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Egorov, D; Hoekstra, Romke; Schlathölter, T

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A comparative VUV absorption mass-spectroscopy study on protonated peptides of different size

D. Egorov, * R. Hoekstra and T. Schlathölter *

The ionization of gas-phase protonated peptides and proteins can induce molecular responses ranging from purely non-dissociative ionization to extensive multifragmentation of the system. In the case of soft X-ray photoionization, a monotonic transition between both regimes occurs in the mass range between 0.5 and 10 kDa. Despite the localized nature of the photoabsorption, excitation energy equilibrates before fragmentation sets in and the transition reflects the increase of the heat capacity with protein size. Here, we have investigated the influence of peptide size on vacuum ultraviolet (VUV) photoionization of protonated proteins, where photoexcitation and ionization are limited to valence electrons rather than inner shell electrons and the photoexcitation contribution is markedly lower. Gas phase protonated peptides with masses ranging from 0.6–2.8 kDa were trapped in a radiofrequency ion trap and exposed to synchrotron radiation. Time of flight mass spectrometry was employed for the investigation of the photoionization and photofragmentation processes. The relationship between peptide fragmentation and peptide size exhibits a similar trend as observed for soft X-ray absorption. Due to the lower excitation energies involved, however, dissociation is already quenched at smaller masses and peptide amino acid compositions, protonation states and ionization potentials lead to deviations from the general trend.

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Introduction

VUV photoabsorption in gas phase biomolecular systems is relevant for research fields including mass spectrometry, VUV microscopy, astrobiology and radiation therapy. In contrast to solid-phase studies,¹ the use of gas-phase molecules rules out effects from accumulated radiation damage. The pioneering experiments in this field were performed by Bari *et al.*² who investigated photoionization of the protonated pentapeptide leucine-enkephalin for VUV photon energies in the 8–40 eV range and by Milosavljević *et al.*³ who focused on the much larger protein cytochrome *C*. For leucine-enkephalin VUV photoionization around 15 eV and higher leads to extensive fragmentation with a mass spectrum dominated by immonium ions as well as smaller sequence ions due to backbone scission.² The photoexcitation range down to the UV range was studied in a complementary study by Rankovic *et al.*⁴ who observe a smooth transition towards a sequence ion dominated spectrum at 6 eV, very similar to what is observed by collision induced dissociation (CID). For the much larger molecule cytochrome *C* VUV photoionization is predominantly a non-dissociative process that can be accompanied by loss of small

neutral groups. A first systematic investigation of the influence of peptide length on VUV photofragmentation by Gonzalez-Magaña *et al.*⁵ has shown that non-dissociative ionization becomes possible for masses larger than about 0.9 kDa. This is in line with a recent study on substance *P* ($M \sim 1.35$ kDa), where VUV photoionization leads to extensive fragmentation, accompanied by significant non-dissociative ionization channels.⁶

In the soft X-ray range, photoionization generally leads to mass spectra similar to those observed in the VUV range, albeit with higher yields of immonium ions and less non-dissociative ionization.⁷ For conventional activation techniques known to trigger ergodic (statistical) behavior of the system (*e.g.* CID,⁸ surface induced dissociation (SID),⁹ thermodesorption¹⁰), backbone scission is typically the dominating process, which leads to the formation of sequence ions.

In a previous near-edge X-ray absorption mass spectrometry study on protonated proteins we found a clear relationship between fragmentation and non-dissociative ionization yields and the protein size.¹¹ The localized inner-shell photoexcitation or photoionization is followed by an Auger de-excitation process. The resulting excitation energy distributions are broad, with average values near 20 eV. Non-dissociative ionization is found to decrease monotonically with heat capacity of the molecule. In large proteins, the photoexcitation energy seems to dissipate predominantly by means of intramolecular vibrational redistribution (IVR), while small peptides undergo extensive fragmentation.

Zernike Institute for Advanced Materials, University of Groningen, Nijenborgh 4,
9747AG Groningen, The Netherlands. E-mail: yegorovdmiriii@gmail.com,
t.a.schlatholter@rug.nl

Interestingly, simultaneous absorption of several 90 eV photons by a large protein (10 fold protonated and 6 fold deprotonated ubiquitin) predominantly leads to formation of immonium cations, hinting at a fast and local fragmentation mechanism.¹²

VUV photoabsorption involves valence electrons rather than inner shell electrons and accordingly excitation energies are on average much lower as compared to soft X-ray absorption. The transition to non-dissociative ionization is thus likely to take place at much smaller masses than for the soft X-ray case. However, as typical peptide charge states are low ($z = 1-3$) the effect of an increase in charge state may enhance fragmentation. This implies that VUV photoionization is expected to be sensitive to details of the molecular structure and the charge state of the molecule.

In the following, we present and discuss VUV photoabsorption data for the following series of protonated peptides with masses ranging from 0.6 to 2.8 kDa: YG₅F (614 Da), YG₁₀F (899 Da), angiotensin I (1.3 kDa), gramicidin A (1.9 kDa), the PK26-P collagen fragment (2.3 kDa) and melittin from honey bee venom (2.8 kDa). To investigate the influence of the charge state effect, data for two mass-over-charge regimes $m/z = 600-750$ and for $m/z = 900-950$ will be presented.

Experiment

Our home built tandem mass-spectrometer setup was interfaced with the U125/2-NIM beamline¹³ of the BESSY II synchrotron at the Helmholtz-Zentrum Berlin. The sketch of the setup is shown in Fig. 1.

All peptides were protonated and transferred into the gas phase by means of electrospray ionization. All electrospray

Table 1 Overview of the electrospray solutions used

Sample	Water (%)	Methanol (%)	Formic acid (%)	Conc. (μM)
YG ₁₀ F	0	99	1	40
YG ₅ F	0	99	1	40
Angiotensin I	55	44	1	40
Gramicidin A	0	99.8	0.2	40
PK26-P	50	49.99	0.01	50
Melittin	0	99.995	0.005	40

solutions were made with HPLC grade methanol, water and formic acid (for the details of the solutions, see Table 1). Angiotensin I, melittin from honey bee venom, and gramicidin A were purchased from Sigma-Aldrich, Netherlands. YG₁₀F, YG₅F were synthesized by JPT peptides, Germany. PK26-P was synthesized by ProteoGenix, France. Gramicidin A is an antibiotic compound containing both the L-amino and D-amino acids. It has the following sequence: HCO-L-X-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-NHCH₂CH₂OH, where X is either Val or Leu. For our experiments we mass select the variant with X = Val.

The experimental technique is described in detail elsewhere.¹¹ Briefly, the electrosprayed droplets entered the first vacuum chamber through a heated capillary. Here, the ions were phase space compressed by means of an RF-ion funnel. The ions were then collected in an RF-octupole, serving as a linear ion trap. A 50–100 ms long pulse of ions was extracted from the octupole and mass-selected by a quadrupole mass filter. Eventually, the ions were transferred into a Paul trap. Trapping was facilitated by a He buffer gas pulse, injected into the trap as a pulse of 100 ms duration. The protonated peptides were then exposed to a monochromatic beam of VUV photons from the U125/2-NIM

Fig. 1 Sketch of the experimental setup. The length of the time of flight (TOF) system between the 3D RF trap and the MCP detector at the end of the TOF tube is not to scale.

beamline for a period of 50–200 ms at typical photon fluxes around $5 \times 10^{12} \text{ s}^{-1}$ for 20 eV VUV photons. In order to ensure single-photon absorption conditions, exposure times were adjusted such that less than 10% of the precursor ions underwent photoabsorption. Under these circumstances, less than 10% of the photoions stem from multiphoton absorption. Photofragmentation is accompanied by kinetic energy release. To cool down energetic photofragment ions, a second He buffer gas pulse was injected into the Paul trap.

Subsequently, the trapped ions were extracted into the TOF spectrometer with resolution $M/\Delta M \sim 300\text{--}400$. In order to account for ESI fluctuation, reference spectra of the precursor ion without photoabsorption (photon beam off) were recorded after two photoionization spectra (photon beam on). The obtained mass spectra are difference spectra, with positive peaks for the photoproducts and a negative peak indicating photo-induced precursor loss.

To account for residual gas contributions, a single photoionization spectrum of the residual gas only was recorded and subtracted from the difference spectrum, if necessary.

In order to acquire a mass-spectrum with sufficient statistics it was necessary to average about 500 cycles. The obtained non-dissociative ionization and fragmentation yields were always corrected for the m/z dependent detection efficiency of the micro channel plate detector.¹¹

An important issue is the purity of the photon beam, *i.e.* the contamination with higher order photons. The quasi-periodic hybrid undulator of the U125/2-NIM beamline has been optimized for higher order suppression¹⁴ but cannot deliver full suppression. Higher-order contaminations are expected to be most significant for photon energies where the photon flux is very low. In a previous publication we have presented a photofragmentation spectrum for protonated leucine enkephalin at $E_{\text{ph}} = 8 \text{ eV}$ at very low photon flux, which is quantitatively very similar to the $E_{\text{ph}} = 8.4 \text{ eV}$ spectrum taken at the SOLEIL synchrotron with a photon beam cleaned from higher order contributions.⁴ This indicates that only negligible contributions of higher orders were present even for $E_{\text{ph}} = 8 \text{ eV}$. To minimize this contribution even more, we have set the lowest photon energy of this study to $E_{\text{ph}} = 14 \text{ eV}$, which is much closer to the flux maximum of the beamline.

Results and discussion

Valence ionization and excitation

VUV photoabsorption leads to excitation of valence electrons into unoccupied states or into the continuum. The process is sketched in Fig. 2. Key parameters are the ionization potential IP, *i.e.* the binding energy of the electrons in the highest occupied molecular orbital (HOMO) and the photon energy E_{ph} . Despite the fact that photoexcitation is a process involving two distinct molecular states, the high density of states in macromolecular systems lets these states appear as a continuum, with the density of states becoming a key parameter. For a given E_{ph} , valence electrons down to binding energies of E_{ph} can be ionized into the continuum. Electrons from deeper lying orbitals

Fig. 2 Energetic ordering of valence orbitals for [leu enk + H]⁺ and histogram representing the density of states. (i) For $E_{\text{ph}} = 14 \text{ eV}$, ionization is only possible from highest occupied molecular orbitals and excitation might even dominate. (ii) For $E_{\text{ph}} = 20 \text{ eV}$, inner valence electrons can be excited and outer valence electrons can be ionized. (iii) For $E_{\text{ph}} = 35 \text{ eV}$, only ionization of valence electrons is energetically allowed and excitation is ruled out.

down to $E_{\text{ph}} + \text{IP}$ can be excited into unoccupied states. The photon energy is thus a crucial parameter determining the balance between excitation and ionization processes.

To probe different excitation/ionization regimes we used photons of 14, 20 and 35 eV. For $E_{\text{ph}} = 14 \text{ eV}$, only electrons from orbitals within the highest HOMOs can be transferred into the continuum and most photoabsorption events will thus lead to excitation.

For higher photon energies around 20 eV, almost all outer valence electrons can be ionized whereas inner valence electrons can only be excited. For photon energies of 35 eV, inner and outer valence electrons can only be ionized.

In the excitation case, the charge state of the molecule does not change and the entire photon energy E_{ph} is converted into electronic excitation of the molecule. In the ionization case the charge state increases by 1 and at maximum $E_{\text{ph}} - \text{IP}$ of energy is available for electronically exciting the molecule. For a given VUV photon energy $h\nu$ this implies that excitation into unoccupied states:

leads to higher excitation of the peptides than does ionization into the continuum:

Some typical values are presented in Table 2 which also indicates the ratio between excitation and ionization. If the

Table 2 Electronic excitation energies for a generic peptide with an ionization potential of 12 eV

Photon energy in eV	Excitation energy in eV		Branching ratio between excitation and ionization
	Excitation	Ionization	
14	14	0–2	Relevant
20	20	0–8	Low
35	35	0–23	Almost zero

initial electronic excitation is followed by internal conversion (IC) and intramolecular vibrational redistribution (IVR), the internal temperature of the molecule increases and statistical fragmentation may set in. For a given photon energy, fragmentation is expected to be more extensive for excitation than for ionization. However, direct, fast fragmentation mechanisms occurring before IC and IVR sets in can lead to different dynamics. Also, the increase in charge state upon photoionization can destabilize the molecule.

To illustrate the photon energy dependence, Fig. 3 displays mass spectra obtained for gramicidin A using photons of 14, 20 and 35 eV. For all three energies qualitatively similar mass spectra are observed, featuring the [gramicidin A + 2H]³⁺ ion formed by non-dissociative ionization, a number of singly and doubly charged intermediate size fragments (sequence ions) due to backbone scission, and immonium ions with their small *m/z*.

(i) For $E_{\text{ph}} = 14$ eV (Fig. 3a), photoionization is only possible from the highest occupied orbitals and therefore only contributes

to a lesser extent to the mass spectrum. Photoionization in this case is accompanied by negligible energy deposition and mainly leads to formation of [gramicidin A + 2H]³⁺ rather than fragment ions. A second and probably stronger channel is the excitation from lower lying orbitals, involving deposition of the entire 14 eV into the molecule. The fingerprint of the excitation channel are singly charged *y*-type and *b*-type sequence ions, extending to relatively large mass: *z*₁⁺(231), *y*₁⁺(248), *b*₃⁺(256), *b*₄⁺(369), *b*₅⁺(441), *b*₆⁺(540), *b*₇⁺(639), *b*₈⁺(738), *y*₇⁺(1146), *y*₈⁺(1245), *y*₉⁺(1345). The complementary fragment pairs [*b*₆, *y*₉], [*b*₇, *y*₈] and [*b*₈, *y*₇] are indicators of single backbone scission with separation of the two [gramicidin A + 2H]²⁺ charges following photoexcitation. The predominance of *b*_{*n*}⁺ fragments has been previously observed for 4.66 eV UV photodissociation (UVPD) by Theisen *et al.*¹⁵ The strong neutral loss channels, particularly loss of 18 and the W sidechain, that are observed at 4.66 eV are absent in our data.

The immonium ion peaks are weak and mainly due to W residues (130, 159), reflecting the high content of this aromatic amino acid in gramicidin A.

(ii) For $E_{\text{ph}} = 20$ eV (Fig. 3b), outer valence electrons are photoionized and only inner valence electrons can be subject to excitation, *i.e.* the excitation channel contributes much less to the observed mass spectrum. As expected, the [gramicidin A + 2H]³⁺ peak is thus markedly higher than for $E_{\text{ph}} = 14$ eV. On the other hand, particularly the signals from singly charged sequence ions from the carboxyl terminal (*e.g.* *y*_{1/7/8/9}⁺) are reduced. Instead, the larger *y*-ions also show up as dications (*y*₇²⁺(574), *y*₈²⁺(623), *y*₉²⁺(673)), which were barely visible at $E_{\text{ph}} = 14$ eV, confirming the dominating role of photoionization at 20 eV. Fragments from the carboxyl terminal are likely to carry the additional charge, because of the low local IP and the high proton affinity of the W residues located in this region.

The yield of W immonium related ion (130) increases strongly, when E_{ph} is raised from 14 to 20 eV.

(iii) For $E_{\text{ph}} = 35$ eV (Fig. 3c), non-dissociative ionization into [gramicidin A + 2H]³⁺ is reduced, reflecting the increasing contribution of inner valence ionization processes, which are accompanied by higher excitation energies. Regarding sequence and immonium ions, the spectrum is barely different from the $E_{\text{ph}} = 20$ eV case.

The purpose of the present study is the investigation of peptide stability after VUV photoabsorption as a function of peptide size. From the gramicidin A data, we can conclude that changes in photon energy only induce drastic qualitative changes in the fragmentation pattern, when E_{ph} is close to the ionization potential of the protonated peptide, *i.e.* when going from 14 eV to 20 eV. Note, that the IPs of protonated peptides and proteins are known to depend on protonation state but also on conformation.¹⁶

For investigating peptide size effects, we have chosen to use a photon energy of 20 eV, as this energy is sufficiently far above the IPs of the systems under study. At the same time, photoionization at this energy is solely due to the removal of outer valence electrons as can be seen from Fig. 2.

Mass spectra

As peptide protonation influences stability, it is important to keep the mass-over-charge ratio *m/z* as constant as possible

Fig. 3 Comparison of photofragmentation mass spectra of [gramicidin A + 2H]²⁺ after exposure to VUV photons of 14, 20 and 35 eV.

Table 3 Common immonium ion (and related) fragments of amino acids as obtained from CID experiments.^{29,30} The masses observed in our experiments are marked in bold

Amino acid	1-letter code	Immonium ion mass (Da)	Related ion masses (Da)
Lysine	K	101	70, 84, 112, 129
Glutamine	Q	101	56, 84, 129
Histidine	H	110	82, 121, 123, 138 , 166
Arginine	R	129	59, 70, 73, 87, 100, 112
Isoleucine	I	86	44, 72
Leucine	L	86	44, 72
Tryptophan	W	159	77, 117, 130 , 132, 170, 171
Tyrosine	Y	136	91 , 107
Phenylalanine	F	120	91

when m is varied. We have done this for two m/z regimes: $m/z \sim 900$ – 950 and $m/z \sim 600$ – 750 to be able to disentangle peptide size effects and Coulombic effects. The formation of immonium ions is an important channel in peptide photofragmentation by energetic photons.^{2,7} Immonium ions are formed by a combination of a -type and y -type cleavages and contain the side chain of the respective amino acid. Masses of immonium ions and their most common fragments for the amino acids relevant to this study are summarized in Table 3. The observed species are marked in bold. In the following, we will first describe the general features of the photofragmentation spectra of a series of peptides before discussing specific peptide size effects. All spectra are normalized to the total photoabsorption yield.

[YG₁₀F + H]⁺

YG₁₀F is a synthetic peptide, which has been previously investigated in the soft X-ray regime.¹¹ This peptide is the smallest one we studied in the $m/z = 900$ – 950 range. The mass spectrum for 20 eV photons is shown in Fig. 4. Immonium ion peaks are much less intense than for soft X-ray absorption but still dominate the spectrum, with highest peaks for Y (107, 136), F (120) and y_1^+-18 (148). Non-dissociative ionization into [YG₁₀F + H]²⁺ is a weak channel. However related peaks to the loss of OH (−17), Y sidechain (−107) or both are more prominent. These dications are all fingerprints of relatively gentle photodissociation processes.⁵ Interestingly, in contrast to the gramicidin only a single strong doubly charged sequence ion (b_{11}^{2+} (368)) is observed, indicating that the protonation site is likely to be located on the N-terminal, as previously observed for leucine-enkephaline.¹⁷

Singly charged sequence ions from both the N-terminal and C-terminal are observed ($c_{11}^+(752)$, $y_1^+(166)$, $y_2^+(223)$, $y_{10}^+(680)$). In contrast, in soft X-ray absorption, fragments with $m/z > 200$ are negligible and non-dissociative ionization is not observed.

[Gramicidin A + 2H]²⁺

Gramicidin A (Fig. 3b) shows a significant increase in non-dissociative ionization as compared to YG₁₀F. As mentioned before, complementary sequence ions are a key feature of the mass spectrum. Photoionization with subsequent production of complementary N- and C-terminal fragments seems a very

Fig. 4 20 eV photofragmentation mass spectrum of [YG₁₀F + H]⁺.

significant fragmentation channel. For soft X-ray ionization of [gramicidin A + 2H]²⁺, non-dissociative ionization was found to be negligible and backbone scission to be weak. The mass spectra were dominated by tryptophan immonium fragments.¹¹

[Melittin + 3H]³⁺

[Melittin + 3H]³⁺ is the largest ion in the $m/z = 900$ – 950 series. For this cation, photoabsorption at $E_{ph} = 20$ eV is predominantly a non-dissociative process, see Fig. 5. The [melittin + 3H]⁴⁺ peak is accompanied by a relatively intense neighbor due to loss of a $m \sim 44$ group. For substance P, which features an amidated C-terminal just as melittin, this loss has been assigned to originate either from R (CH_3N_2 , $m = 43$) or from L (C_3H_7 , $m = 43$).⁶ Alternatively, it could be due to CNOH₃ loss from the amidated C-terminal ($m = 45$). Immonium ion formation is almost fully quenched and backbone scission is strongly suppressed.

A number of sequence ions become apparent when zooming in into the low intensity regime (see lower panel of Fig. 5). Both, N-terminal fragments ($a_4^+(271)$, a_5^+ , $b_8^+(370)$, $a_9^+(413)$, $c_{21}^+(812)$) and C-terminal fragments ($x_5^+(371)$, z_3^+ , $z_6^+(413)$, $y_{16}^+(632)$, $z_{21}^+(812)$, $x_{21}^+(826)$, $x_{22}^+(859)$) are observed, with no apparent preference regarding fragment type. Most N-terminal fragments have complementary C-terminal fragments (see Table 5), *i.e.* the role of complementary pairs is more relevant in melittin than in gramicidin A. No proline effect, *i.e.* cleavage on the N-terminal to proline leading to a strong contribution of y_{13} ions, as occurring in UV photodissociation¹⁵ of [melittin + 5H]⁵⁺ is observed.

At a photon energy of 20 eV, double photoionization is energetically ruled out, *i.e.* the [melittin + 3H]⁵⁺ peak has to be assigned to sequential two-photon absorption. This peak is more than an order of magnitude weaker than the [melittin + 3H]⁴⁺ contribution. The influence of sequential multi photon absorption on the spectrum is thus expected to be negligible. This is

Fig. 5 20 eV photofragmentation mass spectrum of $[\text{melittin} + 3\text{H}]^{3+}$. The red letters in the sequence indicate most likely protonation sites.

also supported by the fact that for pairs of complementary sequence ions, peak intensities are comparable and the total charge adds up to 4+.

Morrison and Brodbelt¹⁸ have shown that the protons in $[\text{melittin} + 3\text{H}]^{3+}$ initially reside at A₄, K₂₁ and R₂₄, indicated as red letters in the sequence shown in Fig. 5. Assuming a charge-directed fragmentation mechanism as invoked in CID and SID,¹⁹ the protonation sites can explain the occurrence of the complementary fragment pair a_4^+/x_{22}^{3+} (C-side of A₄ residue, photoinduced hole located on the C-side of the scission), c_{23}^{3+}/z_3^+ (N-side of the R₂₄ residue, photoinduced hole located on the N-side of the scission) and the doubly charged fragments x_5^{2+} and z_6^{2+} (both sides of K₂₁ residue, photoinduced hole on the N-side of the K₂₁ residue). The complementary fragments a_5^+/x_{21}^{3+} and a_5^+/z_{21}^{3+} are all stemming from the close vicinity of the A₄ residue, with the photoinduced hole on the C-side of the scission.

The y_{16}^{3+} fragment originates far from the three protonation sites and must be related to the location of the photoinduced hole. This is in agreement with the results of Kjeldsen and coworkers who have studied positive charge locations for 4-fold protonated melittin by means of electron capture dissociation (ECD) and found the TGLPALI_{11–17} region a likely candidate for hosting a fourth positive charge.²⁰ Note, that the peak at 632 might also be due to a b_{19}^{3+} ion. However, this is not very likely, as b₁₉ only contains a single protonation site.

$[\text{YG}_5\text{F} + \text{H}]^+$

This is the smallest peptide under investigation in the m/z = 600–750 range. VUV photoionization of this peptide (Fig. 6) is dominated by the formation of the same immonium ions as observed for $[\text{YG}_{10}\text{F} + \text{H}]^+$. The overall intensities are similar, as well. However, the Y immonium ion related peak at m/z = 107 is dominating the other immonium ions, while for $[\text{YG}_{10}\text{F} + \text{H}]^+$

all immonium ion peaks have comparable intensities. Non-dissociative ionization is weak. Moreover the NDI peak at m/z = 307 might contain contributions from a_4^+ and x_3^+ fragments. It is thus only possible to estimate an upper limit for non-dissociative ionization. Backbone scission fragments play a significant role in the spectrum and are related to either the N-terminal: b_4^+ -107(228), a_4^+ (307), c_6^+ (466) or the C-terminal: y_1^+ -18(148), y_1^+ (166), x_3^+ (306), y_4^+ (394).

A characteristic feature of peptides with a Y at the N-terminal is the loss of the Y sidechain (107). For YG₅F the Y sidechain loss peak at mass 507 is less prominent than the corresponding Y loss peak in $[\text{YG}_{10}\text{F} + \text{H}]^+$.

$[\text{Angiotensin I} + 2\text{H}]^{2+}$

For a peptide of mass 1296 Da, this system shows a remarkable high yield of non-dissociative ionization (see Fig. 7). The fragmentation spectrum is dominated by the NDI peak accompanied by tyrosine sidechain loss and by immonium ions and related ions for I and L(86), H(110, 138), Y(136). Backbone scission fragments from both the N-terminal: a_1^+ (88), b_2^+ (272), a_3^+ (343), a_6^+ (379), a_9^+ (380), b_6^+ (393), a_7^+ (428), a_8^+ (501), a_4^+ (507), b_9^+ (584) and the C-terminal: z_2^+ (252), y_2^+ (269), x_3^+ (403), y_4^+ (514), x_9^+ (605) are present, but with a much lower intensity than immonium ions, non-dissociative ionization and accompanying loss of neutrals (44, 107) with the latter being the Y sidechain. A striking feature of the fragmentation pattern is the abundance of a-type ions, previously reported for 6.4 eV photodissociation of angiotensin and other peptides with R residues at the N-terminal or close to it.^{21,22}

The stability of angiotensin can be partly explained by the fact that 3 (one R and two H) out of 10 residues have high proton affinities. A high density of residues with high proton affinity is known to increase peptide stability.¹⁹

From a Coulomb repulsion point of view, most probable protonation sites of this peptide are likely on opposite sides of

Fig. 6 20 eV photofragmentation mass spectrum of $[\text{YG}_5\text{F} + \text{H}]^+$.

Fig. 7 20 eV photofragmentation mass spectrum of $[\text{angiotensin I} + 2\text{H}]^{2+}$.

the molecule, *i.e.* R_2 and H_9 . This assumption is supported by the fragmentation pattern, as the most intense sequence ions are related to bond cleavage at these residues. It is difficult to determine the amino acid, to which the photoinduced hole drifts, since other backbone scission fragments are very weak or blended. Most likely, though, it is the H_6 residue, in agreement with a_6^{2+} and b_6^{2+} being the smallest doubly charged N-terminal fragments observed in the spectrum.

[PK26-P + 3H] $^{3+}$

The spectrum of this 26-residue protein is shown in Fig. 8. The mass spectrum exhibits an extremely low fragmentation yield and is dominated by non-dissociative ionization ($[\text{PK26-P} + 3\text{H}]^{4+}$) and the corresponding CO_2 -loss peak. This is in line with our

earlier results on soft X-ray absorption, 11 where we have observed remarkably high non-dissociative ionization yield. These signs of high stability are readily explained by the structural rigidity of PK26-P due to the high proline content. Proline has much higher structural rigidity compared to other amino acids as the α -carbon is a direct substituent of the side chain. 11 The high proton affinity of the 3 K residues and the relatively high proton affinity of 6 proline residues further enhance the stability of PK26-P.

Only a few fragments are observed, namely the K immonium ion at $m/z = 129$ and the backbone scission fragments $\text{b}_2^+(212)$, $\text{y}_4^+(377)$. A thorough investigation of $[\text{PK26-P} + 3\text{H}]^{3+}$ photofragmentation over a wide range of photon energies can be found in. 23

[Melittin + 4H] $^{4+}$

The mass spectrum of $[\text{melittin} + 4\text{H}]^{4+}$ depicted in Fig. 9 shows a high non-dissociative ionization yield accompanied by the 44-loss peak. The latter is less pronounced than for triply protonated melittin. Most likely, this is because the increased Coulomb repulsion between the protonation sites reduces stability. A reduced stability is also implied by the fragment intensities, which are higher than observed for $[\text{melittin} + 3\text{H}]^{3+}$.

An interesting feature of $[\text{melittin} + 4\text{H}]^{4+}$ photoabsorption is the m/z region between 570 and 705, which is enlarged in Fig. 10.

As for $[\text{melittin} + 3\text{H}]^{3+}$, fragmentation mainly leads to the formation of sequence ions from the N-terminal ($\text{b}_2^+(171)$, $\text{b}_3^+(228)$, $\text{a}_4^+(271)$, $\text{a}_5^+(370)$, $\text{a}_6^+(413)$, $\text{a}_6^+(484)$, $\text{c}_{23}^{4+}(610)$, $\text{a}_{20}^{3+}(660)$, $\text{b}_{20}^{3+}(669)$, $\text{c}_{20}^{3+}(675)$) and from the C-terminal ($\text{x}_5^{2+}(371)$, $\text{z}_3^+(413)$, $\text{z}_6^{2+}(413)$, $\text{y}_{13}^{3+}(542)$, $\text{x}_{13}^{3+}(551)$, $\text{y}_{14}^{3+}(580)$, $\text{x}_{20}^{4+}(592)$, $\text{z}_{21}^{4+}(609)$, $\text{x}_{21}^{4+}(620)$, $\text{x}_{22}^{4+}(645)$, $\text{z}_{17}^{4+}(660)$, $\text{y}_{24}^{4+}(670)$, $\text{x}_{17}^{3+}(675)$, $\text{z}_{25}^{4+}(694)$). Many of these fragments form complementary pairs (see Table 5). In contrast to the case of $[\text{melittin} + 3\text{H}]^{3+}$, y_{13} and x_{13} fragments are present, not ruling out the proline effect.

Fig. 8 22 eV photofragmentation mass spectrum of $[\text{PK26-P} + 3\text{H}]^{3+}$.

Fig. 9 20 eV photofragmentation mass spectrum of $[\text{melittin} + 4\text{H}]^{4+}$.

Fig. 10 20 eV high-mass region of photofragmentation mass spectrum of [melittin + 4H]⁴⁺.

Table 4 Overview of the peptide parameters ionization energy (IE), number of degrees of freedom (DOF), the average excitation energy (E_{dep}) deposited by photoionization by 20 eV photons (22 eV for PK26-P), the final internal energy per degree of freedom (E_{DOF}) and temperature of the peptides. The initial internal energy E_{in} is derived from the temperature of the trapped peptides, i.e., 300 K. All energies are given in eV except for E_{DOF} which is in meV

Peptide	DOF	E_{in}	IE	E_{dep}	E_{DOF}	T (K)
[YG ₅ F + H] ⁺	234	0.95	8.9 ^{2,5}	5.9	29.3	861
[YG ₁₀ F + H] ⁺	339	1.37	8.9 ^{2,5}	5.9	21.3	719
[Angiotensin + 2H] ²⁺	546	2.21	12 ²⁵	4.9	12.9	547
[Gramicidin A + 2H] ²⁺	828	3.34	12 ²⁵	4.9	9.9	475
[PK26-P + 3H] ³⁺	939	3.79	13.1 ²⁵	5.3	9.6	468
[Melittin + 3H] ³⁺	1293	5.22	13.1 ²⁵	4.4	7.5	410
[Melittin + 4H] ⁴⁺	1296	5.24	14.2 ²⁵	3.9	7.0	398

From the observed sequence ions, it can be concluded that the 5 positive charges are located on G₁, KV₇₋₈, PALISWI₁₄₋₂₀, KRK₂₁₋₂₃ and RQQ₂₄₋₂₆. Note, that there is not necessarily a single unique charge distribution. Immonium ions are also present in the spectrum, but with relatively low relative intensity (I, L(86), R, Q(129)), W(130)).

Peptide size effects

Our previous experiments¹¹ showed that the energy per degree of freedom (E_{DOF}) of the peptides after photon absorption is a suited parameter to describe and discuss peptide size effects on non-dissociative ionization and fragmentation yields.

For the determination of E_{DOF} one ideally needs to know the valence density of states and the VUV absorption cross sections for each binding energy. A systematic valence photoemission study²⁴ on various neutral amino acids shows very similar valence density of states distributions for the amino acids in accordance with what is calculated for [leu enk + H]⁺ (cf. Fig. 2). Therefore it is a fair approach to estimate the average energy

deposited E_{dep} by taking the calculated density of states for [leu enk + H]⁺ (cf. Fig. 2) as a generic peptide density of states. For the different peptides this density of states is shifted to match the respective ionization energies (IE).

Accurate values for many of the ionization energies of protonated peptides are unknown. For the singly protonated Y containing peptides YG₅F and YG₁₀F, ionization energies of about 9 eV are expected from theory.⁵ For the multiply protonated systems we estimate the IE by the formula derived by Budnik *et al.*²⁵ from experimental data:

$$\text{IE} = 9.8 + 1.1z$$

where z is the charge state of the peptide. Given the uncertainties in the approximation of E_{dep} we assume a generic energy uncertainty of 1 eV.

With E_{dep} known, we can calculate the peptide's internal energy and temperature after photon absorption in the same manner as in.¹¹ Briefly, the peptide's initial thermal energy per degree of freedom before photoabsorption is calculated in the framework of the harmonic oscillator model.²⁶ The number of vibrational degrees of freedom is equal to $3N - 6$ (N being the number of atoms). The initial energy per degree of freedom is then raised by $E_{\text{dep}}/(3N - 6)$ to obtain E_{DOF} . In a next step the E_{DOF} may be converted back into peptide temperature. In Table 4 the relevant peptide parameters are summarized.

In Fig. 11 the non-dissociative ionization yields (Y_{ndi}) for 20 eV photoabsorption are plotted as a function of E_{DOF} together with the general trends for either soft X-ray excitation or ionization. To complete the picture some additional results for 35 eV photon absorption are added. For 35 eV the E_{DOF} was estimated as for 20 eV. Note that the all yields are defined as the

Fig. 11 Non-dissociative ionization yields (Y_{ndi}) as a function of E_{DOF} for 20 eV VUV photon absorption. Additional data for 35 eV photons are represented by open symbols. The error bars indicate the estimated uncertainty in the average energy deposition, see text. The lines represent the general trends of NDI for soft X-ray absorption in either the excitation (blue, SX_{z+1}) or the ionization (green, SX_{z+2}) regime (Egorov *et al.*¹¹).

sum of the integrals of the respective peaks in the mass spectrum, divided by the photoinduced loss in precursor ions.

For internal energies of 15 meV per degree of freedom and beyond, the non-dissociative ionization yields become smaller than 10% and rapidly drop. The exponential decay is very similar to the one found for soft X-ray excitation (SX_{z+1}) and thus less steep than the one for soft X-ray ionization (SX_{z+2}). This is likely a manifestation of the role of the final charge state of the irradiated peptides. Both in VUV ionization and soft-X-ray excitation the charge state of the peptide is increased by one, whereas in soft X-ray ionization the charge state is increased by two. Therefore the similarity between the present VUV data and the soft X-ray excitation (SX_{z+1}) appears logic. The charge state effect shows up also in the comparison of three- and four-fold protonated melittin. For both photon energies (20 and 35 eV), the non-dissociative ionization yield Y_{ndi} for [melittin + 4H] $^{4+}$ is clearly lower than the one for [melittin + 3H] $^{3+}$ indicating the reduced stability of the peptide with increasing charge state.

In comparing the VUV and soft X-ray photofragmentation on basis of the energy per degree of freedom it is of note that for the soft X-ray data the peptides used were significantly larger than the ones used here. The reason being that for soft X-ray photoabsorption the deposited energy is determined by the subsequent Auger process and amounts to approximately 20 eV on average. This amount of deposited energy is much higher than for VUV photoionization which is on the order of 5 eV only (*cf.* Table 5). Therefore for the present VUV experiments smaller peptides are used to cover the same range of E_{DOF} . An increment in charge state leads to a stronger decrease in m/z for a small peptide than for a large protein. Charge state effects are thus expected to be more pronounced in the present study as compared to the soft X-ray range.

From Fig. 11 it is clear that in the 5–15 meV/DOF range of excitation energies there is a fair amount of scatter in the data. In part this might be related to the presence of neutral loss channels, such as CO_2 , NH_3 or even tyrosine (107). Laskin *et al.*²⁷ have shown that statistical (ergodic) fragmentation of peptide radical cations can involve dissociation pathways where pre-exponential factors differ by up to 7 orders of magnitude despite the fact, that activation energies are comparable. The reason for these differences is that low activation energy channels can be entropically hindered, as they require substantial molecular rearrangement. As mentioned in the results section, for a number of peptides under study, neutral loss channels are prominent in the

Fig. 12 As Fig. 11 but now the summed ionization yields (Y_{z+1}) of non-dissociative ionization (Y_{ndi}) and ionization accompanied by loss of neutral fragments are plotted. The green line is to guide the eye.

spectra, *i.e.* in these cases their dissociation rate can outcompete backbone scission. Other peptides exhibit negligible neutral losses, most likely because the associated dissociation rates are slower than for backbone scission.

Fig. 12 depicts the summed yields (Y_{z+1}) of channels that leave the peptide backbone intact at a charge state of $z + 1$, *i.e.* non-dissociative ionization and ionization accompanied by the loss of neutral fragments. Now, the data cluster more along a general trend line through the data. Two molecules deviate significantly from the trend line: [gramicidin A + 2H] $^{2+}$ and [angiotensin I + 2H] $^{2+}$.

[Angiotensin I + 2H] $^{2+}$ appears to be extraordinarily stable against backbone scission. It was already mentioned, that 3 of the 10 residues have high proton affinity. Furthermore, in angiotensin I the initial protonation sites are on opposite sides of the molecule implying that the internal Coulombic interactions are weak. Neutral loss channels (44, 107, *etc.*) are strong (*cf.* Fig. 7) and clearly outcompete a large fraction of backbone scission channels.

The behavior of [gramicidin A + 2H] $^{2+}$ is the opposite of [angiotensin I + 2H] $^{2+}$. [Gramicidin A + 2H] $^{2+}$ falls below the line implying that the peptide is more likely to undergo severe fragmentation. The gramicidine peptide contains 4 W residues, which amounts to almost half of the peptide mass. W is known to be the amino acid that is most prone to fragmentation.²⁸ Furthermore, for [gramicidin A + 2H] $^{2+}$ only negligible neutral loss channels are observed, *i.e.* these channels are outcompeted by backbone scission.

The last quantity to be discussed are the fragmentation yields Y_F (see Fig. 13, red squares), involving backbone scission, *i.e.* the total yield of sequence ions and immonium ions. These data are complementary to Y_{z+1} shown in Fig. 12.

As obvious from Fig. 13, Y_F can exceed unity for small peptides. The reason lies in the multiplicity of the fragmentation process: up to one (singly) charged fragment per charge $z + 1$ of the photoionized precursor can be formed.

Table 5 Observed pairs of complementary sequence ions in 20 eV photoabsorption of 3 and 4 fold protonated melittin

Light fragment	Heavy fragment	
	Melittin 3+	Melittin 4+
171:b $_2^+$	—	670:y $_{24}^{4+}$
271:a $_4^+$	859:x $_{22}^{3+}$	645:x $_{22}^{4+}$
370:a $_5^+$	826:x $_{21}^{3+}$	620:x $_{31}^{4+}$
413:z $_3^+$	812:c $_{23}^{3+}$	610:c $_{23}^{4+}$
413:a $_9^{2+}$, z $_6^{2+}$	—	675:x $_{17}^{3+}$, c $_{20}^{3+}$
484:a $_6^+$	—	592:x $_{20}^{4+}$

Fig. 13 Peptide fragmentation yields (red squares: total and blue squares: charge state corrected) induced by 20 eV photons as a function of energy per degree of freedom. The blue line represents the trend observed in case of soft X-rays (Egorov *et al.*¹¹). The green curve is determined by 1 minus the ionization yield Y_{z+1} (see Fig. 11).

The blue squares in Fig. 13 correspond to $Y_F^{\text{corr}} = Y_F/(z + 1)$. Note, that a fraction of the respective Y_F values are due to photoexcitation rather than photoionization. Here, the correction factor has to be $1/z$, rather than $1/(z + 1)$. For the large peptides at higher charge state, IPs are high and photoexcitation can be a sizeable contribution to the mass spectrum, whereas for singly charged species, photoexcitation is negligible.

It is obvious from Fig. 13 that Y_F increases monotonically with E_{DOF} and saturates for smaller molecules. This trend is similar to what is observed for soft X-ray absorption. The higher fragmentation yield can be explained by higher destabilization of smaller peptides by the Coulomb repulsion. The effect of the initial protonation state on Y_F^{corr} is obvious for melittin: here, Y_F^{corr} is three times larger for $[\text{melittin} + 4\text{H}]^{4+}$ as compared to $[\text{melittin} + 3\text{H}]^{3+}$.

It is also clear from Fig. 13 that for VUV photoionization it is difficult to define a specific E_{DOF} for a transition from the non-dissociative regime to the regime where fragmentation dominates. In contrast to the case of soft X-ray absorption (blue line), where Y_F exhibits a very steep increase around 10 meV per DOF, the present VUV data scatters widely in the 5–10 meV per DOF range of internal energies. This stresses once again the important role of peptide sequence and charge state in this photon energy range.

Conclusions

We have performed a VUV photoionization study on protonated peptides as a function of peptide size.

For the example of $[\text{gramicidin A} + 2\text{H}]^{2+}$, photofragmentation spectra for 14, 20 and 35 eV show the influence of a transition from predominant photoexcitation *via* valence photoionization to deep valence photoionization on the obtained mass spectra.

A manifestation is the transition of several large singly charged sequence ions to the corresponding doubly charged state. The relative yield of immonium ions strongly increases with photon energy, while non-dissociative ionization increases from 14 eV to 20 eV and decreases when $E_{\text{ph}} = 35$ eV.

For photon energies around 20 eV, we have then systematically studied ionization and fragmentation as a function of peptide size. Despite the fact that there is a general transition from dissociative ionization of very small peptides to non-dissociative ionization of large systems, the relation between molecular survival and molecular size is not monotonic for the molecules under study here. This deviates from our previous observations for the soft X-ray range. The general trend implies that molecular size, *i.e.* heat capacity is a crucial feature and that internal conversion of the photoexcitation energy, followed by intramolecular vibrational redistribution and statistical fragmentation is a fundamental mechanism: as the heat capacity of a given peptide depends on the number of degrees of freedom and thus peptide size, this transition in general reflects the decrease of photoinduced peptide temperature change with peptide size. However, in many systems effects such as molecular conformation and protonation state can more than compensate the pure statistical fragmentation.

For a number of peptides such as angiotensin, melittin and gramicidin A, complementary C- and N-terminal fragments are observed. Most likely protonation sites were discussed and in particular for melittin the location of the photoinduced charge could be determined.

Conflict of interests

There are no conflicts of interest to declare.

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References

- 1 Y. Zubavichus, A. Shaporenko, M. Grunze and M. Zharnikov, *J. Phys. Chem. B*, 2008, **112**, 4478–4480, DOI: 10.1021/jp801248n.
- 2 S. Bari, O. Gonzalez-Magaña, G. Reitsma, J. Werner, S. Schippers, R. Hoekstra and T. Schlathöler, *J. Chem. Phys.*, 2011, **134**, 024314, DOI: 10.1063/1.3515301.
- 3 A. R. Milosavljević, C. Nicolas, J. Lemaire, C. Dehon, R. Thissen, J. Bizau, M. Refregiers, L. Nahon and A. Giuliani, *Phys. Chem. Chem. Phys.*, 2011, **13**, 15432–15436, DOI: 10.1039/c1cp21211g.

- 4 M. L. Rankovic, F. Canon, L. Nahon, A. Giuliani and A. R. Milosavljević, *J. Chem. Phys.*, 2015, **143**, 244311, DOI: 10.1063/1.4939080.
- 5 O. Gonzalez-Magaña, G. Reitsma, S. Bari, R. Hoekstra and T. Schlathölter, *Phys. Chem. Chem. Phys.*, 2012, **14**, 4351–4354, DOI: 10.1039/c2cp23470j.
- 6 F. Canon, A. R. Milosavljević, L. Nahon and A. Giuliani, *Phys. Chem. Chem. Phys.*, 2015, **17**, 25725–25733, DOI: 10.1039/c4cp04762a.
- 7 O. Gonzalez-Magaña, G. Reitsma, M. Tiemens, L. Boschman, R. Hoekstra and T. Schlathölter, *J. Phys. Chem. A*, 2012, **116**, 10745–10751, DOI: 10.1021/jp307527b.
- 8 B. Paizs and S. Suhai, *Mass Spectrom. Rev.*, 2005, **24**, 508–548, DOI: 10.1002/mas.20024.
- 9 M. Mabud, M. Dekrey and R. Cooks, *Int. J. Mass Spectrom. Ion Processes*, 1985, **67**, 285–294, DOI: 10.1016/0168-1176(85)83024-X.
- 10 M. Meotner, A. Dongre, A. Somogyi and V. Wysocki, *Rapid Commun. Mass Spectrom.*, 1995, **9**, 829–836, DOI: 10.1002/rcm.1290090922.
- 11 D. Egorov, L. Schwob, M. Lalande, R. Hoekstra and T. Schlathölter, *Phys. Chem. Chem. Phys.*, 2016, **18**, 26213–26223, DOI: 10.1039/c6cp05254a.
- 12 T. Schlathölter, G. Reitsma, D. Egorov, O. Gonzalez-Magaña, S. Bari, L. Boschman, E. Bodewits, K. Schnorr, G. Schmid, C. D. Schröter, R. Moshhammer and R. Hoekstra, *Angew. Chem., Int. Ed.*, 2016, **55**, 10741–10745, DOI: 10.1002/anie.201605335.
- 13 G. Reichardt, J. Bahrtdt, J. S. Schmidt, W. Gudat, A. Ehresmann, R. Muller-Albrecht, H. Molter, H. Schmoranzner, M. Martins, N. Schwentner and S. Sasaki, *Nucl. Instrum. Methods Phys. Res., Sect. A*, 2001, **467**, 462–465, DOI: 10.1016/S0168-9002(01)00359-X.
- 14 J. Bahrtdt, W. Frentrop, A. Gaupp, M. Scheer, W. Gudat, G. Ingold and S. Sasaki, *Nucl. Instrum. Methods Phys. Res., Sect. A*, 2001, **467**, 130–133, DOI: 10.1016/S0168-9002(01)00236-4.
- 15 A. Theisen, B. Yan, J. M. Brown, M. Morris, B. Bellina and P. E. Barran, *Anal. Chem.*, 2016, **88**, 9964–9971, DOI: 10.1021/acs.analchem.6b01705.
- 16 A. Giuliani, A. R. Milosavljević, K. Hinsien, F. Canon, C. Nicolas, M. Refregiers and L. Nahon, *Angew. Chem., Int. Ed.*, 2012, **51**, 9552–9556, DOI: 10.1002/anie.201204435.
- 17 N. C. Polfer, J. Oomens, S. Suhai and B. Paizs, *J. Am. Chem. Soc.*, 2007, **129**, 5887–5897, DOI: 10.1021/ja068014d.
- 18 L. J. Morrison and J. S. Brodbelt, *Analyst*, 2016, **141**, 166–176, DOI: 10.1039/c5an01819f.
- 19 A. Dongre, J. Jones, A. Somogyi and V. Wysocki, *J. Am. Chem. Soc.*, 1996, **118**, 8365–8374, DOI: 10.1021/ja9542193.
- 20 F. Kjeldsen, M. Savitski, C. Adams and R. Zubarev, *Int. J. Mass Spectrom.*, 2006, **252**, 204–212, DOI: 10.1016/j.ijms.2005.10.009.
- 21 J. P. Reilly, *Mass Spectrom. Rev.*, 2009, **28**, 425–447, DOI: 10.1002/mas.20214.
- 22 R. Parthasarathi, Y. He, J. P. Reilly and K. Raghavachari, *J. Am. Chem. Soc.*, 2010, **132**, 1606–1610, DOI: 10.1021/ja907975v.
- 23 L. Schwob, M. Lalande, D. Egorov, J. Rangama, R. Hoekstra, V. Vizcaino, T. Schlathölter and J.-C. Pouilly, *Phys. Chem. Chem. Phys.*, submitted.
- 24 O. Plekan, V. Feyer, R. Richter, M. Coreno, M. de Simone, K. C. Prince and V. Carravetta, *J. Phys. Chem. A*, 2007, **111**, 10998–11005, DOI: 10.1021/jp075384v.
- 25 B. Budnik, Y. Tsybin, P. Hakansson and R. Zubarev, *J. Mass Spectrom.*, 2002, **37**, 1141–1144, DOI: 10.1002/jms.376.
- 26 L. Drahoš and K. Vekey, *J. Am. Soc. Mass Spectrom.*, 1999, **10**, 323–328, DOI: 10.1016/S1044-0305(98)00156-1.
- 27 J. Laskin, J. H. Futrell and I. K. Chu, *J. Am. Chem. Soc.*, 2007, **129**, 9598–9599, DOI: 10.1021/ja073748r.
- 28 L. J. Hohmann, J. K. Eng, A. Gemmill, J. Klimek, O. Vitek, G. E. Reid and D. B. Martin, *Anal. Chem.*, 2008, **80**, 5596–5606, DOI: 10.1021/ac8006076.
- 29 A. M. Falick, W. M. Hines, K. F. Medzihradsky, M. A. Baldwin and B. W. Gibson, *J. Am. Soc. Mass Spectrom.*, 1993, **4**, 882–893, DOI: 10.1016/1044-0305(93)87006-X.
- 30 I. A. Papayannopoulos, *Mass Spectrom. Rev.*, 1995, **14**, 49–73, DOI: 10.1002/mas.1280140104.