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o-Phthaldialdehyde derivatization of histidine: stoichiometry, stability and reaction mechanism

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Abstract

The irregular behavior of histidine in its reaction with the *o*-phthaldialdehyde (OPA) reagents has been studied. 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. 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 intra-molecular 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 the transformed species. The composition of the initially and transformed OPA derivatives of histidine were identified on the basis of their on-line HPLC–electrospray ionization (ESI) MS spectra and computations. The initially formed species has been identified as the classical isoindole, while the transformed one contains an additional OPA molecule.

Keywords: Derivatization, LC; Histidine; Amino acids

1. Introduction

Stability, stoichiometry and analytical application of the *o*-phthaldialdehyde (OPA) derivatization reaction, in the presence of 3-mercaptopropionic acid (MPA), *N*-acetyl-L-cysteine (NAC) and 2-mercaptoethanol (MCE), with amino acids [1–4,6,9,10] and amines [7,9,10], as well as with amino acids and amines simultaneously [9] were investigated, including review papers [5,7,8]. Stoichiometric studies were followed by simultaneous photodiode array (DAD) and fluorescence (FL) detection [2–4,6,7,9,10]. The mechanism of reactions, except histidine's, was proved on the basis of on-line HPLC–electrospray ionization (ESI) MS measurements [6]. It has been shown that—according to the literature those amino acids and amines that have been declared as the extremely low OPA derivatives providing ones are as stable as all others: however, it is to be taken into account that their initially formed derivatives are transforming to further species. Consequently, their quantitation must be performed on the responses of the total of their derivatives obtained in the corresponding reaction. The common peculiarity of these, more than a single OPA derivative furnishing, primary amino group containing compounds proved to be associated with their intrinsic structural characteristics, i.e. with their original molecular structure: all of them do contain the $–CH_2–NH_2$ moiety [6]. The only exception, that furnishes more than one OPA-derivative, with the initial molecular structure of the $=CH–NH_2$ moiety, proved to be histidine (His). It is worthy of mention that in spite of the outstanding popularity of OPA derivatizations (∼40% of HPLC of amino acids have been performed as OPA derivatives) [5,11,12], to the author's knowledge, only two papers have reported on that His provides two OPA derivatives [13,14], without any acceptable comment to this phenomenon. The aim of this paper was to find the reason and background of this irregular behavior of His.

2. Experimental

2.1. Materials

OPA, MPA, NAC, His, and 3-methylhistidine (MHis) were obtained from Sigma (St. Louis, MO, USA) and

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from Serva (Heidelberg, Germany) (henceforth: collective designation of His and MHis: amino acids). HPLC-grade methanol and acetonitrile were purchased from Romil Chemicals (Leics, UK). All other reagents were of the highest purity available.

2.2. Solutions

Standard solutions of free amino acids were prepared with distilled water in the concentrations of approximately (1–2) $\times 10^{-2}$ M and further diluted before use. Stock solution of OPA contained 0.25 g OPA (weighed with analytical precision) in 50 ml methanol (further on: methanolic OPA solution).

2.2.1. Buffer solution

Borate buffer was mixed in 0.2 M boric acid (dissolved in 0.2 M potassium chloride solution)–0.2 M sodium hydroxide solution (pH 9.9) (50:50, v/v) (further on: buffer solution).

2.2.2. Reagent solutions

OPA/MPA (1:3) reagent was obtained by mixing, in order of listing, 5.0 ml methanolic OPA ($\sim 1.87 \times 10^{-4}$ M OPA), 20.0 ml borate buffer, 49 μL MPA ($\sim 5.6 \times 10^{-4}$ M); if necessary it was adjusted by 1 M sodium hydroxide, to pH 9.3 ± 0.05.

OPA/NAC reagent was prepared from 5 ml methanolic OPA solution, 20.0 ml borate buffer containing ~0.091 g NAC: final pH 9.3 ± 0.05 (for HPLC–ESI–MS studies the OPA/MPA reagent was prepared in the same manner applying five-fold amounts of reactants and/or the OPA/MPA (1:50) reagent [6,7,9,10]).

2.3. Derivatization

2.3.1. 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 (Waters 717, thermostatted for ~4 °C).

2.3.2. Studies with the OPA/MPA(NAC)-amino acids solutions

Derivatizations were performed with reagents prepared at least 90 min earlier before use, and saved no longer as 9 days [2]. The calculated amounts of reagent solutions were mixed with the selected amounts of amino acids, and reacted for 7 min before injection (if not otherwise stated).

2.4. Chromatography

2.4.1. Simultaneous UV and fluorescence detections

The system was a Waters HPLC instrument (Waters Pharmaceutical Division, Milford, MA, USA), consisted of Waters 996 photodiode array and Waters 274 fluorescence detectors, a Waters 600 controller quaternary pump with thermostattable column area, a Waters 717 autosampler, operating with Millennium software (version 2.10, 1992–1995, validated by ISO 9002). Columns were Hypersil ODS bonded phase: 200 mm × 4 mm, or 150 mm × 4 mm,

Table 1
Stability conditions and characteristics of the OPA/MPA and OPA/NAC derivatives of histidine (His) and 3-methylhistidine (MHis)

Derivatives	Retention time (min)	30 °C, 1.8 ml/min, C15 + 2				*R*/*R* value[a]	Retention time (min)	50 °C, 1.0 ml/min, C20 + 2				*R*/*R* value[a]
		FL detection		UV detection				FL detection		UV detection		
		7 m	3 h	7 m	3 h			7 m	3 h	7 m	3 h	
OPA/MPA-MHis[b]	6.88	l00	100	100	100		11.33	100	100	100	100	
Integrator units (pM)		3.47	3.47	0.28	0.27	12.8		3.47	3.47	0.27	0.27	12.8
OPA/NAC-MHis[b]	6.82	100	100	100	100			–	–	–	–	
Integrator units (pM)		2.38	2.48	0.27	0.29	6.2						
OPA/MPA-His1[b]	6.47	70	70	76	75	12.8	10.35	3	3	7	7	10.5
OPA/MPA-His2[b]	8.24	30	30	24	25	23.9	12.38	97	97	93	93	23.4
Integrator units (pM)		5.41	5.31	0.37	0.37			5.67	5.67	025	0.25	
		30 °C, 1.0 ml/min, C15 + 2[c]						50 °C, 1.7 ml/mm, C20 + 2				
		FL detection		UV detection				FL detection		UV detection		
		7 m	3 h	7 m	3 h			7 m	3 h	7 m	3 h	
OPA/MPA-His1[b]	11.03	63	44	76	62	13.2	6.85	8	7	14	14	12.3
OPA/MPA-His2[b]	12.90	37	66	24	38	18.0	8.85	92	93	86	86	24.5
Integrator units (pM)		5.43	5.73	0.36	0.32			5.88	6.46	0.26	0.27	

Indications: C15 + 2 = column: 150 × 4.6 mm + 20 × 4 mm guard; C20 + 2 = column: 200 × 4 mm + 20 × 4 mm guard; pM = picomol/l. *Note*: All values have been calculated to 1.0 ml/min flow rate; (–) no data available.

[a] FL/UV represents the average response ratios of the fluorescence intensities versus the UV ones.

[b] Response (%), expressed in the total.

[c] Taken from [4].

5 μm, used with 20 mm × 4 mm guard columns (supplier: BST, Budapest, Hungary).

Detections were performed simultaneously, applying the DAD and FL systems, connected in order of listing. Blank and test samples were detected between 190 and 400 nm (DAD), evaluated at 334 nm (OPA/MPA(NAC)-amino acids), as well as at the optimum excitation/emission wavelengths of 337/454 nm. The eluent system consisted of two components: eluent A was 0.05 M sodium acetate of pH 7.2, while eluent B was prepared from 0.1 M sodium acetate–acetonitrile–methanol (46:44:10, v/v/v) (titrated with glacial acetic acid or 1 M sodium hydroxide to pH 7.2).

Stoichiometric/stability studies of amino acids were performed in gradient mode as described earlier (0–100% eluent B within 10 min [2], partly applying 1.0 ml/min flow rates at 30 and 50 °C column temperature, partly 1.7 or 1.8 ml/min flow rates at 50 °C column temperature. (*Note*: Data in the tables, without exception, were calculated to 1.0 ml/min elution rate.)

On-line HPLC–ESI–MS studies of amino acids were carried out with reactants of five-fold concentrations.

2.4.2. Simultaneous UV and MS detection (carried out at Central Service for Plant Protection and Soil Conservation Chemical Department, 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 mm × 4 mm, 5 μm, with a 20 mm × 4 mm guard column.

Detections were performed simultaneously, applying the UV 2000 and MSD Finnigan Aqua detectors, connected in order of listing. Blank tests, concentration dependence were recorded between 190 and 900 nm (UV), evaluated at 334 nm (OPA/MPA-amino acids), 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).

The eluent system consisted of two components: eluent A was 0.005 M ammonium acetate of pH 7.2, while eluent B was methanol.

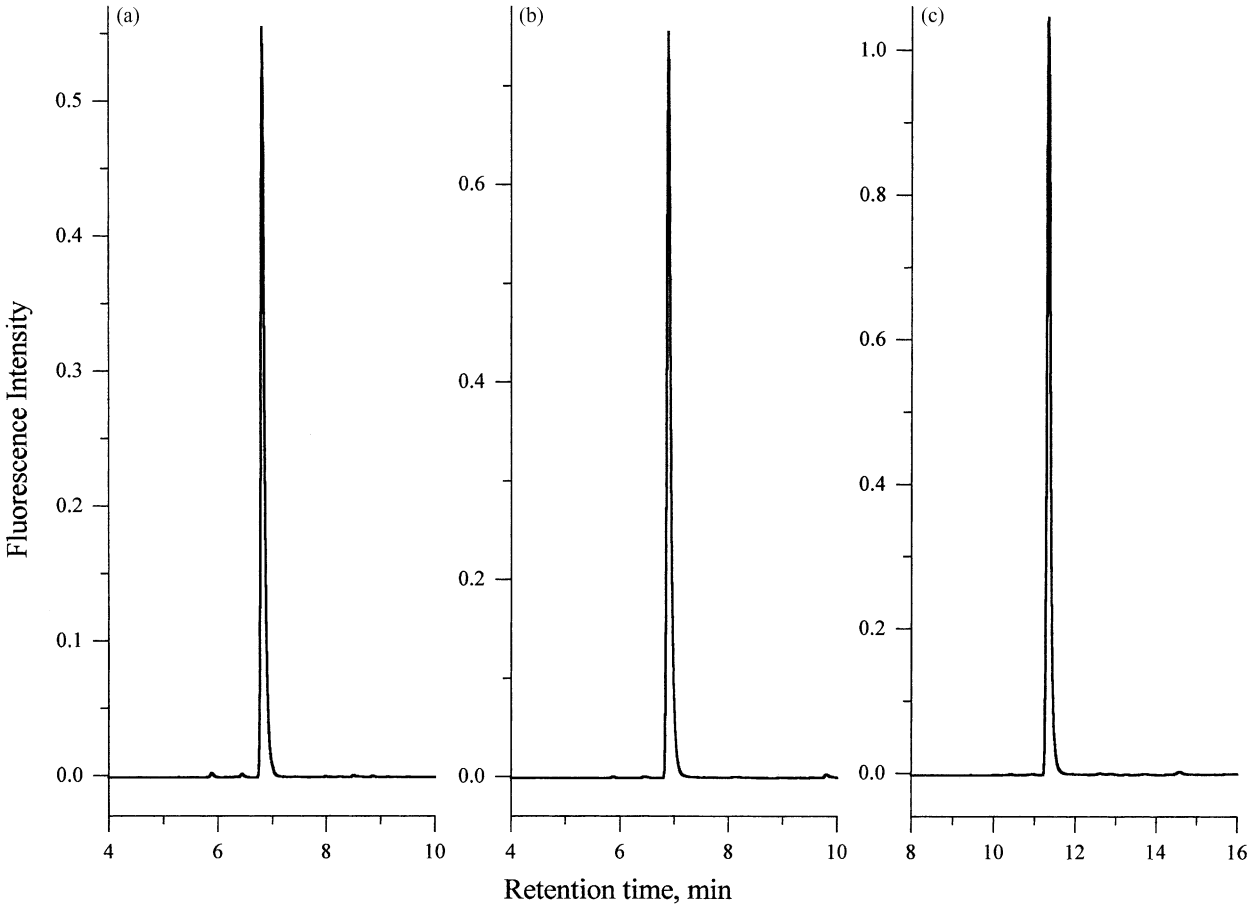

Fig. 1. Fluorescence detected elution profile of 3-methylhistidine (MHis) derivatives obtained with OPA/MPA (1:3): ((a) retention time (t_R) 6.88 min, (c) t_R 11.33 min) and with OPA/NAC (1:3) ((b) t_R 6.82 min) reagents after 7 min reaction time. (a and b) (150 + 20) mm × 4 mm column, elution temperature: 30 °C, flow rate: 1.8 ml/min; (c) (200 + 20) mm × 4 mm column, elution temperature 50 °C, flow rate 1.0 ml/min ([OPA]–[MPA]/[amino acids] = 20:60:1; $1 = 1 \times 10^{-9}$ M).

On-line HPLC–ESI–MS studies of amino acids were performed in gradient mode as follows: 0–60% eluent B within 5 min, hold for 6 min, changed for 100% eluent A within 1 min, hold for 8 min (total elution time: 20 min). Eluent flow rate was 1 ml/min, at room temperature.

3. Results and discussion

3.1. Stability studies of the OPA/MPA derivative of 3-methylhistidine (MHis)

Literature data revealed that MHis provides a single OPA derivative [14–16].

Our investigations, carried out as a function of the reaction time and elution temperature, confirmed that (i) the reaction of MHis leads to a single and stable OPA derivative with the OPA/MPA and OPA/NAC reagents, at 30 and at 50 °C elution temperatures, equally (Table 1, Fig. 1a–c). The UV maximum of its derivative corresponds with 334 nm; (ii) the response (integrator unit/pmol) and response ratio (RR) values of MHis, similarly to other single derivative furnishing amino acids [2–4] proved to be independent of the reaction time and column temperatures (Table 1).

3.2. Stability studies of the OPA/MPA derivatives of histidine (His)

3.2.1. Results of earlier investigations

According to our earlier studies [2–10] we were aware of the fact that His provides more than one OPA derivative, the first eluting His species (His1) provides the characteristic UV maximum of the OPA-amino compounds at 334 nm and the characteristic MS spectra of its isoindole [OPA/MPA-His1 ($m/z = MH^{+} = 360$], while the UV maximum of its longer retention time version (His2) is shifted to 353 nm. Unfortunately, the optimum conditions of His2's ionization recently [6] were not yet found. Consequently, in order to clarify its composition further stoichiometric and mass spectrometric studies were needed.

3.2.2. Stoichiometric studies at 30 and 50 °C elution temperatures

Elutions at different column temperatures applying various flow rates (Table 1, Fig. 2a–c), as well as, elution profile

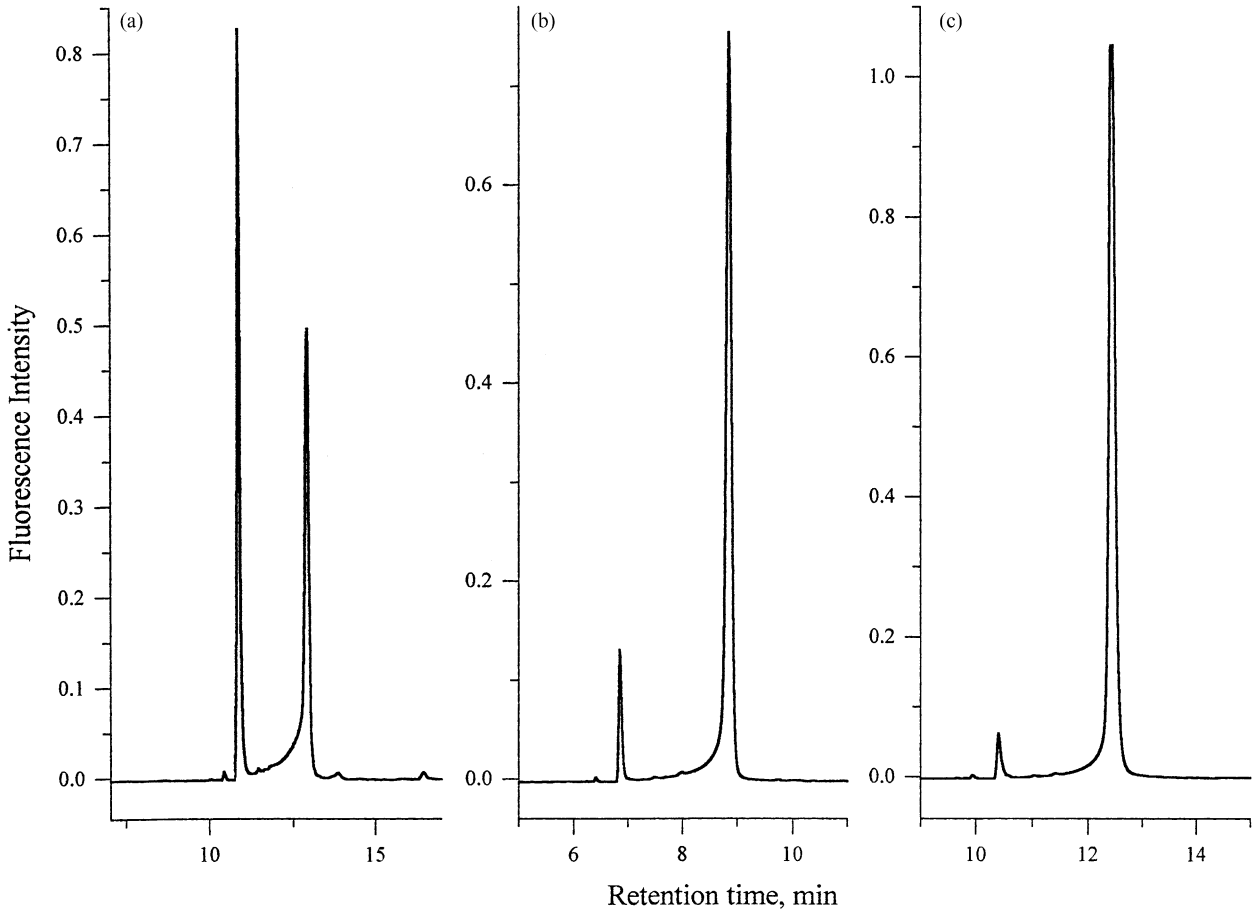

Fig. 2. Fluorescence detected elution profile of histidine1 (His1) and histidine2 (His2) derivatives obtained with the OPA/MPA reagent after 7 min reaction time applying a column of (150 + 20) mm × 4 mm (a) and (200 + 20) mm × 4 mm (b and c): (a) 30 °C, flow rate 1.0 ml/min, t_R His1, 11.03 min, t_R His2, 12.90 min; (b) 50 °C, flow rate 1.7 ml/min, t_R His1, 6.85 min, t_R His2, 8.85 min; (c) 50 °C, flow rate 1.0 ml/min, t_R His1, 10.35 min, t_R His2, 12.38 min ([OPA]/[MPA]/[amino acids] = 20:60:1; $1 = 1 \times 10^{-9}$ M).

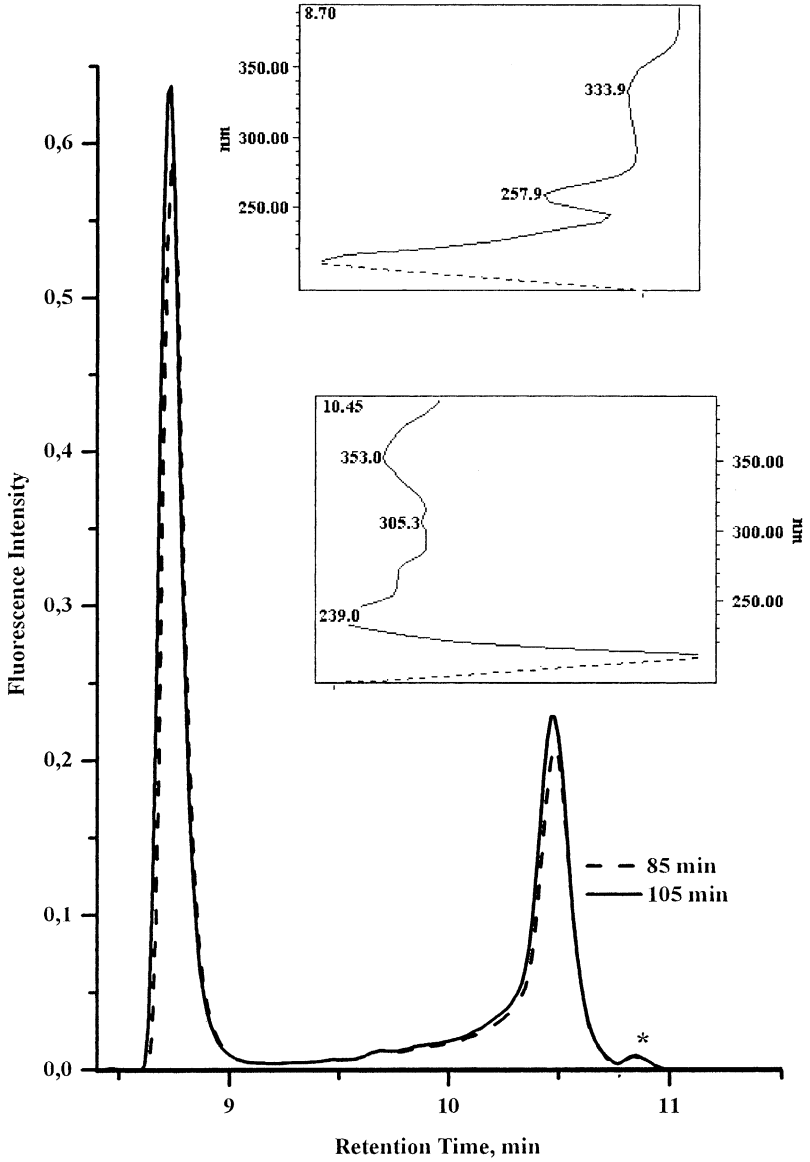

Fig. 3. Fluorescence detection and DAD profiles of histidine1 (His1: retention time 8.70 min) and histidine2 (His2: retention time 10.45 min) derivatives obtained with the OPA/MPA = 1:50 reagent after 85 and 120 min reaction times applying a column of (150 + 20) mm × 4 mm, flow rate 1 ml/min ([OPA]/[MPA]/[amino acids] = 20:1000:1; 1 = 5×10^{-9} M).

of derivatives obtained with varied reactant concentrations (Fig. 3) furnished experiences that proved to be unambiguously concordant: both with the below detailed, assumed and finally also by computations affirmed possible mechanism(s) of His's OPA derivatization (Figs. 4–6) and with the on-line HPLC–ESI–MS data (Figs. 7–9). Namely, the transformation rate of the initially formed classical, OPA-His derivative of isoindole structure proved to be dependent on the temperature (Table 1, Fig. 2a–c). The temperature dependence of transformation rate was proved not only by the considerably different ratios of the initially formed His1 and its transformed species His2 (Table 1), eluted at different column temperatures (30 and 50 °C), but also at the same elution temperature, i.e. at 50 °C applying various flow rates; The longer the time of exposition of derivatives at 50 °C temperature the higher the transformation of His1 to His2 at 30 °C (Table 1, Fig. 2a–c: ratios of His1/His2, expressed in percentages, in order of listing at 30 °C: 63/37 with 1.0 ml/min and 70/30 at 1.8 ml/min, while at 50 °C: 8/92 with 1.7 ml/min and 3/97 with 1.0 ml/min flow rates). The mole ratios of His1 and His2 proved to be [10] independent both of the reactant concentrations (∼five-fold) and that of the mole ratios of reagent's constituents (Fig. 3: [OPA]/[MPA]/[His] = 20:1000:1; 1 = 5.9×10^{-9} M per injection; His1/His2, expressed in percentages, in order of listing, after 85 and 105 min: 60:40 and 64:36, respectively).

3.2.3. On-line HPLC–ESI–MS studies

The composition of the OPA derivatives of MHis and His was confirmed on the basis of on-line HPLC–ESI–MS mea-

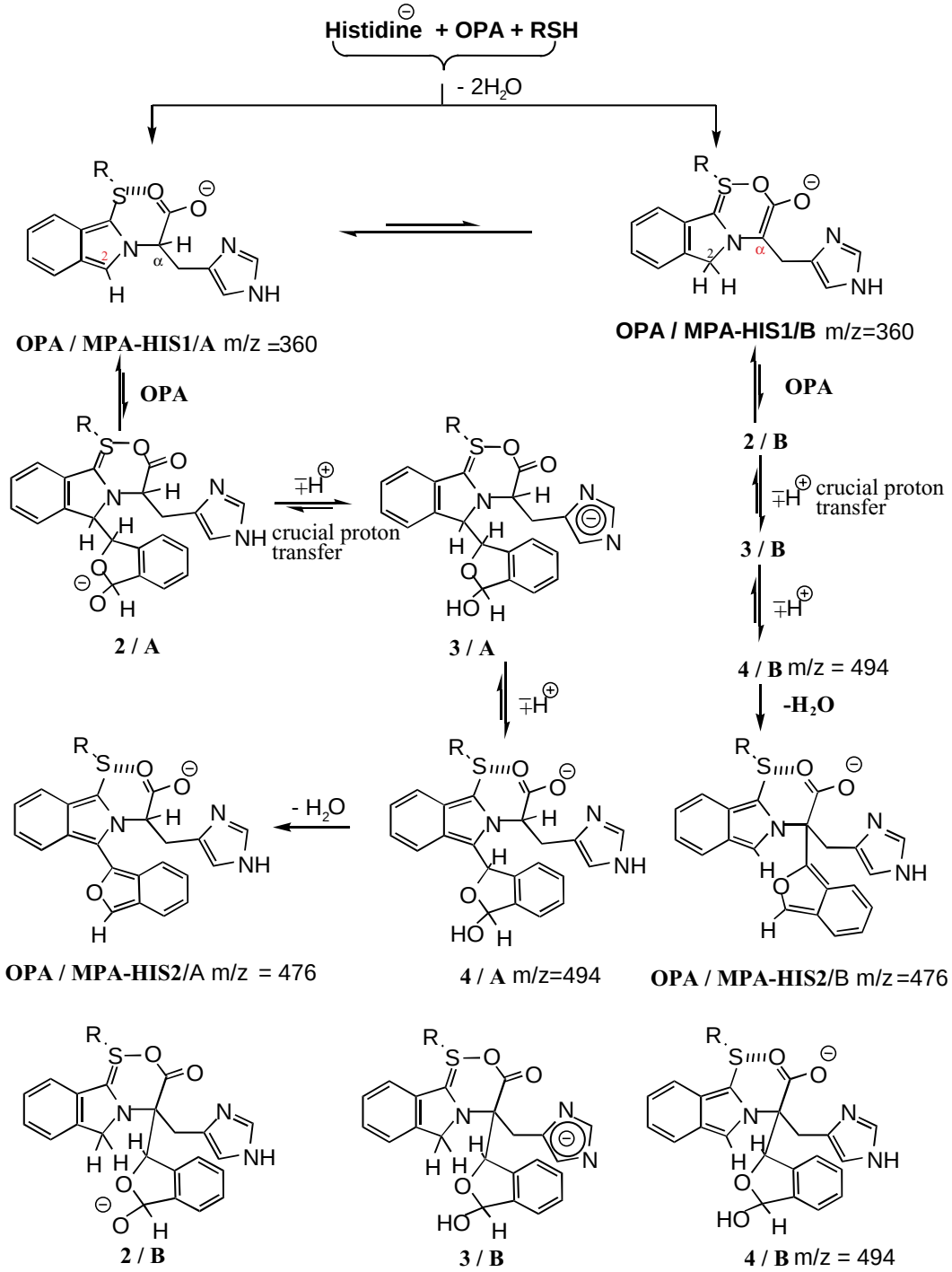

Fig. 4. Reaction mechanism resulting in the formation of OPA/MPA–His1 and OPA/MPA-His2 derivatives.

surements (Figs. 7–9) and leaded to discernment of two possible mechanisms concerning the OPA derivatization of His, depicted in Figs. 4–6 (detailed description in Section 3.2.4).

(i) The MS spectra of the single peak of OPA/MPA-MHis proved to be the classical isoindole (Fig. 7a–d): (a) UV, (b) TIC chromatograms, (c) the area of the molecular ion, $MH^+ = m/z = 374$ and (d) the area of its cationized version $MNa^+ = m/z = 396$.

(ii) As to the composition of the OPA/MPA-His derivatives, it has been repeatedly proven [6], that MS spectra of the transformed OPA/MPA-histidine2, from their TIC chromatograms, could not be obtained (in spite of the fact that reactants were applied in five-fold concentrations). Therefore, selected ion monitoring (SIM) elutions (Figs. 8 and 9) were performed with derivatives obtained both with the OPA/MPA (1:3) (Fig. 8a–e) and with the OPA/MPA (1:50) reagents (Fig. 9a–e). As

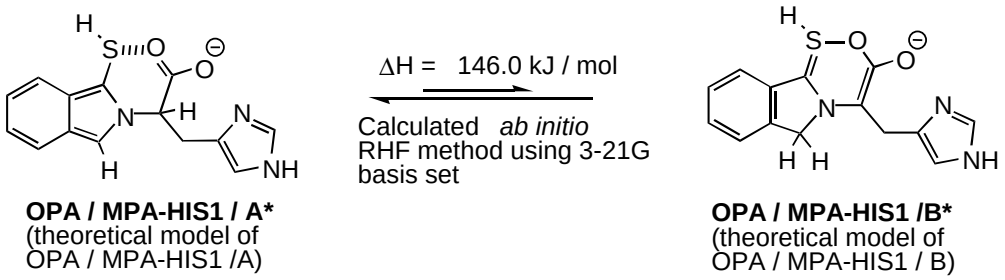

Fig. 5. Theoretical approach explaining the more possible structure of OPA/MPA-His1 tautomer.

seen, SIM chromatograms provided results quite independent on the reagent's composition: in both cases two peak pairs were obtained with different retention times and with the same *m*/*z* masses.

(iii) On the one part, the classical isoindole, composed from its protonated ($MH^+ = m/z = 360$) and cationized ($MNa^+ = m/z = 382$) molecular ions noted at 3.22–3.37 min (OPA/MPA-His1) and at 9.06–9.23 min (OPA/MPA-His1′), while, on the other part the species, containing one additional OPA molecule, i.e. the protonated molecular ion ($MH^+ + OPA = m/z = 494$) and its cationized version ($MNa^+ + OPA = m/z = 516$) were obtained at 3.94–4.03 min (OPA/MPA-His2) and at 9.97–10.35 min (OPA/MPA-His2′), respectively. The appearance of these two peak pairs can be attributed to the parallel virtual tautomerization of His molecule resulting in species, containing the $–CH_2–NH_2$ moiety, ready to interact with an additional OPA molecule (computation approach: Section 3.2.4).

To explain the possible origin of these two peak pairs composed of same masses, computation approach proved to be convincing (Figs. 4–6). As to the identification of the second peak pair on the basis of their SIM spectra (Figs. 8 and 9: His1′, His2′), we assume that their appearance can be explained by their distinguished ionization properties, i.e., by their particularly high sensitivity, compared to the first peak pairs (Figs. 8 and 9: His1, His2).

(iv) Namely, under conditions of our stoichiometric studies followed by simultaneous DAD and FL detections the second peak pair have not been found: probably being present in infinitesimally low concentration.

3.2.4. The possible mechanisms for the uptake of two OPA molecules

According to the proposed mechanism (Figs. 4–6) the primarily formed isoindole OPA/MPA-His1/A may undergo equilibrium tautomerization involving Cα → C2 proton

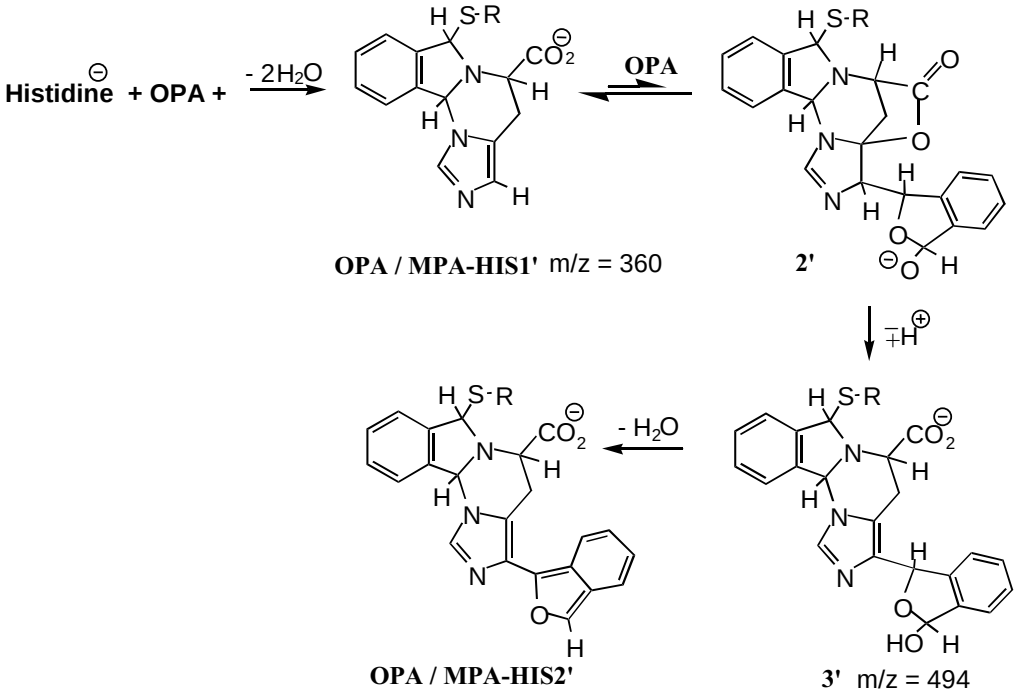

Fig. 6. Reaction mechanism resulting in the formation of OPA/MPA-His1′ and OPA/MPA-His2′ derivatives.

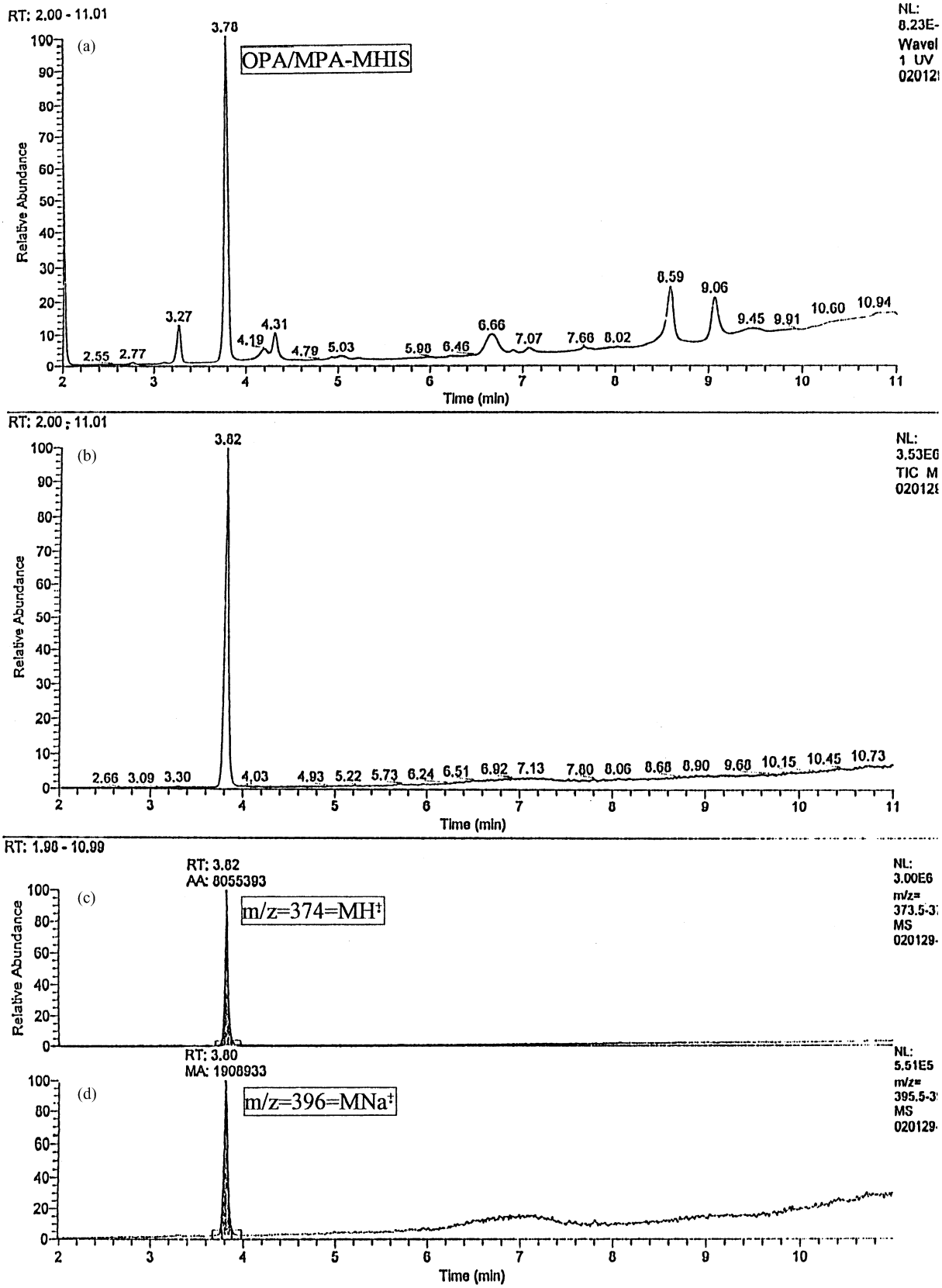

Fig. 7. UV/MS chromatograms of the OPA/MPA derivative of 3-methylhistidine (MHis): (a) UV, (b) total ion current (TIC) chromatograms, (c) the area of the molecular ion, $MH^+ = m/z = 374$ and (d) the area of its cationized version $MNa^+ = m/z = 396$.

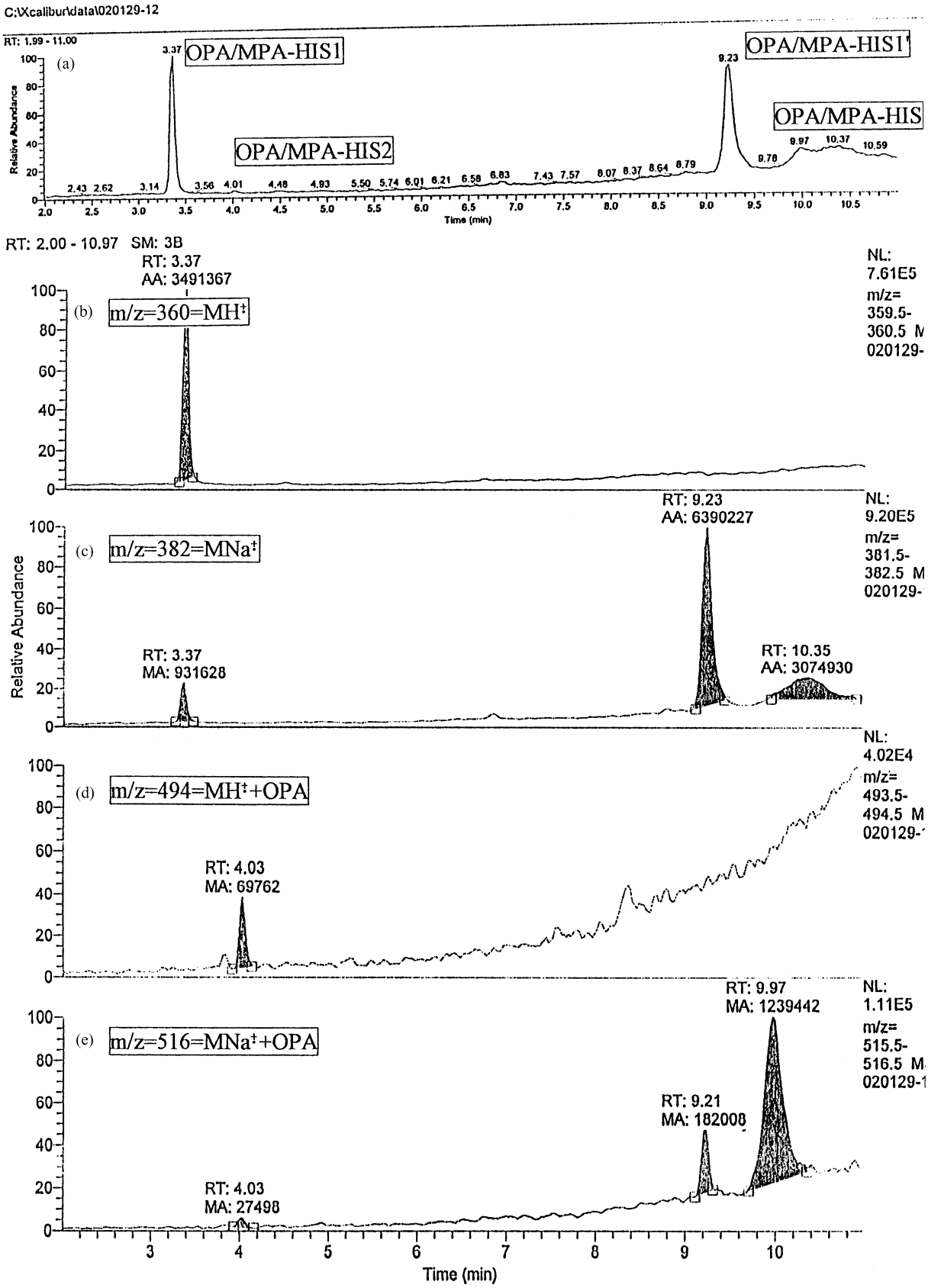

Fig. 8. (a) Selected ion monitoring (SIM) chromatograms of the OPA/MPA derivatives of histidine (His), obtained with OPA/MPA = 1:3 reagent, composed of the masses of *m/z* 360 (MH^+), *m/z* 382 (MNa^+), *m/z* 494 (MH^+ + OPA), and *m/z* 516 (MNa^+ + OPA). Peak areas of (b) *m/z* 360 (MH^+), (c) *m/z* 382 (MNa^+), (d) *m/z* 494 (MH^+ + OPA) and (e) *m/z* 516 (MNa^+ + OPA).

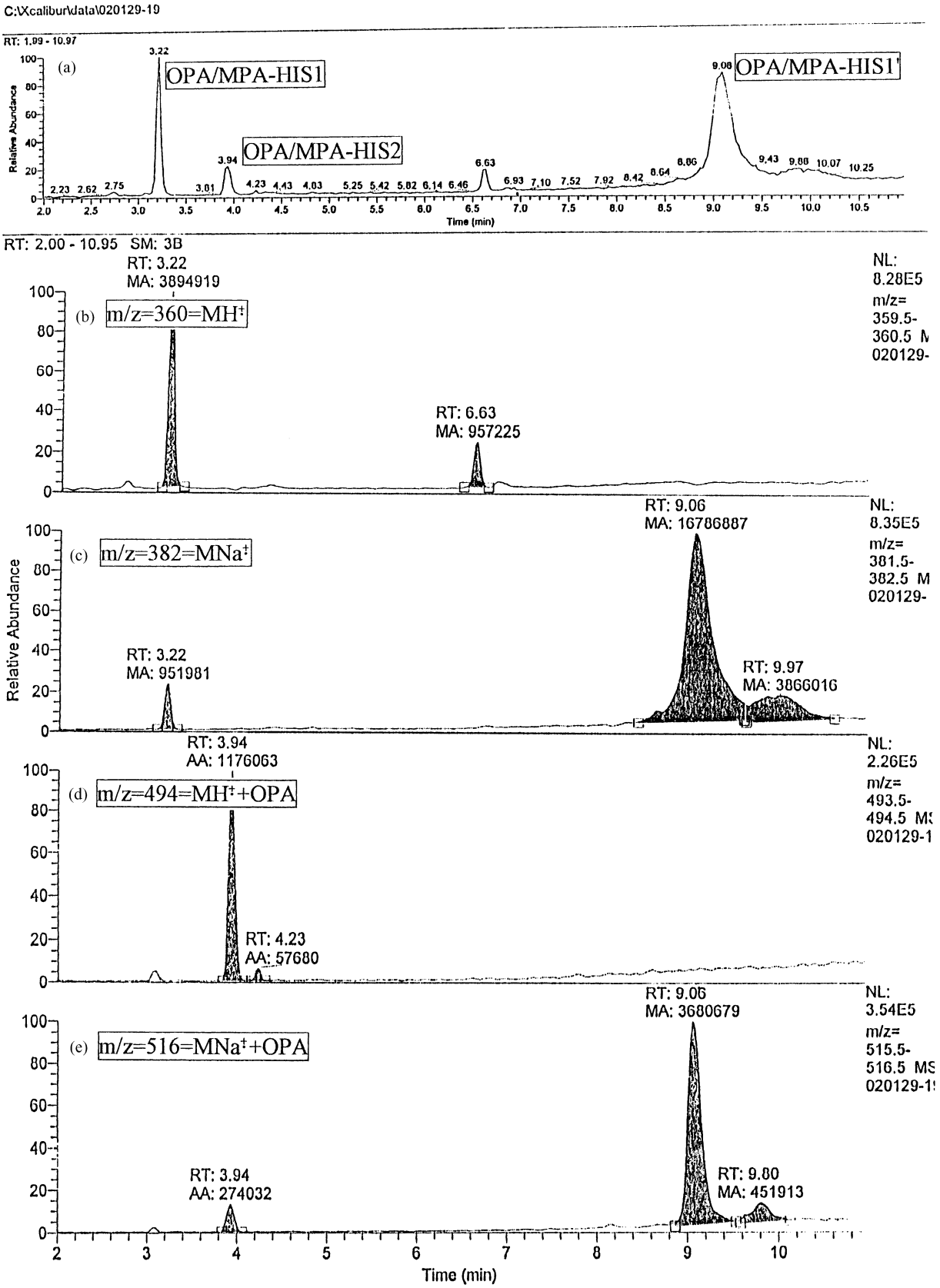

Fig. 9. (a) SIM chromatograms of the OPA/MPA derivatives of histidine (His), obtained with the OPA/MPA (1:50) reagent, composed of the masses of *m/z* 360 (MH^+), *m/z* 382 (MNa^+), *m/z* 494 (MH^+ + OPA), and *m/z* 516 (MNa^+ + OPA). Peak areas of (b) *m/z* 360, (c) *m/z* 382, (d) *m/z* 494 and (e) *m/z* 516.

transfer to give intermediate OPA/MPA-His1/B (Fig. 4) (as discussed later, both of these tautomers can be stabilized, more or less, by intramolecular S–O close contact interaction [17]). The second molecule of OPA is then attacked either by the C2 or Cα atoms to give alcoholate-type intermediates 2/A or 2/B, respectively. Since the equilibria affording 2/A or 2/B are presumably shifted towards OPA/MPA-His1/A and OPA/MPA-His1/B the uptake of the second OPA molecule can only be completed by subsequent proton-transfer from C2 to the negatively charged oxygen atom leading to 4/A and 4/B, respectively, which are suitable for the irreversible water-loss resulting in OPA/MPA-His2/A or OPA/MPA-His2/B species. However, it must be pointed out that either of these presumed crucial proton-transfers must be catalyzed by the imidazole unit involving intermediate 3/A or 3/B. This view is supported by the fact that 3-methylhistidine analogue of OPA/MPA-His1 does not take up an additional OPA molecule. Moreover, on the basis of its acidity, imidazole ($pK_a = 6.9$) seems to be a good source of mobile protons, in low concentration, at pH 9 available for the above mentioned crucial proton-catalysis. Finally, it is important to note that on the exclusive basis of MS data, the detected peak with *m*/*z* 476 may correspond either to OPA/MPA-His2/A or OPA/MPA-His2/B. However, the theoretical modeling carried out on Hartree–Fock (HF) level of theory using 3-21G basis set for OPA/MPA-His1/A* and its isomer OPA/MPA-His1/B* (the theoretical models representing OPA/MPA-His1/A and its tautomeric form OPA/MPA-His1/B: Fig. 5) show that tautomer A is more stable by 146.0 kJ/mol than tautomer B suggesting that 4/A, the derivative of OPA/MPA-His1/A, is much more possible structure for *m*/*z* 494 than 4/B with the same *m*/*z* value.

On the other hand, it is necessary to underline—according to the calculations—the above mentioned S–O close contact interaction must play an important role both in OPA/MPA-His1/A and OPA/MPA-His1/B. This view is firmly supported by theoretically optimized structures obtained for OPA/MPA-His1/A* and OPA/MPA-His1/B* with S–O distances 2.92 and 2.88 Å, respectively. The sum of van der Waals radii of sulfur and oxygen is about 3.2 Å [17], consequently, these distances are convincing values. The comparison of the two values also suggests that the strengthening of the S–O interaction can be considered as a component of the driving force in each elementary steps associated with the saturation of C2 atom, that is, in the formation of intermediates 2–4/A or 2–4/B. The computations were performed by Gaussian 98 program package [18].

An alternative route proposed for the construction of bis-OPA derivative with the same molecular weight as have OPA/MPA-His2/A and OPA/MPA-His2/B is outlined in Fig. 6. In this mechanism, the reaction starts with the formation of tetracyclic intermediate OPA/MPA/His1′ (*m*/*z* = 360). The second OPA molecule is then bonded to the imidazole ring and the resulted intermediate 2′ is stabilized by proton-migration (2′ → 3′) followed by subsequent water-elimination finally affording OPA/MPA-His2′ (*note*: the NMR confirmation of the above mechanism, as completion to the MS spectra), would be an additional evidence. However, due to the isolation difficulties of the OPA derivatives of His, as well as the lack of the very new, on-line HPLC–NMR–MS system, NMR completion proved to be impossible).

4. Conclusion

In summary, it can be stated that the background and reason of the special behavior of the OPA derivatives of His has been clarified.

(1) 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 predestinated to react with an additional OPA molecule.
(2) The ratio of its initially formed and transformed OPA derivatives depends on the temperature, but independent of the composition of the OPA reagents: very likely due to the fact that elevated temperature favors the formation of the $–CH_2–NH_2$ moiety containing His tautomers.
(3) The behavior of 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.
(4) 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.
(5) From analytical point of view, it proved to be unambiguous that the consequences of virtual tautomerization cannot 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.

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