dpia1–4). (Detailed composition of fragments in the text.)

fragment at $m/z = 230$ (Table 6). This observation is in thorough accord with the fact that the mixed compounds exist also in the one additional OPA molecule containing species (Orn4 and Lys4) due to their formation from the $–CH_2–NH_2$ moiety containing, i.e., from the C5 neighboring amino group.

3.5.5. *Analytical consequences*

Based on stoichiometric studies summarized in Sections 3.1–3.4 (Tables 1–3 and 5, Figs. 1–3), analytical reproducibility of BAs, together with Diah, Orn and Lys have been investigated with various concentrations, applying the OPA–ET–FMOC (1:10:0.4) reagent, in 80% (v/v) MET containing medium, at pH 9.30 (Table 7).

As seen, two-step derivatization of Orn, Lys, Put, Cad, Spd and Spm, applying optimum analytical condition furnished acceptable reproducibility (RSD values ≤ 4.7%) and limit of quantitation (∼3 pM).

Table 6
Fragmentation possibilities of the simple mixed compound of ornithine (Orn): Orn5 = {[OPA][ET][FMOC][Orn]–H_2O}$^{+\cdot}$ = m/z = 497.5

Cleavage between	Possible fragments of Orn5 = m/z = 497.5	
	α-Amino group	δ-Amino group
C2 and C3	FMOC m/z = 281.24 OPA/ET m/z = 219.1	FMOC m/z = 233.2 OPA/ET m/z = 295.24
C3 and C4	FMOC m/z = 267.24 OPA/ET m/z = 215.1	FMOC m/z = 247.2–H_2O = 229.2 OPA/ET m/z = 309.24
C4 and C5	FMOC m/z = 239.24 OPA/ET m/z = 187.0	FMOC m/z = 275.2 OPA/ET m/z = 347.24

Indications: as in Fig. 9A–D.

3.5.6. *Practical utility of the simultaneous quantitation of Orn, Lys together with Put, Cad, Diah, Spd and Spm in biological tissues*

In this context, our main concern was associated with the elimination of the matrix effect (high salt and hydroxide ion concentrations). Preliminary studies confirmed, that without any extraction/isolation steps, Orn, Lys, Put, Cad, Spd and Spm content of mouse tissues could be quantitated in the presence of the matrix containing considerable amount of potassium perchlorate, originated from the deproteinization by perchloric acid, followed by neutralization with potassium hydroxide (Fig. 10).

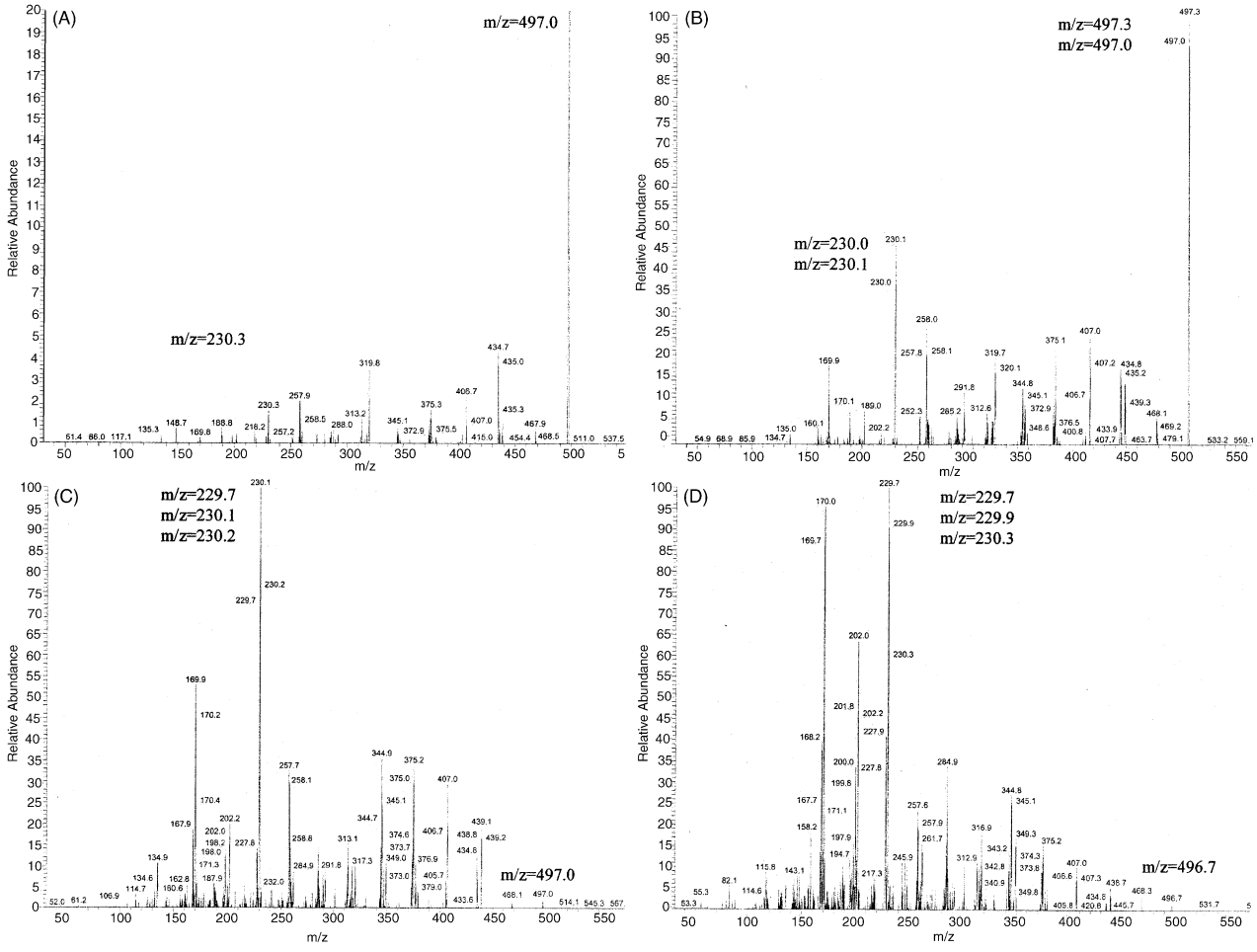

Fig. 9. MS–MS fragmentation study of the OPA–ET–FMOC-Orn5 derivative obtained as a function of the applied voltage: (A: 10 eV, B: 20 eV, C: 30 eV, D: 40 eV). (Detailed composition of fragments in the text.)

Table 7
Quantitation of different amounts of the OPA–ET–FMOC derivatives of biogenic amines, ornithine and lysine in model solutions on the basis of their fluorescence intensities (Ex/Em = 337/454) and on their UV absorbance at 334 nm; reagent: OPA–ET–FMOC (1:10:0.4), 80% (v/v) methanol content, pH 9.3 (Fig. 10)

Amino acid/amine	Retention time (min)	Integrator units/1 pmol A*									
		440	220	110	55	27.5	13.75	6.87	3.43	Average #	RSD (%)
On the basis of fluorescence detection											
Ornithine (Orn)	3.47–3.66	2.75	2.78	2.85	3.07	3.62	–	–	–	2.79	1.8
Lysine (Lys)	3.86–4.12	5.51	5.38	5.18	4.83	4.14	3.73	2.87	1.87	–	–
Putrescine (Put)	9.75–979	5.21	5.18	5.12	5.04	4.97	4.99	4.00	3.65	5.09	2.0
Cadaverine (Cad)	10.15–10.18	10.33	10.37	10.34	10.44	10.45	10.17	9.22	10.07	10.35	1.0
1,7-Diaminoheptane (Diah)	11.05–11.08	11.26	11.41	11.52	11.69	11.50	11.27	10.25	9.83	11.44	1.4
Spermidine (Spd)	11.32–11.37	7.34	6.94	7.38	7.23	7.37	7.08	7.16	7.07	7.20	2.2
Spermine (Spm)	12.54–12.60	5.17	4.97	5.21	4.98	4.67	5.34	4.69	4.98	5.00	4.8
On the basis of UV detection											
Ornithine (Orn)	3.36–3.72	0.58	0.52	0.45	0.35	0.25	0.21	0.32	0.20	–	–
Lysine (LysS)	3.85–4.12	0.58	0.53	0.44	0.41	0.41	0.40	0.40	0.22	–	–
Putrescine (Put)	9.75–9.78	0.82	0.81	0.81	0.81	0.80	0.80	0.73	0.66	0.81	0.93
Cadaverine (Cad)	10.15–10.17	0.82	0.82	0.82	0.83	0.82	0.82	0.75	0.67	0.82	0.50
1,7-Diaminoheptane (Diah)	11.05–11.07	0.84	0.84	0.85	0.86	0.83	0.84	0.82	0.73	0.84	1.2
Spermidine (Spd)	11.31–11.36	0.63	0.62	0.65	0.64	0.62	0.60	0.60	0.64	0.63	2.9
Spermine (Spm)	12.53–12.60	0.45	0.44	0.48	0.46	0.46	0.44	0.40	0.41	0.44	6.0

Indications as in Tables 1–6; italic printed values: have been omitted from the mean, they are read and used from calibration curves; (–) no utilizable data available.

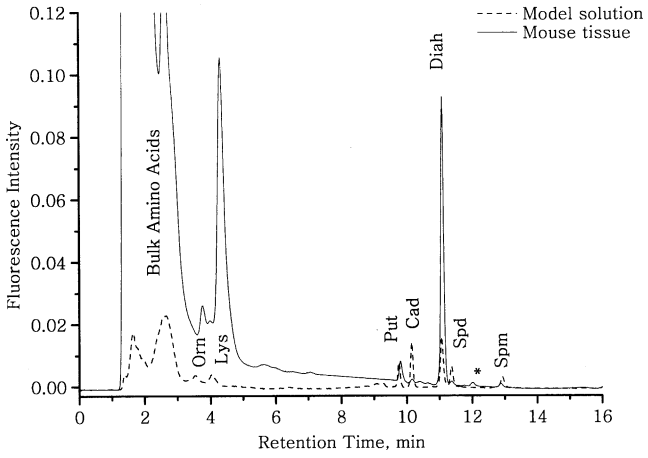

Fig. 10. Fluorescence detected chromatogram of model solution (A: containing 13–14 pM Orn, Lys, Put, Cad. Diah, Spd and Spm of each), overlayed with a mouse tissue sample (B: obtained from 77 mg freeze dried tissue, containing in total, expressed in μg, Orn (23.8), Lys (27.7), Put (0.40), Cad (0.04), Diah (internal standard, 4.43), Spd (0.13) and Spm (0.32); derivatized and eluted under optimum conditions; (*) unknown.

4. Conclusion

(1) A new principle, the two-step derivatization of biogenic amines has been introduced, applying the OPA–ET–FMOC reagent.
(2) The behavior and characteristics of the OPA–ET–FMOC derivatives of Orn, Lys, Put, Cad, Diah, Spd and Spm have been studied both from analytical point of view and with respect to their composition.
(3) Stoichiometric studies, carried out in order to optimize analytical conditions have been followed by simultaneous DAD and FL detections, as a function of the reagent's composition, pH value of the medium and the time of reactions. Regarding the stability of the OPA–ET–FMOC derivatives and the extent of side reactions, the impact of derivatization conditions proved to be of primary importance. Under optimum conditions, applying the 80% MET containing OPA–ET–FMOC reagent at pH 9.3, leaded to simultaneous quantitation of Orn, Lys, Put, Cad, Diah, Spd and Spm in the presence of the rest of other protein amino acids, within 18 min (reproducibility = 2.1% RSD).
(4) On the basis of on-line HPLC–ESI-MS measurements, it has been proved that Spd and Spm derivatives are the expected ones, i.e., $(OPA–ET)_2$FMOC-Spd and $(OPA–ET)_2(FMOC)_2$-Spm were formed.
(5) In the case of Orn and Lys, unexpectedly, mixed species have been identified, labeled both with the OPA–ET and with the FMOC group.

Acknowledgement

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