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Triuret as a Potential Hypokalemic Agent: Structure Characterization of Triuret and Triuret-Alkali Metal Adducts by Mass Spectrometric Techniques

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Abstract

Triuret (also known as carbonyldiurea, dicarbamylurea, or 2,4-diimidotricarbonic diamide) is a byproduct of purine degradation in living organisms. An abundant triuret precursor is uric acid, whose level is altered in multiple metabolic pathologies. Triuret can be generated via urate oxidation by peroxyxynitrite, the latter being produced by the reaction of nitric oxide radical with superoxide radical anion. From this standpoint, an excess production of superoxide radical anions could indirectly favor triuret formation; however very little is known about the potential *in vivo* roles of this metabolite. Triuret's structure is suggestive of its ability to adopt various conformations and act as a flexible ligand for metal ions. In the current study, HPLC-MS/MS, energy-resolved mass spectrometry, selected ion monitoring, collision-induced dissociation, IRMPD spectroscopy, Fourier transform-ion cyclotron resonance mass spectrometry and computational methods were employed to characterize the structure of triuret and its metal complexes, to determine the triuret-alkali metal binding motif, and to evaluate triuret affinity toward alkali metal ions, as well as its affinity for Na⁺ and K⁺ relative to other organic ligands. The most favored binding motif was determined to be a bidentate chelation of triuret with the alkali metal cation involving two carbonyl oxygens. Using the complexation selectivity method, it was observed that in solution triuret has an increased affinity for potassium ions, compared to sodium and other alkali metal ions. We propose that triuret may act as a potential hypokalemic agent under pathophysiological conditions conducive to its excessive formation and thus contribute to electrolyte disorders. The collision- or photo-induced fragmentation channels of deprotonated and protonated triuret, as well as its alkali metal adducts, are likely to mimic the triuret degradation pathways *in vivo*.

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Keywords

Triuret; Uric acid degradation; Hypokalemia; Alkali metal adducts; Isomers; Electrospray ionization; Photodissociation; Collision-induced dissociation; IRMPD spectroscopy

Introduction

First discovered in 1870 by Ernst Schmidt [1], triuret ($C_3H_6N_4O_3$) is a nitrogen-rich compound which can be viewed as one of the most concentrated forms of peptide bonds. Surprisingly, very little is known about the biochemical and physiological significance of this molecule, although for many years it has been hypothesized that triuret (also known as carbonyldiurea, dicarbamylurea, or 2,4-diimidotricarbonic diamide) represents a byproduct of purine degradation in living organisms [2–6]. Small single crystals of triuret were observed in the cytoplasm of protozoans [6–12], where this purine derivative excretory product is contained in crystalline inclusions. It is uncertain whether other animals, phylogenetically unrelated to protozoans, are able to produce such large amounts of triuret, and if so, what conditions are conducive to its formation. An even more intriguing question concerns the potential roles of triuret in healthy and diseased states: in other words, is triuret content in biological samples of value as a biomarker?

In humans, higher primates and in a particular dog breed, Dalmatian, the end-product of purine catabolism is uric acid [13–20]. Most of this metabolite is excreted, with a small fraction being targeted by various reactive species, including free radicals. Consequently uric acid can be further degraded, e.g. into allantoin and urea. In other mammals, the activity of uricase further catabolizes uric acid into allantoin. In a number of living organisms, purine metabolic pathways do not stop at allantoin, but progress to simpler products, such as urea, carbon dioxide, and water. From a biochemical and structural point of view, triuret is an intermediate between allantoin and allantoate on one side, and their respective degradation products on the other side. It is therefore logical to expect that triuret precursors should be urate, allantoin, and allantoate (Scheme 1). It cannot be completely excluded that some organisms may possess an enzymatic activity for generating/degrading triuret. Literature analysis suggests that *Corynebacterium michiganense* may have an enzyme generically named *triuretase* that is able to degrade triuret [21]. The current lack of structural data concerning this putative *triuretase* makes it impossible to establish whether it is truly an individual enzyme, or if the observed activity is simply the result of an already known component of the purine metabolic pathway. In vertebrates, the chain of purine-degrading enzymes (uricase, allantoinase, allantoicase, ureidoglycolate lyase and urease) has been progressively truncated as a result of evolutionary pressure; however, the adaptive value promoting this change remains elusive [17, 22–24].

Under carefully controlled conditions, certain purines may generate triuret as a result of chemical, electrochemical or photo-oxidative degradation [6, 25–28]. A remarkable non-enzymatic pathway of triuret production has been previously reported, namely the peroxynitrite-mediated oxidation of urate [29]. This reaction can also occur at physiological pH, even in the presence of human plasma components or some biologically relevant antioxidants [30, 31], making it a plausible *in vivo* event. It was suggested that peroxynitrite may aid further triuret degradation to the aminocarbonyl radical, the latter being a reactive species and as such, hazardous for living organisms [29, 32]. Evaluation of triuret binding selectivity toward alkali metal ions revealed that this molecule preferentially produces a potassium adduct [33]. Naturally, this raises the question about possible triuret roles, if any, in altering potassium levels *in vivo* and its potential contribution to yet another process with harmful health consequences, hypokalemia, or potassium deficiency.

Taken together, the above mentioned observations seem to support the idea that triuret is an exclusively harmful molecule, either intrinsically or by virtue of its ability to generate dangerous degradation products. Insufficient knowledge concerning the biological relevance of triuret and the conditions of its *in vivo* formation make such a conclusion premature. In the 1950s it was reported that triuret may have antimalarial [34] and antineoplastic properties [35]. The functional groups, conformational flexibility, and the presence of peptide bonds in the triuret moiety make it a potentially interesting molecular "prototype" in designing new mimetic compounds of therapeutic value. To our knowledge, there are only a few reports concerning the properties of the unsubstituted triuret, whereas its substituted analogs have not been systematically investigated as potential inhibitors or chelating agents [6, 34, 35]. Synthesis of substituted triuret libraries and their testing in drug discovery studies holds promise, especially if the *in vivo* fluctuations of this metabolite prove to be of prognostic value.

In this work we conducted a series of experiments to investigate the chelating properties of triuret and its intrinsic reactivity. One of our goals was to determine the preferential coordination site of this compound, since there are multiple possibilities for triuret to bind metal ions and consequently to form various isomers. The most intriguing challenge and the goal of this research were to evaluate the binding motifs of the triuret-potassium and -sodium adducts in order to understand the chemistry underlying the potential hypokalemic effects of triuret *in vivo*.

Materials and methods

1. Chemicals and solvents

Triuret (CAS registry number: 556-99-0) was purchased from the Sigma-Aldrich library of rare chemicals (St. Louis, MO, USA). Urea, ammonium hydroxide (30%), ammonium acetate, glacial acetic acid, and HPLC grade solvents (acetone, methanol, and water) were from Fisher Scientific (Pittsburgh, PA, USA). Lithium chloride (LiCl), sodium chloride (NaCl), potassium chloride (KCl), rubidium chloride (RbCl), cesium chloride (CsCl), thiosemicarbazide and semicarbazide hydrochloride were obtained from Acros Organics (part of Thermo Fisher Scientific). Crown ether 12-crown-4 was purchased from Sigma, and 18-crown-6 was purchased from Aldrich. All the reagents were used as received without further purification.

A stock solution of 5 mM triuret was prepared as follows. 14.6 mg triuret in 20 mL water combined with 50 μ L ammonium hydroxide (30%) was heated gently and vortexed vigorously or sonicated (~ 45 min) in an ultrasonic bath (model FS30, Fisher Scientific) with warm water until the compound dissolved completely. As triuret dissolves slowly, it was necessary to alternate vortexing and gently heating the solution. Caution: increasing the concentration of ammonium hydroxide will improve the solubility of triuret; however, this procedure and/or increased heating of the solution can also induce triuret degradation.

2. Liquid chromatography/tandem mass spectrometry

The liquid chromatography/tandem mass spectrometry (LC-MS/MS)³ experiments were performed with an Agilent 1100 liquid chromatography system (Agilent Technologies, Palo Alto, CA, USA) and an TSQ 7000 triple-quadrupole mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) interfaces operated in both positive- and negative-ion detection modes. The high performance liquid chromatography (HPLC) system comprised a quaternary solvent delivery system, an on-line degasser, a thermostated autosampler, and a column temperature controller. The Luna 5 μ C18(2) 100A (150 mm $\times$ 4.6 mm) column used in this study was purchased from Phenomenex (Torrance, CA, USA). The mobile phase consisted of 5 mM ammonium acetate with 0.1% acetic acid (solvent A) and methanol (solvent B). The linear gradient elution mode was used to elute triuret and related species. The HPLC flow rate was 0.3 mL min⁻¹, and the autosampler injection volume was set at 10 μ L.

In the TSQ 7000 instrument, high pressure nitrogen (Airgas, Gainesville, FL, USA) was used as both the sheath (80 psi) and the auxiliary (15 units) gas. The ion transfer heated metal capillary temperature was maintained at 200 °C. In the ESI interface, the spray voltage was set at 4.5 kV. In the APCI interface, the vaporizer was kept at 500 °C, and the corona current was set to 4 μ A by applying approximately 4 kV to the corona needle. The second quadrupole of the mass spectrometer was used as a collision cell with ultra high purity argon (Airgas, Radnor, PA, USA) as a collision gas at a pressure of 2.5 mTorr. The HPLC-MS/MS system is controlled by Xcalibur software, which is a flexible Windows NT PC-based data acquisition system that allows complete instrument control.

Triuret was studied in both positive- and negative-ion modes using the ESI and the APCI interface, while triuret-alkali metal adducts were generated by the ESI ion source operated in positive-ion detection mode. The post-column complexation selectivity method used for evaluating the binding selectivity of triuret toward alkali metal ions was implemented on a HPLC-ESI-MS/MS TSQ-7000 instrument operated in selected ion monitoring (SIM) mode, and equipped with a mixing tee for infusion of post-column reagent mixtures. A programmable syringe pump (Harvard Apparatus, Holliston, MA, USA) delivered the post-column reagent mixture at the selected flow rate.

3. Fourier transform-ion cyclotron resonance mass spectrometry

A custom Fourier transform-ion cyclotron resonance (FT-ICR) mass spectrometer at the FOM Institute for Plasma Physics Rijnhuizen in Nieuwegein, The Netherlands was used for the infrared multiple photon dissociation (IRMPD) experiments. The FT-ICR mass spectrometer is based on a 4.7 T actively shielded superconducting magnet (Cryomagnetics, Oak Ridge, TN, USA) with a 128 mm i.d. horizontal bore, vacuum system and an open, cylindrical ICR cell [36]. Instrument control and data acquisition was performed using a PXI-based data station adapted from that described previously [37]. The instrument was equipped with a commercial Z-spray electrospray ionization source (Micromass, Manchester, UK) that produces ions at atmospheric pressure in a spray plume orthogonal to a sampling cone. Solution flow rates ranged from 20 to 30 μ L min⁻¹. Specific ESI operating

³Abbreviations used

APCI, atmospheric pressure chemical ionization; CID, collision-induced dissociation; E_{CM} , collision energy (center-of-mass frame); E_{lab} , collision energy (laboratory frame of reference); EPD, electrostatic potential distribution; ERMS, energy-resolved mass spectrometry; ESI, electrospray ionization; ESP, electrostatic potential; FELIX, free electron laser for infrared experiments; FT-ICR, Fourier transform-ion cyclotron resonance; FWHM, full width at half maximum; HPLC, high performance liquid chromatography; IRMPD, infrared multiple photon dissociation; L, ligand; LC-MS/MS, liquid chromatography/tandem mass spectrometry; MS, mass spectrometry; SCF, self consistent field; SIM, selected ion monitoring; H_K^+ , potassium ion affinity; H_{Na}^+ , sodium ion affinity.

parameters such as spray voltage, cone voltage, and ion transfer lens voltages were adjusted to maximize formation and transfer of ions to the ICR cell.

4. Infrared multiple photon dissociation

IRMPD spectra were obtained by photoexcitation of mass-selected trapped ions with tunable pulsed radiation from the “Free Electron Laser for Infrared eXperiments” (FELIX) at the FOM Institute for Plasma Physics in Nieuwegein, The Netherlands [38, 39] as a function of laser wavelength. Data were recorded over the wavelength range 19.4 μm (520 cm^{-1}) to 5.5 μm (1820 cm^{-1}). Pulse energies were around 40 mJ per macropulse of 5 μs duration. The full width at half maximum (FWHM) bandwidth of the laser was typically 0.5% of the central wavelength.

IRMPD occurs through non-coherent absorption of tens to hundreds of IR photons when the laser frequency matches a vibrational mode of the gas phase ion. The energy is distributed over all vibrational modes by intramolecular vibrational redistribution, which allows the energy of each photon to be “relaxed” prior to the absorption of the next photon, and thus allows promotion of the ion’s internal energy to the dissociation threshold [40]. The IRMPD product ions and un-dissociated precursor ion intensities were measured using the excite/detect sequence of the FT-ICR mass spectrometer after irradiation. The IRMPD yield was normalized to the total ion current, and corrected over the spectral range for variations in laser power.

5. Computational methods

The HyperChemTM (Hypercube, Inc., Gainesville, FL, USA) suite of programs was used to determine the 56 most stable conformational isomers of the triuret-alkali metal adducts. All the stable conformers were imported into the Gaussian 03 (Gaussian, Inc., Pittsburgh PA, USA) software [41], where geometry optimizations were performed using the B3LYP/6-311++G(d,p) level of theory. The final structures were then compared for total energy and structural similarity, which reduced the number of different conformers under consideration to 25 (for each alkali metal adduct). To verify that all conformations were minima, frequency calculations at the same level of theory were performed. Using the predicted frequencies, simulated IR spectra in the range of 0-4000 cm^{-1} were produced for each conformer. Plotting the corresponding calculated IR spectra for the two lowest energy conformers showed that the spectral bands for each are distinct enough to allow comparison to IRMPD spectra and to differentiate which particular conformation may exist in experimental conditions. Electrostatic potential (ESP) surface diagrams of each isomer were generated from the self consistent field (SCF) total electron density from the ground state calculation by using the GaussView 3.09 (Semichem, Inc., Shawnee Mission, KS, USA) software.

Results and discussion

1. On the structure of triuret and its adducts

The triuret molecule has a certain degree of flexibility, allowing it to exist in multiple conformeric forms. Some conformations can be stabilized or favored by replacing the hydrogen atoms with alkyl side groups. For example, hexaethyltriuret assumes symmetric and asymmetric helical conformations in solution. Moreover, interconversion of these forms was observed and reversal of helicity was shown in a variable-temperature NMR spectroscopy study [42]. Flexibility and presence of functional groups allow the triuret molecule to act as a ligand for metal ions or even coordinatively unsaturated metal centers. Triuret’s flexibility is able to facilitate the coordination needs of metal ions, although one can easily imagine that only certain triuret conformations would be selectively stabilized. In

crystals triuret assumes the planar conformation [43] schematically represented in Scheme 1. Our calculations also showed that this symmetrical conformer is the most stable *in vacuo*, followed closely by the other planar asymmetrical conformer, the difference between them being only 0.5 kcal mol⁻¹. The electrostatic potential distribution (EPD) was calculated for these two isomers and is shown in Figure 1. Regardless of the assumed conformation, the neutral triuret molecule possesses centers of pronounced negative electrostatic potential with an outward orientation. These structural characteristics define the potential of the triuret as a ligand toward metal ions. In adducts with alkali metal ions or in protonated species, triuret prefers different conformations compared to those of the bare triuret molecule. For example, Figure 2 shows six of the most stable conformers of sodiated triuret. Excluding two of the most stable conformers of the protonated triuret or triuret-alkali metal adducts (e.g., structures A and B in Figure 2), other conformations predicted by computational optimizations (Supplementary material) were less stable and were not expected to contribute to the IRMPD spectral signature; therefore they were not included in the subsequent analysis.

It should be noted that the properties of the triuret molecule (flexibility, EPD, ability to form hydrogen bonds) make it a probable non-covalent interaction partner for diverse biological molecules. Further discussion of triuret interactions with biomolecules is beyond the scope of this study.

2. Mass spectrometry of protonated and deprotonated triuret

The first report concerning the detection of the product of the oxidative reaction of uric acid with peroxynitrite in a positive-mode ESI mass spectrum was published a decade ago [44]. The detected ion [product + H]⁺ has a *m/z* of 147. Besides this ion, the mass spectra also showed peaks corresponding to [product + NH₄]⁺ (*m/z* 164) and [product + Na]⁺ (*m/z* 169) [44]. Soon thereafter another report provided additional evidence that triuret is indeed the product of peroxynitrite-mediated oxidation of urate [29]. By employing positive-mode ESI mass spectrometry, these authors detected the dominant ion [triuret + H]⁺ (*m/z* 147) and the adduct [triuret + Na]⁺ (*m/z* 169), as well as fragment ions that aided in the elucidation of the structure. Mass spectral data were gathered in single MS mode [29].

To investigate the mass spectrometric behavior of triuret, we employed a triple-quadrupole LC-MS/MS instrument with ESI and APCI ion sources. Both ion sources were operated in two different ion modes, positive and negative. Regardless of any given operation mode, the experimental parameters were adjusted in such a way as to ensure the "gentlest" possible experimental conditions. Therefore, positive-ion mode operation produced [triuret + H]⁺ (*m/z* 147) as the most abundant ion for both ESI and APCI sources. "Gentle" ionization conditions not only ensured minimal fragmentation rates, but also allowed adduct ions such as [triuret + NH₄]⁺ (*m/z* 164), [triuret + H₂O + H]⁺ (*m/z* 165), [triuret + CH₃OH + H]⁺ (*m/z* 179), and [2(triuret) + H]⁺ (*m/z* 293) to be preserved and detected.

Post-column injection of a highly diluted aqueous solution of ammonium hydroxide not only led to a significant increase in the yield of [triuret + NH₄]⁺ (*m/z* 164) ions, but also allowed for the detection of triuret-dimer and triuret-trimer adducts, [2(triuret) + NH₄]⁺ (*m/z* 310), and [3(triuret) + NH₄]⁺ (*m/z* 456) respectively. Protonated triuret fragmentation pathways (Scheme 2) were determined based on tandem mass spectra and energy-resolved mass spectrometry (ERMS) experiments (Figure 3). As shown, the dissociation of protonated triuret under collision-induced dissociation (CID) conditions primarily occurs following the consecutive loss of neutral ammonia (NH₃) or isocyanic acid (HNCO) in different combinations. Some of these reactions are accompanied by hydrogen atom migration. Detection of a fragment ion [OCNCO]⁺ (*m/z* 70) that failed to retain a single hydrogen atom, was surprising (Scheme 2). Structure and reactivity of this ion have been repeatedly

investigated in the past [45–52]. Our CID MS/MS experiments of the mass-selected ion with m/z 70 produced following the fragmentation of protonated triuret revealed that its structure is "linear" as shown on Scheme 2 and its MS/MS spectrum is consistent with that reported earlier for a similar structure [47, 48].

In the negative-ion mode, deprotonated triuret $[\text{triuret} - \text{H}]^-$ (m/z 145) is the main ion detected and the peak corresponding to the deprotonated dimer $[2(\text{triuret}) - \text{H}]^-$ (m/z 291) can also be seen. Surprisingly, in one of the experiments the ion $[\text{triuret} + \text{Cl}]^-$ (m/z 181 for ^{35}Cl and m/z 183 for ^{37}Cl) was seen, with the Cl^- later proving to be a contaminant (mass spectra not shown). This serendipitous observation shows that triuret is capable of coordinating both cations and anions.

CID MS/MS and ERMS experiments (Figure 3c) showed that deprotonated triuret generates fewer fragments (Scheme 3) than its protonated counterpart. The fragmentation scheme of deprotonated triuret seems to plausibly reflect its potential degradation pathways under physiological conditions. One could hypothesize that certain extramolecular factors would be capable of degrading deprotonated triuret into biuret, urea, isocyanic acid, and ammonia, as represented on Scheme 3. Additionally, it cannot be excluded that triuret may be able to generate the aminocarbonyl radical following its oxidation reaction with peroxynitrite, as proposed before [29].

In conclusion, ESI and APCI mass spectra recorded in both positive- and negative-ion modes in combination with CID MS/MS and ERMS data correlate well with the triuret structure and predicted properties. These mass spectrometric characteristics will serve as reference data for the analytical methods for triuret detection and quantification in a wide range of samples, including complex biological and clinical specimens.

3. ESI mass spectrometry of triuret-alkali metal adducts

ESI MS is recognized as a soft ionization method, which allows for the transfer of ionic species preexisting in solution to the gas phase [53]. When a mixture of triuret solution and alkali metal chloride solution (1:1) was electrosprayed, species corresponding to $[\text{triuret} + \text{Li}]^+$ (m/z 153), $[\text{triuret} + \text{Na}]^+$ (m/z 169), $[\text{triuret} + \text{K}]^+$ (m/z 185 for ^{39}K and m/z 187 for ^{41}K), $[\text{triuret} + \text{Rb}]^+$ (m/z 231 for ^{85}Rb and m/z 233 for ^{87}Rb), and $[\text{triuret} + \text{Cs}]^+$ (m/z 279) were generated. The relatively high peak intensities seen for these product ions indicate that triuret must have affinity for this group of metal cations. When experiments were performed with sodium, potassium, rubidium and cesium cations, peaks due to two additional types of ions were seen: the dimer adduct $[2(\text{triuret}) + \text{alkali metal}]^+$, and the trimer adduct $[3(\text{triuret}) + \text{alkali metal}]^+$.

4. Evaluation of triuret binding selectivity toward alkali metal ions in solution

Structural characteristics of triuret, such as presence of electronegative functional groups, flexibility of its molecular moiety, and electrostatic potential distribution (EPD) make this molecule a good candidate for a polydentate chelator for metal cations. To estimate the binding selectivity of triuret toward alkali metal ions in solution, a rapid method based on an HPLC-MS/MS instrument operated in the selected ion monitoring mode was employed. In this method, the solution of the ligand (triuret) was introduced by an autosampler via the chromatographic system. The post-column reagent mixture consisting of alkali metal chlorides (LiCl , NaCl , KCl , RbCl , and CsCl at 1 mM each in water) was injected from the syringe pump at a rate of 1, 5, 10, 20, 50, or 100 $\mu\text{L min}^{-1}$ through a tee union upstream from the ESI interface. Flow rates and concentrations of triuret and alkali metal salt solutions were adjusted as needed by the design of each particular experiment and the resultant data were isotope corrected. Competitive complexation of the alkali metal cations

with triuret revealed that the $[\text{triuret} + \text{K}]^+$ adduct prevailed over all other species ($[\text{KL}]^+$ $[\text{CsL}]^+ [\text{RbL}]^+ > [\text{NaL}]^+ > [\text{LiL}]^+$; where L = triuret). Varying the amount of the reagent mixture furnished to the reaction by changing the injection flow rate did not alter this outcome, and the potassium-triuret adduct remained the most abundant product. Furthermore, the same result was reproduced when performing the experiment with a reagent mixture containing equimolar amounts of sodium and potassium chlorides (Figure 4). These results suggest that in solution triuret has an increased affinity for potassium compared to other alkali metal cations. Assuming that the SIM mass spectrometric technique under HPLC-ESI-MS/MS experimental settings is a true reflection of the coordination chemistry of triuret solutions, it can be concluded that triuret will prefer to bind potassium over sodium (or other alkali metals). This property suggests that triuret may be able to deplete potassium under certain *in vivo* conditions and should therefore be considered as a potential hypokalemic agent.

5. Validation of the post-column complexation selectivity method

The post-column complexation selectivity method used for evaluating the binding specificity of triuret toward alkali metal ions in solution was implemented on a HPLC-ESI-MS/MS TSQ-7000 instrument (selected ion monitoring mode) equipped with a mixing tee for infusion of post-column reagent mixtures.

The mass spectra of the compounds containing polyisotopic elements are difficult to interpret if there is an overlapping of isotope clusters of ions. On the other hand, if such overlapping does not occur, the presence of polyisotopic elements with their distinctive signature greatly facilitates the assignment of the isotope clusters in a mass spectrum [54]. For quantitative measurements, one specific m/z peak was selected from the isotope cluster for each analyte (triuret-alkali metal adduct) and its SIM response was integrated. Next, an isotope correction of area counts of each SIM response was performed by using calculated isotope distributions.

The control experiments were performed with a well-studied molecule, the crown ether 18-crown-6 with previously determined binding constants for alkali metal ions [55–57]. First, the 18-crown-6 solution was premixed with alkali metal chloride solutions in equimolar amounts (1 mM each), electrosprayed, and the distribution of crown ether/alkali metal complexes in the ESI mass spectra was examined. Next, the experiment was performed by employing the post-column complexation method and the ESI mass spectra of crown ether/alkali metal complexes generated in this way were compared to those obtained with premixed solutions. The host molecule 18-crown-6 (10 μL of 1 mM solution in methanol) was introduced by the autosampler of the HPLC system at a flow rate of 0.3 mL min^{-1} . The post-column reagent mixture (solution of LiCl, NaCl, KCl, RbCl, and CsCl at 1 mM each in water) was delivered through the mixing tee at a flow rate of 0.03 mL min^{-1} . The results of both measured distribution sets were similar and in agreement with previously reported theoretical and experimental data for 18-crown-6/alkali metal complexes [55–57].

6. CID MS/MS characterization of the monomer triuret-alkali metal adducts

As shown above, ESI MS is a suitable method for generation of triuret-alkali metal adducts. To characterize mass-selected adducts of interest, the CID MS/MS approach with argon as the collision gas was employed on a triple-quadrupole mass spectrometer. Low-energy CID MS/MS spectra recorded at collision energy (laboratory frame of reference) $E_{\text{lab}} = 10 \text{ eV}$ are shown in Figure 5. The data show that at this collision energy cesium, rubidium, and potassium monomer adducts display identical qualitative behaviors: the dissociation of the metal ion-organic ligand bond occurs with the release of the intact triuret molecule and the bare metal cation. Quantitative assessment shows that a larger radius of the alkali metal ion

($\text{Cs}^+ > \text{Rb}^+ > \text{K}^+$) correlates with a higher dissociation rate (Figure 5). This dissociation path is the most energetically convenient, in agreement with the weak dipole-ion nature of the interaction between triuret and these alkali metal cations.

Under similar experimental conditions, in the gas phase, monomer triuret adducts of sodium and lithium display completely different behaviors. Under low-energy CID conditions mass-selected $[\text{triuret} + \text{Na}]^+$ and $[\text{triuret} + \text{Li}]^+$ ions fragment with the release of neutral ammonia (Figure 5). Firstly, this process can be explained by the smaller radii of Li^+ and Na^+ compared to those of other alkali metal ions, leading to higher charge densities and therefore stronger metal ion-ligand bonds. Consequently, these bonds survive the low-energy collision conditions and the more convenient fragmentation channel (release of ammonia) is thus favored. The newly activated fragmentation channel presumably does not have a high energy barrier for dissociation.

In the condensed phase, e.g. in solution, with a permanent dynamic environment offering solvation conditions satisfying the coordinative needs of metal ions, the stability of triuret-alkali metal adducts is expected to be different. The solvation sphere exerts a stabilizing effect on adducts by coordinatively saturating the metal center and by promoting the non-covalent interactions of the coordinated solvent molecules with the organic moiety of the adduct. Previous reports have conclusively shown the role of solvation phenomena in the selective stabilization of adducts of some metal ions over others [58].

Increasing the collision energy to values of $E_{\text{lab}} > 15$ eV led to a higher abundance and diversity of fragment ion products derived from $[\text{triuret} + \text{Na}]^+$ and $[\text{triuret} + \text{Li}]^+$ adducts (Figure 5). The resulting fragment ions can be rationalized either by sequential releases of neutral molecules such as ammonia (NH_3 , 17 u) and isocyanic acid (HNCO , 43 u), or by newly formed adducts of biuret, urea, and isocyanic acid with sodium and lithium ions (for the sodium ion: $[\text{biuret} + \text{Na}]^+$ (m/z 126), $[\text{urea} + \text{Na}]^+$ (m/z 83), $[\text{HNCO} + \text{Na}]^+$ (m/z 66); and for the lithium ion: $[\text{biuret} + \text{Li}]^+$ (m/z 110), $[\text{urea} + \text{Li}]^+$ (m/z 67), $[\text{HNCO} + \text{Li}]^+$ (m/z 50)).

7. Influence of the degree of metal ion solvation on the collision-induced fragmentation pathways of triuret complexes

Investigation of the CID behavior of mass-selected trimers $[\text{metal} + 3(\text{triuret})]^+$ ($\text{metal}^+ = \text{Na}^+, \text{K}^+, \text{Rb}^+, \text{Cs}^+$) showed that dissociation occurs similarly to the monomer entities of K^+ , Rb^+ and Cs^+ , releasing an intact triuret molecule. This was the only observed fragmentation channel, regardless of the metal ion investigated ($\text{Na}^+, \text{K}^+, \text{Rb}^+, \text{Cs}^+$). Likewise, dimer adducts $[\text{metal} + 2(\text{triuret})]^+$ were also characterized by a single fragmentation channel, leading to the release of a triuret molecule and formation of triuret-metal monomers, $[\text{metal} + \text{triuret}]^+$ ($\text{metal}^+ = \text{Na}^+, \text{K}^+, \text{Rb}^+, \text{Cs}^+$).

Monomers produced from trimers and dimers have similar fragmentation patterns to those of monomers generated directly by the ESI source, mass-selected in the first quadrupole and subjected to fragmentation conditions in the second quadrupole. Characterization of monomers under CID conditions was described above and it was shown that Cs^+ , Rb^+ , and K^+ adducts eliminate the intact ligand (triuret), whereas Na^+ and Li^+ monomer adducts dissociate via different pathways, with triuret fragmentation into smaller organic molecules (Figure 5).

With the increase in degree of solvation for the alkali metal ion due to coordination of two or three triuret molecules (dimer or trimer adducts) the only fragmentation channel for all analyzed metals is the dissociation of metal ion-organic ligand bonds. Monomer adducts, which are coordinatively unsaturated ionic species, i.e. with a lower solvation degree than

dimers or trimers, follow different fragmentation pathways. This observation indicates that with increases in solvation degree for the alkali metal ions, their charge densities acquire a more "diffuse" character, with CID reactions resulting in expulsion of intact ligands. In coordinatively unsaturated ionic species the interaction metal ion-ligand is different in nature. As shown in CID experiments, charge densities are metal-specific and become a defining factor for these metal ion-ligand interactions in monomer triuret adducts. In larger metal ions such as Cs^+ , Rb^+ , and K^+ charge densities are distributed more diffusely and therefore their interaction with the ligand even in the monomer complexes is relatively weak in the gas phase, exhibiting behaviors similar to trimer and dimer triuret complexes with sodium. In monomer complexes of triuret with sodium (and lithium), the ions possess higher charge densities and therefore ligand fragmentation is induced. The mechanism whereby these metal-specific fragmentation pathways occur is of special interest. At least some of the fragmentation pathways could be governed by intramolecular rearrangements preceded by ligand insertion. Their elucidation is relevant for understanding the intramolecular catalytic function of metal ions during such rearrangements. Their discussion is beyond the scope of this study and will not be pursued further.

8. Triuret affinity for K^+ and Na^+ relative to other organic ligands

We have used the Cooks' kinetic method [59, 60] to evaluate triuret affinity for K^+ and Na^+ relative to other organic ligands (L). Besides triuret, ligands such as urea, semicarbazide, thiosemicarbazide, acetone, and crown ether 12-crown-4 were arbitrarily chosen. The kinetic method experiments involved ESI generation of gaseous $[\text{L}' + \text{metal} + \text{L}'']^+$ hetero-dimers and subsequent low-energy collision-induced dissociation (target gas: Ar) of these species to the potassiated (or sodiated) monomers according to Scheme 4 ($\text{metal}^+ = \text{K}^+$ or Na^+).

The signal of the hetero-dimers was optimized by adjusting the ESI conditions. The $[\text{L}' + \text{metal} + \text{L}'']^+$ ion of interest was mass-selected with the first quadrupole (Q1) and was subjected to CID in the collision chamber, the second quadrupole (Q2). The dissociation product ions, monomers $[\text{L}' + \text{metal}]^+$ and/or $[\text{L}'' + \text{metal}]^+$, were analyzed by the third quadrupole (Q3). No secondary fragments were observed. All experiments were repeated three times for reproducibility. The results of these experiments are presented in Table 1. Potassium ion affinity (H_{K}^+) order for the studied ligands may be deduced from the relative abundances (acetone < urea < semicarbazide < thiosemicarbazide < triuret < 12-crown-4) of the product ions shown in Table 1. These experiments show that for the investigated organic molecules, the relative ordering of sodium ion affinities (H_{Na}^+) is the same as for H_{K}^+ . The triuret molecule binds K^+ (or Na^+) stronger than oxygen-containing ligands: acetone, urea, semicarbazide. This observation could be explained plausibly by the ability of triuret to act as a bidentate ligand in the hetero-dimers under investigation, thus forming a chelate with the metal ion. Obviously, chelates tend to be more stable than complexes between monodentate ligands and metal ions, provided that the coordination involves the same type of donor atoms. As expected, crown ether 12-crown-4 binds alkali metal cations stronger than triuret due to the presence of a macrocyclic effect during coordination. Deduced potassium (or sodium) ion affinity order for the studied ligands (acetone < urea < semicarbazide < thiosemicarbazide < triuret < 12-crown-4) may be anchored to the known values of binding energies either experimentally determined or theoretically calculated [61–64]. This procedure allows for the estimation of unknown binding energies. Evidently, binding energies for triuret-alkali metal adducts will be lower than those for crown ether adducts, but higher than those of alkali metal adducts with acetone, urea, semicarbazide or thiosemicarbazide.

9. Probing the best binding motif in triuret-potassium and -sodium adducts by IRMPD

IRMPD spectroscopy is a highly sensitive technique successfully employed to obtain detailed binding information on metal ion-organic ligand complexes [65–68]. Calculated IR spectra (Supplementary material, Figures S1–S6) for the two lowest energy conformers of the protonated triuret or triuret-alkali metal adducts (e.g. structures A and B in Figure 2) showed that the spectral bands for each of them are distinct enough to allow comparison to IRMPD spectra and to differentiate which particular conformation may exist under a given set of experimental conditions. This spectroscopic method revealed that IRMPD spectra of triuret-potassium and -sodium adducts are very similar (Figure 6a and 6b), suggesting that their structures should also be similar. The experimental spectra (Figure 6) and those predicted for the respective lowest energy conformers of studied adducts clearly represent the best match. These data indicate that the best binding motif for both sodiated and potassiated adducts is a bidentate association involving two carbonyl oxygens (e.g. structure A in Figure 2). This conformer represents a charge-solvated structure. The IRMPD spectrum of the protonated triuret (Figure 6c) differs to some extent from that of metal adducts. The difference is due to the presence of corresponding metal ions in adducts. Our findings about the favored coordination sites (carbonyl oxygens) in the triuret-potassium and -sodium adducts are consistent with the gas phase infrared fingerprint spectroscopy and theoretical studies conducted on sodiated or potassiated di-, tri-, tetra- and pentapeptides which show that the amide carbonyl oxygen in such complexes is always chelated in the low-energy conformer [69–77].

Photodissociation of $[\text{triuret} + \text{K}]^+$ results in the loss of the intact triuret to form the atomic metal cation, consistent with CID data (Figure 5) for $[\text{triuret} + \text{K}]^+$, $[\text{triuret} + \text{Rb}]^+$ and $[\text{triuret} + \text{Cs}]^+$. For the sodiated adduct (m/z 169) photodissociation and CID spectra exhibit two more low-energy channels, one corresponding to loss of ammonia (signal at m/z 152), and the other to formation of the urea-sodium adduct (m/z 83). It is intriguing to speculate whether analogous decompositions of triuret may be induced under physiological conditions. Detection of the m/z 152 and 83 fragments suggests that a rearrangement involving hydrogen atoms occurs in $[\text{triuret} + \text{Na}]^+$ prior to photo- or collision-induced fragmentation. It cannot totally be excluded that a more extensive collision-induced isomerization may have occurred partially during the CID experiment. The two new competing dissociation channels may also have originated from the partial isomerization of the sodiated adduct ions during the last step of ion desolvation in the ESI process. Such phenomena (rearrangement versus desolvation during ESI) were previously reported for some metal ion adducts [78]. Further experiments and theoretical calculations will be necessary to support this hypothesis as applied to the studied $[\text{ligand} + \text{metal}]^+$. Low-energy CID of the ESI-generated sodiated dimer $[2(\text{triuret}) + \text{Na}]^+$ resulted in the loss of one intact triuret. The sodiated trimer $[3(\text{triuret}) + \text{Na}]^+$ generates the dimer and the monomer by consecutive losses of the intact triuret. The monomer produced by CID of mass-selected dimer or trimer behaves as described above, releasing ammonia and producing the urea-sodium adduct and bare metal cation.

Concluding remarks

Investigations dedicated to uric acid reactivity, its degradation products and physiological relevance proved that this metabolite possesses unique and diverse biochemical properties. Depending on the physiological milieu, it may act as either antioxidant or pro-oxidant [79–88]. Multiple metabolic pathologies, both genetic and acquired, are associated with altered uric acid content [89–104]. From this standpoint, the study of triuret as a byproduct of uric acid degradation becomes even more important, especially considering that triuret can be generated via urate oxidation by peroxynitrite (ONOO^-), the latter being produced by the reaction of nitric oxide radical ($\cdot\text{NO}$) with superoxide radical anion ($\text{O}_2^{\cdot-}$) [105]. It is

therefore enticing to hypothesize that an excess of superoxide radical anions could lead to a transient increase in triuret production. Nevertheless, very little is known about the biochemistry and physiological significance of this molecule.

The triuret molecule has diverse structural characteristics. Flexibility of its molecular moiety, electrostatic potential distribution and ability to form hydrogen bonds make it a probable non-covalent interaction partner for a variety of bioorganic molecules. Moreover, our experimental data revealed that triuret is capable of coordinating both anions (Cl^-) and cations (H^+ , NH_4^+ , metal ions). IRMPD spectroscopy results combined with computational methods suggest that the best triuret-alkali metal binding motif is a bidentate association involving two carbonyl oxygens. The monomeric triuret-alkali metal chelates represent a coordinatively unsaturated and charge-solvated structure. In solution, solvent molecules will satisfy the coordination needs of coordinatively unsaturated species and also will stabilize the triuret adducts by generation of hydrogen-bonding interactions.

While evaluating triuret affinity for K^+ and Na^+ relative to other organic ligands by using the Cooks' kinetic method, it was established that the triuret molecule binds these alkali metal cations stronger than monodentate ligands (e.g. acetone, urea, semicarbazide, thiosemicarbazide) do. This behavior is attributed to the ability of triuret to act as a bidentate ligand and form a chelate with the metal ion. Compared to monodentate ligands and triuret, crown ether 12-crown-4 exhibited a higher affinity for both cations. Evidently, the multidentate binding of a metal ion is a strong stability factor of the complexes.

The complexation selectivity method implemented on a HPLC-ESI-MS/MS instrument using the selected ion monitoring mode of detection revealed an increased triuret affinity for potassium compared to other alkali metal cations in solution. This observation suggests that triuret may be able to deplete potassium under certain pathophysiological conditions and should therefore be considered as a potential hypokalemic agent. Hypokalemia is an electrolyte disorder that has been associated with a plethora of pathologic conditions: cardiovascular, neuromuscular, renal, and metabolic [106–109]. A hypokalemic state results from either an increased K^+ shift into cells and/or excessive net loss of K^+ . Due to potential encounters of triuret with such alkali metals as sodium and potassium ions under physiological conditions, elucidation of the coordination chemistry of metal-triuret adducts becomes of special interest. It would be enticing to hypothesize that excessive triuret production could, at least to some extent, impact the ion balance and hence convey relevance to its elevated content in certain pathologies. Specialized studies would be necessary to define the conditions conducive to *in vivo* triuret formation in amounts significant enough for exerting such a hypokalemic effect, as well as to clarify whether such phenomena can indeed be associated with pathological metabolic conditions.

The observed and described collision- or photo-induced fragmentation channels of deprotonated and protonated triuret, as well as its alkali metal adducts, are likely to mimic the triuret degradation pathways under physiological conditions. As shown in the current study, low-energy collision-induced dissociation of the investigated ionic species ($[\text{triuret} - \text{H}]^-$, $[\text{triuret} + \text{H}]^+$, $[\text{triuret} + \text{metal}]^+$) produced some of the following neutral fragment molecules: ammonia (NH_3 , 17 u), isocyanic acid (HNCO , 43 u), urea ($\text{OC}(\text{NH}_2)_2$, 60 u), biuret ($\text{H}_2\text{N}(\text{CO})\text{NH}(\text{CO})\text{NH}_2$, 103 u). One could easily envision certain extramolecular factors being able to degrade triuret *in vivo* into the enumerated molecules. Another neutral species ($\text{C}_3\text{H}_3\text{N}_3\text{O}_3$, 129 u) was formed under CID conditions, where both $[\text{triuret} + \text{Li}]^+$ and $[\text{triuret} + \text{Na}]^+$ released ammonia, whereas the 129 u fragment remained coordinated to the metal cation (ions with m/z 136 for Li^+ and with m/z 152 for Na^+). A similar process, loss of ammonia from $[\text{triuret} + \text{metal}]^+$, was observed when the sodiated adduct was subjected to photodissociation. This neutral fragment ($\text{C}_3\text{H}_3\text{N}_3\text{O}_3$, 129 u) coordinated to the

cation may have either “linear” or cyclic structure (cyanuric acid). A summary of the possible triuret degradation pathways under physiological conditions is presented on Scheme 1.

The mass spectrometric characteristics described in this study provide reference data for analytical methods designed for identification and quantification of triuret and its adducts in a variety of samples, including biological and clinical.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

The electrostatic potential distribution for two of the most stable triuret conformers: (a) the planar symmetrical (most stable) and (b) the planar asymmetrical conformer. The difference between them is only $0.5 \text{ kcal mol}^{-1}$. Values in dark blue indicate strong, positive electrostatic potential, whereas those in light green correspond to a strong, negative electrostatic potential.

Figure 2.

Six of the most stable conformers of sodiated triuret (in order of stability). Metal ion is shown in purple, oxygen in red, nitrogen in blue, carbon in green and hydrogen in white. Relative energies were computed using the density functional theory quantum mechanical method at the B3LYP/6-311++G(d,p) level.

Figure 3.

Energy-resolved mass spectrometry (ERMS) data. (a) Total ion intensity plotted as a function of the laboratory collision energy (E_{lab}) in the experiment performed in the positive-ion mode using APCI ionization source and the solution of triuret as analyte. Argon was used as a collision gas. (b) ERMS plot for the protonated triuret (m/z 147) and CID product ions. This plot is normalized to the total ion intensity (Figure 3a) for each energy value. (c) ERMS plot for the deprotonated triuret (m/z 145) and CID product ions. This plot is normalized to the total ion intensity (not shown) for each energy value.

Figure 4.

Competitive complexation of Na^+ and K^+ by triuret in solution. Data was measured in septuplicate with the post-column complexation selectivity method using the selected ion monitoring mode of detection (HPLC-ESIMS/ MS instrument) and isotope corrected. In all experiments 5 μL of triuret (5 mM) was introduced via the HPLC system operated at a flow rate of 300 $\mu\text{L min}^{-1}$ using methanol as mobile phase. The post-column reagent mixture (solution of NaCl and KCl at 2 mM each in water) was delivered through the mixing tee at a flow rate: A) 5 $\mu\text{L min}^{-1}$; B) 10 $\mu\text{L min}^{-1}$; C) 20 $\mu\text{L min}^{-1}$.

Figure 5.

Collision-induced dissociation mass spectra of triuret-alkali metal adducts: (a) $[\text{triuret} + {}^{133}\text{Cs}]^+$ ($E_{\text{lab}} = 10\text{ eV}$), (b) $[\text{triuret} + {}^{85}\text{Rb}]^+$ ($E_{\text{lab}} = 10\text{ eV}$), (c) $[\text{triuret} + {}^{39}\text{K}]^+$ ($E_{\text{lab}} = 10\text{ eV}$), (d) $[\text{triuret} + {}^{23}\text{Na}]^+$ ($E_{\text{lab}} = 10\text{ eV}$), (e) $[\text{triuret} + {}^{23}\text{Na}]^+$ ($E_{\text{lab}} = 20\text{ eV}$), (f) $[\text{triuret} + {}^7\text{Li}]^+$ ($E_{\text{lab}} = 10\text{ eV}$), and (g) $[\text{triuret} + {}^7\text{Li}]^+$ ($E_{\text{lab}} = 20\text{ eV}$). Precursor ions were generated via ESI and mass-selected prior to subjecting them to CID using triple-quadrupole mass spectrometer. Argon was used as the collision gas.

Figure 6.

Experimental IRMPD spectra (red line) of the potassium-triuret adduct (a), sodium-triuret adduct (b) and protonated triuret (c) compared to DFT frequency calculations convoluted with a 30 cm⁻¹ FWHM Gaussian band profile (black line) for the respective predicted most stable conformers.

Scheme 1.

Triuret precursors (uric acid, allantoin and allantoic acid), triuret and its degradation products (ammonia, cyanuric acid, urea, biuret, isocyanic acid and aminocarbonyl radical). Progression through each arm of the pathway requires specific chemical or physical agent(s) and appropriate medium conditions. Some degradation channels may occur under physiological conditions with/or without participation of enzymes.

Scheme 2.

Main collision-induced fragmentation pathways for protonated triuret (m/z 147) based on APCI-MS/MS data.

Scheme 3.

Main collision-induced fragmentation pathways for deprotonated triuret (m/z 145) based on APCI-MS/MS data.

Scheme 4.

Table 1

Low energy CID MS/MS experiments for hetero-dimeric $[L' + \text{metal} + L'']^+$ precursor ions generated via ESI and mass-selected prior to subjecting them to CID.

Precursor ion $[L' + \text{metal} + L'']^+$	E_{lab} (eV) ^a	E_{CM} (eV) ^b	Sole or major MS/MS product ion ^c
[triuret + K + urea] ⁺ (<i>m/z</i> 245)	5–10	0.70–1.40	[triuret + K] ⁺ (<i>m/z</i> 185)
[triuret + Na + urea] ⁺ (<i>m/z</i> 229)	5–10	0.74–1.49	[triuret + Na] ⁺ (<i>m/z</i> 169)
[triuret + K + acetone] ⁺ (<i>m/z</i> 243)	5–10	0.71–1.41	[triuret + K] ⁺ (<i>m/z</i> 185)
[triuret + Na + acetone] ⁺ (<i>m/z</i> 227)	5–10	0.75–1.50	[triuret + Na] ⁺ (<i>m/z</i> 169)
[urea + K + acetone] ⁺ (<i>m/z</i> 157)	5–10	1.01–2.03	[urea + K] ⁺ (<i>m/z</i> 99)
[urea + Na + acetone] ⁺ (<i>m/z</i> 141)	5–10	1.10–2.21	[urea + Na] ⁺ (<i>m/z</i> 83)
[semicarbazide + K + acetone] ⁺ (<i>m/z</i> 172)	5–10	0.94–1.88	[semicarbazide + K] ⁺ (<i>m/z</i> 114)
[semicarbazide + K + thiosemicarbazide] ⁺ (<i>m/z</i> 205)	5–11	0.82–1.79	[thiosemicarbazide + K] ⁺ (<i>m/z</i> 130)
[thiosemicarbazide + K + urea] ⁺ (<i>m/z</i> 190)	5–11	0.87–1.91	[thiosemicarbazide + K] ⁺ (<i>m/z</i> 130)
[triuret + K + semicarbazide] ⁺ (<i>m/z</i> 260)	5–11	0.67–1.46	[triuret + K] ⁺ (<i>m/z</i> 185)
[triuret + Na + semicarbazide] ⁺ (<i>m/z</i> 244)	5–11	0.70–1.55	[triuret + Na] ⁺ (<i>m/z</i> 169)
[triuret + K + thiosemicarbazide] ⁺ (<i>m/z</i> 276)	6–11	0.76–1.39	[triuret + K] ⁺ (<i>m/z</i> 185)
[triuret + Na + thiosemicarbazide] ⁺ (<i>m/z</i> 260)	6–11	0.80–1.46	[triuret + Na] ⁺ (<i>m/z</i> 169)
[12-crown-4 + K + semicarbazide] ⁺ (<i>m/z</i> 290)	8–12	0.97–1.45	[12-crown-4 + K] ⁺ (<i>m/z</i> 215)
[12-crown-4 + Na + semicarbazide] ⁺ (<i>m/z</i> 274)	8–12	1.02–1.53	[12-crown-4 + Na] ⁺ (<i>m/z</i> 199)
[12-crown-4 + K + thiosemicarbazide] ⁺ (<i>m/z</i> 306)	8–12	0.92–1.39	[12-crown-4 + K] ⁺ (<i>m/z</i> 215)
[12-crown-4 + Na + thiosemicarbazide] ⁺ (<i>m/z</i> 290)	8–12	0.97–1.45	[12-crown-4 + Na] ⁺ (<i>m/z</i> 199)
[12-crown-4 + K + triuret] ⁺ (<i>m/z</i> 361)	8–14	0.80–1.39	[12-crown-4 + K] ⁺ (<i>m/z</i> 215)
[12-crown-4 + Na + triuret] ⁺ (<i>m/z</i> 345)	8–14	0.83–1.45	[12-crown-4 + Na] ⁺ (<i>m/z</i> 199)

^aCollision energy (laboratory frame of reference).

^bCollision energy (center-of-mass frame) calculated according to the formula $E_{\text{CM}} = E_{\text{lab}} \cdot N/(N + m)$, where E_{lab} represents laboratory frame collision energy, N and m are the masses of the neutral target molecule and the ion to be activated, respectively.

^cThe MS/MS product ion abundances are compared to establish a relative order of potassium affinities (HK^+) and sodium affinities (HNa^+).