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Variable Electrospray Ionization and Matrix-Assisted Laser Desorption/ Ionization Mass Spectra of the Bisquaternary Ammonium Salt Ethonium

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Abstract

Determination of a single "fingerprint" mass spectrum of a biologically active compound, required for compound identification in mixtures and biological materials in such applied tasks as ecological monitoring and biological imaging, may become a nontrivial problem for labile compounds whose mass spectra depend strongly on the applied experimental conditions. In the present communication, qualitatively different mass spectral patterns obtained for the bisquaternary ammonium salt ethonium Cat2+•2CI- under varied conditions of electrospray ionization and matrixassisted laser desorption/ionization (MALDI) are described and systematized. It is shown that qualitative changes in the electrospray mass spectra of ethonium occur upon a cone voltage increase from 10 V to 100 V, which are caused by the subsequent appearance and destruction of several primary ions: the intact dication Cat2+, the dication-counterion cluster Cat²⁺•Cl⁻ and[Cat - H]⁺ and [Cat - CH₃]⁺ ions. Novel experimental evidence of survival of the gas-phase dication Cat²⁺ with the shortest possible distance of ca 4 Å between the quaternary nitrogen atoms provided by two CH, groups and Cat²⁺ fragmentation with preservation of the doubly charged state of the fragments are revealed under soft electrospray conditions. The interpretation of the MALDI mass spectrum is made taking into account the formation of a salt of the bisquaternary ammonium base and 2,5-dihydroxybenzoic acid, which results in the absence of any chlorine-containing ions in the mass spectra and a fragment ion distribution that is different from that observed under electrospray ionization conditions. The main mass spectral features revealed for ethonium may aid in the identification of other types of bisquaternary ammonium compounds.

Keywords: Bisquaternary ammonium compounds; Ethonium; Electrospray; Matrix-assisted laser desorption/ionization; Organic dication

Introduction

Widening of the scope of mass spectrometry from straightforward identification of compounds to studies of interactions in supramolecular assemblies of fragile biomolecules and to biological imaging brings to life new requirements to the mass spectra obtained by soft ionization techniques. The trivial task of determining the "fingerprint" mass spectrum characteristic of an individual compound can be significantly complicated by the chemical reactivity, lability, and degradability of some types of compounds, which are reflected in their mass spectra. High sensitivity of such compounds to variation of external conditions leads to noticeable qualitative differences in their mass spectral patterns obtained not only by different ionization/desorption methods, but on variation of experimental parameters in the framework of a single ionization technique as well. In the present work, we report a case of a representative bisquaternary ammonium compound (BQAC), ethonium (Scheme 1), which produces qualitatively different mass spectral patterns under different mass spectrometric conditions (Scheme 1)

$$\begin{bmatrix} O & CH_3 & CH_3 & O \\ CH_3 - (CH_2)_9 - O - C - CH_2 - N & -(CH_2)_2 - N & -(CH_2)_2 - CH_3 \\ CH_3 & CH_3 & CH_3 & CH_3 \end{bmatrix} \bullet 2CI^-$$

Scheme 1: Structure of the bisquaternary ammonium salt ethonium.

Bisquaternary ammonium salts, including gemini surfactants and bolaforms, find many useful applications as disinfectants, herbicides, industrial detergents and emulsifiers, chemotherapeutic agents, components of various pharmacological and cosmetic formulations, building blocks of nanosized materials, and others [1-7]. Surfactants based on BQACs used as chemotherapeutic antimicrobial agents [6-9] possess such advantages as low toxicity towards humans, development. Ethonium is used as an antimicrobial agent for wound healing in surgical and stomatological practice [10,11]. In our previous molecular biophysical studies [12-14] it was shown that the molecular mechanisms of action of ethonium and similar membranotropic agents involve non-covalent intermolecular interactions with phospholipids of microbial cell membranes. To continue investigations on the interactions of ethonium with its molecular targets by means of soft ionization mass spectrometry [14] knowledge of its mass spectrometric characteristics is required. In this context, the aim of the present work was to systematize the experimental conditions-dependent features of electrospray (ESI) and matrix-assisted laser desorption/ionization (MALDI) mass spectra of ethonium. A brief summary of basic information on the peculiarities of mass spectra of BQACs, obtained earlier by various techniques [15-31] and relevant to the subject of the present investigation, is as follows. The main types of primary ions expected in the soft ionization mass spectra of BQACs are the salt dication Cat2+ and its cluster with the salt anion Cat2+ Anion-. Doublycharged ions are a rarity in liquid secondary ion mass spectrometry (SIMS), fast atom bombardment (FAB), laser desorption/ionization (LDI), and MALDI, but are detected under field desorption (FD) and

practical absence of mutagenic effects, and slow microbial resistance

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ESI conditions. The stability of the dication Cat²⁺, and, consequently, its abundance in the mass spectra, is determined by structural parameters of Cat²⁺ such as the distance between the two quaternary nitrogen atoms. It is believed that the distance-dependent Coulombic repulsion between two positive charges is the main driving force for dication fragmentation via charge separation or charge elimination. The extent of so-called dequaternization leading to the transformation of quaternary groups to tertiary amines, which is similar to the main thermal degradation pathway of quaternary compounds, can be considered as a measure of the thermal excitation acquired by the BQAC under given conditions. Under ESI conditions the mass spectral pattern of a BQAC depends strongly on such a parameter of the electrospray ion source as the cone voltage (CV) (called also nozzle-skimmer potential or orifice potential) [28,31]. Usually, at low CV values the salt dication Cat²⁺ dominates, while at higher CV it is destroyed and replaced by the dication-counterion cluster Cat2+•Anion- [26-28,31]. Since the pathways of fragmentation of Cat²⁺ and Cat²⁺ • Anion⁻ are different, the spectra obtained at low and high CV values may appear as spectra of two different compounds [31].

In our more recent quantum chemical modeling of alkylammonium BQACs [29,32,33] some new features of the electronic structure of quaternary compounds have been revealed. It was shown that there is no single positive charge located at the quaternary nitrogen atom but that the charge is smeared over the hydrogen atoms of the CH₃ and CH₂ groups adjacent to the quaternary nitrogen. Due to this phenomenon the density of the delocalized charge becomes lower (as compared to the single unit charge), which, in turn, decreases the magnitude of the electrostatic repulsion between the alkylammonium groups and provides a higher stability to the BQAC dication. The stabilization of the BQAC dication in the gas phase was further supported in SIMS [30] and ESI [31] studies of the BQAC decamethoxinum whose main structural distinctions from ethonium are in a much longer spacer of ten CH₂ groups and menthyl rings in the side chains. Namely, along with the expected observation of the Cat²⁺ stability due to the relatively large distance (ca 14 Å [29]) between the quaternary nitrogen atoms, an unexpected feature of preferential Cat2+ fragmentation with preservation of the doubly-charged state of the fragments has been revealed. Further, stable gas-phase noncovalent clusters of two tetramethyl ammonium (TMA⁺) cations which model two quaternary sites of BQAC with the Cl⁻ counterion were produced by soft ionization techniques [33]. This demonstrates that the repulsion between two closely located N⁺(CH₃)₄ cations is not sufficient for their separation even in a noncovalent complex.

As for the MALDI technique, the necessity to account for the effect of possible chemical reactions of the 2,5-dihydroxybenzoic acid (DHB) matrix compound with basic analytes on the mass spectra has been demonstrated [34]. In particular, for quaternary ammonium salts it was proved that the competitive substitution of an inorganic anion of the salts by organic (DHB - H)⁻ anion in the course of sample preparation leads to formation of a new salt Cat⁺•(DHB - H)⁻ or (Cat²⁺•2(DHB - H)⁻ ion) [34]. Due to this effect the MALDI mass spectra contain the latter new salt ion species and are characterized by ions related to the cation of the initial salt in the positive ion mode and [DHB - H]⁻ anions in the negative ion mode [34,35].

In the present study of ethonium by ESI and MALDI techniques some additional novel features are revealed. The intact dication Cat²⁺ is detected under ESI conditions for the BQAC with the minimal possible distance provided by two methylene groups between the two quaternary nitrogen atoms. Moreover, the fragmentation of this dication is observed with preservation of the doubly-charged state of its fragments. Qualitative changes in the ethonium ESI mass spectral pattern with CV increase are revealed and explained by the consecutive appearance and destruction of at least three types of primary ions. For MALDI the effect of formation of the salt consisting of bisquaternary ammonium base and DHB acid on the MALDI mass spectral pattern is confirmed.

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Materials and Methods

ESI mass spectra were obtained with a triple quadruple (QqQ) Micromass Quattro micro mass spectrometer (Waters, Manchester, UK). The electrospray ion source temperature was 120°C (393 K), while the desolvation temperature was 200°C. The capillary potential was 3.5 kV. For obtaining the dependences of the ESI mass spectral pattern on cone voltage (CV) the latter was varied from 10 V to 100 V with a step of 10 V. Data acquisition and processing were performed using MassLynx 4.1 software (Waters, Manchester, UK).

Collision-induced dissociation (CID) measurements were performed using an Autospecoa- TOF mass spectrometer (Micromass, Manchester, UK) which was equipped with an ESI ion source. The instrument was comprised of a double-focusing stage of *EBE* configuration coupled to an orthogonal acceleration time-of flight (oaTOF) analyzer for MS/MS experiments. Nitrogen was used both as bath gas (100°C; 250 L/h) and as nebulizing gas (15 L/h). The ESI source was operated at 4 kV. CID spectra were obtained at a laboratory frame energy (E_{lab}) of 400 eV using Xe as collision gas and by reducing the precursor ion beam to 70% of its original value Data acquisition and processing were performed using OPUS V3.1X software. The 10⁻⁴ M analyte solutions in methanol were infused into the mass spectrometer by a syringe pump (Harvard Apparatus, South Natick, MA, USA), employing a 500 µL syringe, at a constant flow rate of 5 µL/min.

MALDI experiments were performed using an Autoflex II time-offlight mass spectrometer (Bruker Daltonik GmbH, Germany) equipped with a nitrogen laser (wavelength 337 nm). Positive and negative ion spectra were recorded in the linear mode with ion acceleration up to 20 keV with the delayed extraction time set to 20 ns. The energy of the laser beam was attenuated down to 40% of the full laser power. The digitizer acquisition rate was 2 GS·s⁻¹. Spectra were recorded by summing 100 laser shots. Data were processed using the acquisition software FlexControl 2.2 (Bruker Daltonik GmbH, Germany). For sample preparation equal volumes of 10^{-2} M ethonium and saturated DHB solutions in deionized water were mixed in an Eppendorf vial. A 1 µL droplet of the mixture was deposited on the standard metal sample holder and dried at ambient conditions.

Ethonium ($C_{30}H_{62}N_2O_4Cl_2$, monoisotopic molecular mass 584.4 Da) was synthesized at the Institute of Organic Chemistry of the National cademy of Sciences of Ukraine (Kiev, Ukraine). Methanol was acquired from Reanal (Budapest, Hungary). 2,5- dihydroxybenzoic acid (DHB) was purchased from Sigma-Aldrich (St. Louis, MI, USA).

Results and Discussion

Electrospray mass spectra

The structure of ethonium (Scheme 1) can be schematically represented as $\operatorname{Cat}^{2+} \bullet 2\operatorname{Cl}^- \operatorname{or} [\operatorname{R}_1 - (\operatorname{CH}_3)_2 \operatorname{N} - \operatorname{R}_2 - \operatorname{N}(\operatorname{CH}_3)_2 - \operatorname{R}_1]^{2+} \bullet 2\operatorname{Cl}^-$ where R_2 is a short polymethylene chain $(\operatorname{CH}_2)_2$ spacer (linker) between two quaternary nitrogen atoms and the two side substituents R_1 are composed of rather long polymethylene chains CH_3 -(CH_3)₉-(marked further as "terminal" R_1 radicals) attached by ester linkages.

The pattern of electrospray mass spectra of ethonium dissolved

in methanol shows a strong dependence on CV variation (Figure 1a-1h). It is remarkable that the mass spectral pattern not only reflects the expected quantitative changes consisting in an increase of fragmentation (in-source decay) on increase of energy of primary ions collisions with the residual gas, but also undergoes qualitative transformations. It can be seen that the ESI mass spectral patterns of ethonium look qualitatively different at a CV of 10 V (Figure 1a), 30 V (Figure 1c), and CV \geq 50 V (Figure 1e-1h). In general, the qualitative changes observed on CV increase can be attributed to the consecutive appearance and destruction of several primary (precursor) ions: the intact dication Cat²⁺ (*m*/*z* 257) (occurring in the CV range from 10 V to 40 V), the dication-counterion cluster Cat²⁺•Cl⁻ (*m*/*z* 549/551) (CV from 10 V to 70 V), and two ions [Cat – H]⁺ (*m*/*z* 513) and [Cat – CH3]⁺ (*m*/*z* 499) (CV from 30 V to 100 V).

The first type of the mass spectral patterns is determined by the ethonium dication Cat²⁺ and its fragmentation. At low CV values below 10 V (Figure 1a) the peak of the intact dication Cat^{2+} (*m*/*z* 257) is the most abundant one in the mass spectrum. This behavior is consistent with the known preference of preservation of multiply charged species at low CV [28], but it is unexpected taking into account the small distance (of about 4 Å [12,29]) between the quaternary nitrogen atoms, which is believed to be the cause of a dramatic destabilization of dications. The fragmentation of $\mbox{Cat}^{\mbox{\tiny 2+}}$ is absent at a CV of 10 V. On gradual increase of CV two interconnected processes are observed: the decrease of Cat²⁺ abundance down to its practical disappearance at a CV of 40 V (Figure 1d) and consecutive appearance, growth and decline of its fragments in the CV range from 20 V to 50 V (Figure 1b-1e). The main pathways of the Cat²⁺ fragmentation, confirmed by CID data (see "Experimental"), consist in the loss of one or two terminal radicals R₁ (141 Da) accompanied by hydrogen transfer: (Equation 1)

Cat²⁺
$$(Cat - R_t + H)^{2+} + (R_t - H)$$
 (1)
m/z 187
 $(Cat - 2R_t + 2H)^{2+} + 2(R_t - H)$ (1')
m/z 117

The pathway (1) of elimination of one R, radical dominates at a CV of 20 V (Figure 1b) and is gradually replaced by the loss of two R radicals (1') on further CV increase up to 30 V (Figure 1c). The fragment $[Cat - 2R_{+} + 2H]^{2+}$ becomes the most abundant one in the spectrum at a CV of 40 V (Figure 1d) and almost disappears at a CV of 50 V (Figure 1e). Thus, it can be concluded that the ethonium bare dication and its fragments are completely destroyed at a CV of 50 V. The low CV peak observed at m/z 257, which corresponds to the doubly charged ion and contains proper isotopic satellites at half mass values (Figure 2a), is replaced by a peak at m/z 256 corresponding to a singly charged ion (to be interpreted below) starting from a CV of 40 V (Figure 2b). It is remarkable that the products of fragmentation of the dication Cat²⁺ ((1) and (1')) preserve the doubly charged state. A peculiar fragment observed at m/z 373 at the lowest CV only (Figure 2b) corresponds the loss of R,⁺; to be singly charged it must have a zwitterionic structure. It should be noted also that several rather exotic clusters (not shown in the mass range of Figure 1), which demonstrate a trend of self-assembling of the ethonium surfactant, are present at a low CV and disappear on its increase, as, for example, the singly charged aggregate 2Cat²⁺•3Cl⁻ (monoisotopic mass 1131.8 Da, the most abundant peak in the group at m/z 1133.8) and a doubly charged one $3Cat^{2+} \cdot 4Cl^{-}$ (monoisotopic mass 1683.3 Da, the most abundant peak in the doubly charged ion group at m/z 842.6). The second group of changes in the mass spectral pattern is connected with the transformations of the Cat²⁺•Cl⁻ (m/z549/551) ion. This ion appears in the mass spectra at the lowest CV value and starts to decrease from a CV of 20 V on (Figure 1b) because of the decomposition of the Cat2+•Cl- ion and is practically complete at a CV approaching 70 V (Figure 1g). In the CV range from 20 V to 40 V the mass spectra are a superposition of the fragments of both Cat²⁺ and Cat²⁺•Cl⁻ precursors. The main pathway of fragmentation known for dication-anion clusters of BQACs is the so called dequaternization process, which is similar to the pathway of thermal degradation of Cat⁺•Cl⁻ and Cat²⁺•2Cl⁻ salts [15,16,24,29,30]. The dequaternization via dealkylation of alkylammonium ion consists in the elimination of any substituent at a quaternary nitrogen ⁻ CH₃ (2), R₁ (2'), and R₁-N(CH₃)₂-R₂ (2") in the present case – in combination with the Cl⁻ anion with formation of a neutral tertiary nitrogen: (Equation 2)

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$$[Cat - CH_3]^* + CH_3CI$$
 (2)
m/z 499

$$\operatorname{Cat}^{2+}\operatorname{CI}^{-} \longrightarrow \left[\operatorname{R}_{1}-\operatorname{N}(\operatorname{CH}_{3})_{2}-\operatorname{R}_{2}-\operatorname{N}(\operatorname{CH}_{3})_{2}\right]^{+} + \operatorname{R}_{1}\operatorname{CI}$$
(2')
m/z 315

$$\vdash [R_1-N(CH_3)_2-R_2-CI]^+ + (CH_3)_2-N-R_1$$
(2")
m/z 306/308

The chlorine attack at the N-C bond with the R₂ spacer leads to a pair consisting of a neutral $(CH_3)_2$ -N-R₁ product and a chlorinated charged fragment $[R_1$ -N $(CH_3)_2$ -CH₂-CH₂-Cl]⁺ (*m*/*z* 306/308) (2[°]). Elimination of one charge can also be achieved *via* elimination of a HCl neutral leading to formation of a [Cat – H]+ (*m*/*z* 513) ion: (Equation 3).

$$\operatorname{Cat}^{2+}\operatorname{Cl}^{-} \longrightarrow [\operatorname{Cat} - H]^{+} + HCI$$
 (3)

Analysis of CID data aimed at supporting the fragmentation pathways observed for the Cat²⁺•Cl⁻ precursor (Figure 3). Interestingly, the CID experiments reveal an unexpected feature in that the product of the loss of the smallest substituent (pathway (2)), known as the preferential route for alkylammonium compounds [15], is absent, as well as that of the reaction (3). There is a relatively small contribution of the products of pathways (2') and (2"), and a more noticeable contribution of fragments with m/z 270 and 242. The latter ions appear to be the main CID products of the [Cat - H]⁺ precursor as well. In this connection, it can be suggested that the [Cat - H]+ product ion of the reaction (3) occurring under CID appears to be highly excited and further undergoes a more deep fragmentation resulting in the fragments with m/z 270 and 242. Since the most probable location of the Cl⁻ anion in the Cat²⁺•Cl⁻ cluster is in the vicinity of the positively charged alkylammonium groups, the loss of HCl must destabilize the bonds adjacent to nitrogen atoms resulting in cleavage of N-C bonds [24] and release of either $[R_1-N(CH_2)_2-CH=CH_2]^+$ (m/z 270) or $[CH_2=N(CH_3)-R_1]^+$ (m/z 242) singly charged fragments and their neutral counterparts. The abundance of the fragment ions at m/z 270 and 242 increased significantly in relation to that of the Cat2+•Clprecursor in the CV range from 20 V to 70 V (Figure 1b-1g).

The above described discrepancies between the CID and the expected in-source decay of Cat²⁺•Cl⁻ can be explained by a difference in the precursor species generated in the two methods: under CID the bare isolated precursor is subjected to collisions with the collision gas, while precursor-solvent clusters can be present in the skimmer-nozzle space and produce specific dissociation products on collisions with the residual gas. The [Cat - H]+ fragment ion produced from the Cat2++Clprecursor via pathway (3) under CID may be instable and undergo secondary fragmentation as described above. The [Cat - H]⁺ species produced as primary ions on the desolvation of the analyte-solvent clusters under in-source decay may be more stable in comparison with the identical fragments of CID origin due to "desolvational cooling". Possible origins of the [Cat - H]+ ion involving analyte-solvent clusters has been discussed earlier [25,31,36]. The absence of a [Cat - CH₂]⁺ product ion in the CID mass spectra of the Cat²⁺•Cl⁻ precursor is not clear; it can be tentatively suggested that a rather short R, spacer



is in some way responsible for the suppression of pathway (2). The appearance and growth of the [Cat - CH₂]⁺ ion in the ESI mass spectra at a CV higher than 30 V leads us to suggest that it is formed upon decomposition of the analyte-solvent clusters as well, where the solvent molecules may assist the separation of CH₂Cl from the Cat²⁺•Cl⁻ precursor. The growth of abundances of [Cat - H]⁺ and [Cat - CH₂]⁺ ions with increase of CV, which facilitates desolvation, supports a desolvation origin of these ions. Thus, the third group of changes of the mass spectral pattern at higher CV values is related to [Cat - H]+ and [Cat - CH₂]⁺ precursor ions originating directly from the analytesolvent clusters. Both ions appear at a CV of 30 V (Figure 1c) and survive up to a CV of 100 V (Figure 1h). Products of [Cat - H]⁺ fragmentation with m/z 270, 242 continue to contribute to the abundance of the corresponding peaks at a CV higher than 80 V, where the Cat²⁺•Cl⁻ precursor is already practically destroyed. In accord with CID data the most abundant fragment of $[Cat - CH_3]^+$ precursor is at m/z 256; it can be formed via cleavage of a N-R, bond. This fragment accompanies its precursor from a CV of 40 V on (Figure 1d), but grows significantly starting from a CV of 60 V (Figure 1f) indicating an increase of the precursor decomposition. Note, that the absence of a primary fragment $[Cat - CH_{2}]^{+}$ (2) in the CID mass spectrum of the Cat²⁺•Cl⁻ precursor (Figure 3) agrees with the practical absence of its secondary fragment at m/z 256. More deep fragmentation of practically all fragment ions consists in the loss of a (R, - H) moiety similar to the primary reaction (1). The loss of 140 Da from the precursors at m/z 270, 256 and 242 results in the fragments at m/z 130, 116 and 102, respectively. The chlorine containing precursor ion at m/z 306/308 (2") produces the chlorine-containing fragment at m/z 166/168. It should be noted that in ESI experiments with ethanol as a solvent the ratio of abundances of Cat²⁺ and Cat²⁺•Cl⁻ ions of ethonium are much smaller than in the case of methanol solvent at the same low CV values. A similar effect was observed earlier for decamethoxinum [14] and other BQAC [27,28], and is explained by a difference in ion pair stability in solvents with different polarity [27]. The existence of several different types of primary ions and their characteristic fragments, cation-anion clustering, and strong dependence on the CV applied are factors that may hamper detection of ethonium in a sample by means of ESI. Therefore, in further ESI studies of ethonium interactions with biomolecules, low CV values necessary for survival of the ethonium dication that enable the observation of noncovalent complexes involving the dication need to be applied.

MALDI mass spectrum

The MALDI mass spectrum of ethonium obtained by the common method of drying a drop of an aqueous solution of ethonium and the DHB matrix mixture is presented in Figure 4. It can be seen that the MALDI mass spectral pattern does not match with any of the ESI patterns obtained under varying CV values.

The most abundant precursor ion in the upper mass region of the MALDI mass spectra is $[Cat - H]^+$ (m/z 513). Two other intense peaks at m/z 242 and 270 correspond to its characteristic fragments, as evidenced by the CID data for the gas-phase $[Cat - H]^+$ ion obtained under ESI conditions. There is also a low abundant precursor $[Cat - CH^3]^+$ (m/z 499) ion with its characteristic fragment at m/z 256. The most intense peak in the mass spectrum at m/z 315 corresponds to the fragment $[Cat - R_1]^+$. Chlorine-containing ions are not detected, nor is Cat^{2+} observed. The absence of the intact dication Cat^{2+} was expected in connection with the rarity that multiply charged species survive under ordinary MALDI conditions [37,38].

The observed MALDI pattern of ethonium is consistent with the

features of MALDI mass spectra of quaternary ammonium compounds obtained with DHB matrix, which were reported in our previous paper [34]. In accord with these findings, a chemical substitution reaction of the Cl⁻ anion of the quaternary ammonium salt ethonium for the (DHB – H)⁻ anion of organic acid DHB must take place in water solution at the stage of sample preparation. As a result, a new salt Cat²⁺•2(DHB – H)⁻ (instead of the initial Cat²⁺•2Cl⁻) is precipitated upon sample drying and the mass spectrum obtained corresponds to this new compound. The MALDI mass spectra of ethonium satisfy completely this rule: formation of the new salt composed of Cat²⁺ and (DHB – H)⁻ leaves no space for Cat²⁺•Cl⁻ and its chlorine-containing fragments; protonated and cationized DHB molecules have a rather low abundance in the positive ion mode (Figure 4), but there is an abundant [DHB – H]⁻ (m/z 153) anion in the negative ion mode.

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The absence of the Cat²⁺•(DHB – H)⁻ ion in the MALDI mass spectrum (Figure 4) points to a low stability of the ethonium dication cluster with the relatively large organic anion in the gas phase. At the same time the presence of the $[Cat - H]^+$ ion confirms our suggestion made above on the basis of CID of electrospray-generated ions concerning the origin of $[Cat - H]^+$ ion from the analyte-solvent (or DHB in the present case) clusters. Indeed, under MALDI conditions the release of the ethonium dication to the gas phase from the laser-sputtered material necessitates its "desolvatation" from both DHB anions and residual neutral DHB matrix molecules. The latter are the only, in the sample available, candidates for a proton subtraction from the dication in the course of $[Cat - H]^+$ formation.

The $[Cat - CH_{3}]^{+}$ ion and abundant $[Cat - R_{3}]^{+}$ ions can be formed following decomposition of precursors containing Cat²⁺ and the $(DHB - H)^{-}$ organic anion in dealkylation reactions similar to (2) and (2') proposed for dequaternization of the Cat²⁺ clusters with the inorganic Cl- anion. In this context, MALDI data obtained on decamethoxinum [34] revealed a similar noticeable increase of the relative abundance of the $[Cat - R_1]^+$ fragment of decamethoxinum under MALDI with DHB matrix in comparison with other techniques. Furthermore, an increased abundance of the [Cat - R₁]⁺ ion was observed in the liquid SIMS mass spectra of an ethonium-sodium dodecylsulfate (SDS) mixture, in which the precursor consisting of the ethonium dication and the SDS anion was present [39]. This set of data allows us to assume that the pathway of elimination of R₁ is strongly enhanced by the preceding clustering of the dication with organic anions as compared with the pathway (2') of R,Cl elimination for the ethonium-chlorine cluster.







Figure 3: Collision induced dissociation mass spectrum of Cat²⁺-Clprecursor ion of ethonium. Collision gas is Xe.



It may be concluded that the absence of the Cat²⁺ and chlorinecontaining ions in the MALDI mass spectra of ethonium, as well as its interactions with the DHB matrix, complicates *de novo* identification of a given compound on the basis of its conventional MALDI mass spectrum only. Interaction of ethonium with organic acids in the complex mixtures may influence its MALDI mass spectral pattern as well. At the same time the $[Cat - H]^+$ ion and a set of abundant peaks of fragments at *m*/*z* 242, 270, 315 and 499 in the MALDI mass spectrum can support the presence of the ethonium dication in the sample.

Comparison of data for ethonium and decamethoxinum

It is of interest to compare the ESI mass spectra of ethonium with those obtained earlier for the BQAC decamethoxinum [12,29,31,35,39], which differs from ethonium by a longer R_2 spacer composed of ten CH_2 groups and by R_1 radicals comprised by menthyl rings. The idea of comparison is connected with the search of distinctions in the mass spectra caused by differences in the so-called inter-charge distance [16-19], that is the distance between the two quaternary groups of the BQAC, which was estimated as 14 Å and 4 Å for decamethoxinum and ethonium, correspondingly [12,29].

The dependence of the ESI mass spectra of decamethoxinum on CV [31] is qualitatively similar to that revealed for ethonium in the present work. However, the specific quantitative changes in the spectra occur in different CV ranges for the two compounds. The dication Cat^{2+} of decamethoxinum, appearing at the lowest CV, begins to fragment at a higher, as compared with ethonium, CV value of about 75 V (*vs* 20 V for ethonium). The Cat^{2+} of decamethoxinum and its fragments disappear from the spectra at a CV above 100 V *vs* 50 V for ethonium. The Cat^{2+} of decamethoxinum survives up to the highest applied CV of 225 V, while it vanishes at a CV of 70 V for ethonium. The

higher CV-dependent energy required for destruction of the dication of decamethoxinum points to its higher stability in comparison with that of ethonium. This behavior is expected on the basis of literature data describing the decrease of stability of the dications of BQACs with decrease of the intercharge distance in the dications [16-19].

An unexpected feature of the ESI mass spectra of ethonium is the survival of its dication with the shortest possible distance between two quaternary nitrogen atoms provided by two CH₂ groups only. Another uncommon feature is the preservation of the doubly charged state of the fragments in pathways (1) and (1') of the Cat²⁺ decay, characteristic of both ethonium and decamethoxinum. In the early literature on BQACs the main driving force leading to destabilization of the gasphase dications was believed to be the Coulomb repulsion between two positive charges, according to which the main pathway of Cat²⁺ fragmentation should be "charge separation" or "charge minimization", leading to a complete destruction of dications with a small inter-charge distance [16-19]. In this connection, the occurrence of the decay pathways (1) and (1') for the decamethoxinum dication was rather surprising [29,31], and for the ethonium dication it was even more surprising. Among possible reasons of the BQAC dication stabilization in the gas phase is the effect of delocalization (smearing) of the unit positive charge over the hydrogen atoms of the alkylammonium quaternary groups [29], mentioned in the introduction, which can reduce the electrostatic repulsion as compared to that between two localized point charges.

It is worth noting that the fission of the labile ether linkages (1) and (1') observed for the gas-phase dications of ethonium and decamethoxinum follows the direction of their biodegradation in that it appears to be more favored than that involving "charge separation". As to biodegradation, the drug design of antimicrobial agents of this type includes the incorporation of the ester linkage into the structure of their dications to allow for their proper metabolization in the living organism [40,41], an approach that is similar to that utilized currently in the design of some biodegradable polyesters [42].

Internal energy dependence on cone voltage

To obtain insights into the survival of the intact ethonium dication under ESI conditions and to evaluate the contribution of the Coulomb repulsion to the destabilization of the dication, the internal energy acquired by Cat²⁺ at different CV values has been estimated. It is known that the extent of in-source fragmentation under ESI is governed by the internal energy acquired by an ion on its collisions with the residual gas in the orifice-skimmer region. An approach to the quantitative evaluation of the dependence of the internal energy of ions on CV was developed in a number of works [43,44] for small organic molecules and peptides. Evaluation of large sets of ESI data obtained with instruments of the same design as that used in the present work (Quattro Micromass) resulted in an empirical equation for the mean internal energy < $E_{int} > [44]$:

$$\langle E_{int} \rangle = [405 \times 10^{-6} - 480 \times 10^{-9} (DOF)] (CV)T + E_{therm}(T)$$
 (4)

where (CV) is the cone voltage value, T (in Kelvin scale) is the source temperature, DOF is the number of degrees of freedom (determined as 3N-6, where N is the number of atoms in the ion), and $E_{therm}(T)$ is the mean internal energy corresponding to the source temperature at zero CV value. The authors of the equation (4) [44] stressed that the values of the numerical parameters (405×10^{-6} and 480×10^{-9}) may depend on pressure variation in the ion source. Since in the framework of the present investigation we were interested in comparing the relative internal energies of certain ions rather than in measuring their exact

values, we have used equation (4) as it is, assuming slight variations of standard pressure values in the instrument settings.

We have applied the equation (4) to estimate the dependence of internal energy of singly and doubly charged ions of ethonium and decamethoxinum on CV value. The value of $E_{therm}(T)$ at the source temperatures of 300 K and 393 K were derived from the values tabulated in the paper [45] on the base of calculations made by *MassKinetics* software [46]. The results for the ethonium dication Cat²⁺ (98 atoms, DOF=288) and [Cat – H]⁺ cation (97 atoms, DOF=285), as well as for the decamethoxinum dication Cat²⁺ (118 atoms, DOF=348) and [Cat – H]⁺ cation (117 atoms, DOF=345) are presented in Figure 5a and 5b, respectively. The trend of the plot for the singly charged cluster Cat•Cl⁺ (not shown) is similar to that of the [Cat – H]⁺ ion.

The numerical values of the mean internal energy obtained by the dications of ethonium and decamethoxinum at several CV values, which are connected with the qualitative changes in the ESI mass spectra patterns of the two compounds, are presented in Table 1.

It should be reminded that, although the mass values of the dication Cat^{2+} and the singly charged ion $[Cat - H]^+$ differ in one atomic mass unit only, the kinetic energy gained by the dication during its movement in the electric field, being proportional to the charge of the ion, is twice as high than that of the singly charged ions. This results in different slopes of the dependences for Cat^{2+} and $[Cat - H]^+$ (or $Cat \cdot Cl^+$) in the plots in Figure 5 and explains mechanistically, why the dication is destroyed at lower CV values than the mono-cations. For example, while the Cat^{2+} of ethonium obtains an internal energy of 12.5 eV (at 393 K) at a CV value of 50 V, sufficient for its complete decomposition, the $[Cat - H]^+$ and $Cat \cdot Cl^+$ acquire the same energy at a CV of about 100 V.

It should be noted that equation (4) considers the number of the atoms (DOF) in the ion only and does not take into account any of its structural features. Nevertheless, the data of the plots in Figure 5 provides interesting information related to the above stated problem of the dication stability in dependence of the inter-charge distance. It can be seen that the internal energies gained by the dications of the two compounds with different inter-charge distance are comparable at the same CV values. However, the decomposition of Cat2+ of ethonium takes place at a CV value of 50 V, which is much lower than a CV of 100 V, required for complete decomposition of the decamethoxinum dication [31]. It can be assumed that the destruction of the ethonium dication at a CV value in 50 V lower than that required for the decamethoxinum dication is facilitated by the contribution of the energy of electrostatic repulsion between the two closely located quaternary groups of ethonium. Very rough estimates show that addition of Coulomb repulsion energy, evaluated for the ethonium dication as about 4 eV, to the internal energy of 9 eV, acquired by the dication at a CV of 50 V at 300 K (Table 1), gives a product comparable to the internal energy of 15.4 eV acquired by the decamethoxinum dication at the same conditions.

On the other hand this means that the Coulomb repulsion between the quaternary groups *per se* is not sufficient for fragmentation even in the dications with the smallest possible distance between the quaternary groups. The intact ethonium dication can be transferred to the gas phase under very gentle conditions, such as under electrospray at the minimal CV value, and it survives in the gas phase in the absence of additional excitation. The gain in internal energy of 3-4 eV acquired by the ethonium dication at the lowest CV value of 10 V is not sufficient to initiate fragmentation neither. It can be speculated on this basis, that the failure to detect the dications with small inter charge distance in

CV, V	Mean internal energy <e<sub>int > ,eV</e<sub>			
	Ethonium		decamethoxinum	
	300 K	393 K	300 K	393 K
10	2.7	4.1	2.8	4.3
20	4.3	6.2	4.2	6.1
50	9.0	12.5	8.4	11.7
100	16.8	23.0	15.4	21.1





Figure 5: Dependence of mean internal energy $\langle E_{int} \rangle$ on cone voltage (CV) value for doubly charged Cat²⁺ and singly charged [Cat – H]⁺ ions of ethonium (a) and decamethoxinum (b). Ion source temperature is 393 K (+120°C).

other ionization/desorption techniques is caused by the acquisition of an internal energy higher than 10-15 eV by the sputtered/desorbed ions. In particular, the patterns of the ethonium mass spectra obtained under fast atom bombardment (FAB) [29] and liquid SIMS [29,39] conditions resemble the pattern obtained under electrospray at a CV of 40-50 V (Figure 1d and 1e) with the exception of fragments resulting from Cat²⁺ (which are never generated for ethonium under FAB/ SIMS). Such a similarity supports the assumption that in both cases the

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mass spectral pattern is determined by the Cat²⁺•Cl⁻ primary ion and its characteristic fragments, while the Cat²⁺ does not survive. It can be tentatively suggested that the energy necessary for fragmentation of the Cat²⁺•Cl⁻ cluster obtained upon collisions with the residual gas under ESI at a CV of 40-50 V and upon collisions with the matrix molecules in the excited zone during the sputtering event under FAB/SIMS is of the same order of magnitude. The distinction between the electrospray mass spectral patterns generated upon significant CV increase (Figure 1f) and those observed for FAB/SIMS is consistent with the appearance of the new precursor ions and their fragments at more energetic conditions which are not achieved under FAB/SIMS.

As to the mono-cations of comparable mass, Cat•Cl⁺ and [Cat – H]⁺, which must gain comparable energies upon in-source decay, the destruction of Cat•Cl⁺ at a CV value of 70 V and survival of [Cat – H]⁺ at much higher CV values add support to the hypothesis of different sources of these two types of ions. Namely, we have proposed that the [Cat – H]⁺ ion may originate from larger, presumably solvated species, which acquire the internal energy necessary for their decay with [Cat – H]⁺ formation at relatively high CV values.

Conclusions

A representative of bisquaternary ammonium salts, ethonium, produces qualitatively different mass spectra in dependence of the experimental conditions applied. It is shown that the distinctions of electrospray mass spectral patterns in different cone voltage ranges are caused by subsequent appearance and destruction of several primary ions: the intact dication Cat^{2+} (occurring in the CV range from 10 V to 40-50 V), the dication-counterion cluster $Cat^{2+} \cdot Cl^-$ (survival range 20 V-80 V), and two ions $[Cat - H]^+$ and $[Cat - CH_3]^+$ (30 V-100 V). In the overlapping CV ranges the spectra are a superposition of the spectra originating from several precursor ions. From the practical point of view, namely, for ethonium identification in mixtures and extracts using electrospray ionization, the established types of ions are to be looked for and monitored when applying different CV values.

From a basic point of view, the survival of the dication of noncyclic BQACs with the shortest possible distance between the quaternary nitrogen atoms is a nontrivial feature, as well as the dominant pathways of fragmentation of Cat²⁺ resulting in formation of the doubly charged fragments instead of more common singly charged "charge separation" products. We provide an explanation for this behavior on the basis of analysis of the mean internal energy acquired by the ethonium ions in the electrospray ion source at different CV values and structural features of the organic dication of ethonium. The higher internal energy acquired by a dication as compared to singly charged species allows to explain its destruction at comparably low CV values. At the same time the survival of Cat²⁺ means that the energy of Coulomb repulsion between the charged groups of the dication (ca 4 eV), which is believed to be the main driving force of its fragmentation, appears to be insufficient for the decomposition of the Cat²⁺ of ethonium at the lowest CV values. The failure of the dication to fragment in spite of the electrostatic repulsion is explained by the delocalization of the unit positive charges over the hydrogen atoms of alkylammonium quaternary groups. An additional gain in internal energy of about 10-12 eV is required for the fragmentation of the ethonium dication. It could be speculated on this basis that the absence of intact dications of BQACs with short inter-charge distance in other desorption/ionization techniques is caused by the acquisition of internal energy exceeding the threshold determined in the present work. A low internal energy enables the survival of the dication's doubly charged fragments [Cat - $R_{+} + 2H]^{2+}$ and $[Cat - 2R_{+} + 2H]^{2+}$ as well. The weakest bonds involved in the formation of the above fragments appear to be the ester bonds.

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The mass spectral pattern of ethonium observed under MALDI with DHB matrix is in agreement with the revealed earlier formation of salts between an ammonium base and acidic matrix compound at the stage of sample preparation. Formation of such a salt hampers the observation of both diagnostic chlorine-containing precursor and fragment ions of the initial salt. Energetic conditions of the laser-generated plume do not permit the dication survival. Thus, the MALDI mass spectral pattern of ethonium does not match any of its electrospray patterns obtained in the whole range of CV values. The diagnostic ions which are to be looked for in the case of ethonium identification by MALDI are $[Cat - H]^+$ and $[Cat - CH_3]^+$ precursors and abundant fragments at m/z 242, 270, and 315.

The main mass spectral features revealed for ethonium may be applied to the identification of other types of BQACs compounds that are similar in structure.

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