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Mapping the tandem mass spectrometric characteristics of citrulline containing peptides

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RATIONALE Protein citrullination (deimination) is a post-translational modification of proteins converting arginine(s) to citrulline(s). “Overcitrullination” could be associated with severe pathological conditions. Mass spectrometric analysis of modified proteins is hindered by several problems. A comprehensive study of fragmentation of deiminated peptides is not yet available. In this paper we have made an attempt to describe the characteristics of these processes, based on the studies of epitope model oligopeptides derived from clinically relevant proteins.

METHODS Solution of purified model peptides containing either one or two citrulline residues as well as their native variants were injected directly to the electrospray source of a high accuracy and resolution quadrupole-time of flight instrument and were analysed by tandem mass spectrometry using low-energy collision induced dissociation.

RESULTS Loss of isocyanic acid from citrulline residues is a preferred fragmentation route for deiminated peptides, which yields ornithine residues in the sequence. However, simultaneous detection of both the isocyanic acid loss and sequence fragments is often compromised. A preferential cleavage site was observed between citrulline and any other following amino acids yielding intensive complementary b and y type ions. Also, citrulline positioned at the *C*-termini displays a preferential cleavage *N*-terminal to this residue yielding characteristic y_1 ions. These phenomena are described here for the first time and are referred to as the “citrulline effect”.

CONCLUSIONS We found that the citrulline effect is very pronounced and could be used as a complementary tool for the confirmation of modification sites in addition to losses of isocyanic acids from the protonated molecules or from fragment ions. Low collision energy applied to peptide ions having partially mobile protons reveal the site of modification by generating specific and intensive fragments of the sequence. On the other hand, fragmenting parent ions with mobile protons usually allow full sequence coverage, although citrulline-specific fragments may exhibit lower intensities compared to other fragments.

Keywords: citrulline, citrullination, deimination, isocyanic acid, citrulline effect, MSMS, peptide sequencing

Introduction

Protein citrullination (deimination) is a post-translational modification of proteins through which arginine residues (Arg, R) in certain sequentially well-defined positions are converted to citrulline (Cit, X)[1] (Figure 1). Citrulline is a genetically non-coded amino acid and has a neutral side chain under physiological conditions. Deimination is catalysed by the peptidyl-arginine deiminase (PAD) enzymes and results in a monoisotopic mass increment of 0.9840 Da, isobaric with deamidation of asparagine and glutamine residues.

'Overcitrullination' is associated with several pathological conditions such as autoimmune diseases[2], numerous neurodegenerative diseases[3,4,5] and even tumour growth[6]. Nevertheless, only immunological (functional) or mass spectrometric (structural) methods could be sensitive and specific enough for the detection of the modified species in complex biological samples due to the low abundance of deiminated proteins[7]. Nowadays, immunodetection is used as a routine method to verify the presence of this post-translational modification in proteins e. g. for rheumatoid arthritis[8], however this approach is unable to determine the modification site. Locating the site of deimination is only possible with high resolution and high mass accuracy tandem mass spectrometry which lacks high specificity and therefore still needs improvement[9]. In the last few years, several methods have been developed to improve the detection of deiminated polypeptides, including a specific and sensitive reaction with the modified residues[10] or the enrichment of citrullinated peptides on beads with phenylglyoxal derivatives[11,12]. Detection of modified proteins is a key step for the diagnosis, monitoring and staging of diseases associated with faulty deimination.

A selective loss of isocyanic acid (HNCO) has been reported for a few citrulline containing model peptides using collision induced dissociation (CID)[13] (Figure 2A). Creese et al. utilised the loss of HNCO for selecting deiminated peptides in a complex sample and further fragmenting them with electron transfer dissociation (ETD) to obtain a good sequence coverage[14].

However, the behaviour of citrulline containing peptides upon CID has not been a target for systematic investigation so far. Our study is therefore focused on unravelling the major characteristics of fragmentation of citrullinated peptides. To obtain clinically relevant results we selected protein epitopes which play role in rheumatoid arthritis (RA)[15-18]. RA is a systemic autoimmune disease in which citrullinated autoantigens are generated and are recognised by antibodies, resulting in a chronic inflammatory condition, destroying joints and causing severe pain to the patients[19]. For these studies several oligopeptide variants were synthesised and analysed to get insight into the major characteristics of CID fragmentation of deiminated peptides.

Materials. Formic acid, distilled water and acetonitrile for MS analyses were from VWR (Radnor, PA, USA). Leu-enkephalin was used as a reference material for accurate mass measurements.

Methods. Model peptides GER, TRGRS, VERHQS, GPRVVER and GYRARPAK were selected from the epitope regions of fibrin, filaggrin and collagen, which are substrates of PADs and are involved in rheumatoid arthritis[15-18]. These model peptides represent a variety of amino acids, e.g. basic, acidic, aromatic and neutral side chains. All peptides were synthesised with free *N*- and *C*-termini by a Syro2000 peptide synthesiser using Fmoc-^{t}Bu strategy. Native Arg containing peptides were prepared along with their Cit containing variants by systematic replacement of Arg to Cit. Ala-containing peptides were also synthesised as control compounds by substituting all arginines with Ala. Full list of the peptides is shown in Table 1.

Peptides were purified by preparative RP-HPLC (Phenomenex Luna 250x10 mm C18 column, 5 µm, 100 Å), with gradient elution (eluent ”A”: 0.1% (*v*/*v*) trifluoroacetic acid in water; eluent “B”: 0.1% (*v*/*v*) trifluoroacetic acid, 20% (*v*/*v*) water in acetonitrile; 0 min: 2% “B”, 2 min: 2% “B”, 30 min: 97% “B”). Peptides were freeze-dried and subsequently analysed by tandem mass spectrometry with a high mass accuracy and resolution Waters QTOF Premier hybrid mass spectrometer equipped with electrospray ionisation source (ESI). Samples were directly injected into the ESI source from a solvent mixture of acetonitrile-water (1:1, *v*/*v*) with 0.1 % formic acid content at a flow rate of 10 µL/min.

Results

This research is focused on the collision induced dissociation (CID) of oligopeptides containing citrulline residues to identify their preferred fragmentation routes. For this, fragmentation characteristics were compared by using oligopeptides and their substituted analogues of 3-8 amino acid residues containing one or two arginines. In these compounds, Arg residues were replaced either by Cit or by Ala (Table 1).

Major fragmentation characteristics of the peptides can be well interpreted by the mobile proton theory[20], which we find useful for Cit-peptides as well. Based on this, peptides could be categorised as having either 1) no mobile protons (where the number of arginines is equal to or greater than the number of ionising protons within the sequence), 2) partially mobile protons (where the number of ionising protons is greater than that of arginines but fewer than that of or equal to the overall basic residues and *N*-terminus) or 3) mobile protons (where the number of ionising protons is greater than that of the overall basic sites)[21]. It should also be noted that this categorisation is only for practical purposes and sometimes lower charge states are missing from the single stage MS spectra. This is the case for large non-tryptic peptides or the ones containing several slightly basic amino acids (His, Lys, Cit). Protons of peptide ions with low charge states in this category sometimes behave like as if they were non-mobile ones (e.g. the singly protonated GPXVVEX peptide, data not shown).

We found that charge state distribution in single stage MS is altered and shifted to lower values by substituting Arg residues with Cit ones, suggesting the neutral characteristic of citrulline under electrospray ionization (Figure S1). This is in agreement with previous studies.[9] In case of the pentapeptides, however, higher charge states were not necessarily absent, only less intensive, indicating that Cit could be partially charged under MS conditions at least for a limited number of peptides. On the other hand, Cit could be considered a neutral residue due to lack of proton sequestration ability in our MSMS experiments.

Charge states of model peptides having no mobile protons usually tend to produce fragments corresponding to the neutral loss of isocyanic acid as base peak or as the second most intensive peak at low collision energies (Figure S2). HNCO loss of citrulline residues yields ornithines in the sequence. High intensity of HNCO loss can be explained by the proton sequestration of arginine residues disabling low-energy protons to migrate through the sequence inducing alternate decomposition pathways. Enhanced collision energy has a beneficial impact on generating sequence ions but along with hardly identifiable combinations of complex neutral losses. This latter feature was especially pronounced in case of the threonine containing pentapeptide series. It is, however, important to note that the intensities of parent ions having no mobile protons are usually low amongst peptides derived from enzymatic cleavage of proteins.

Parent ions having partially mobile protons also show highly abundant loss of HNCO, often being base peaks at low energies (Figure 3C, Figure S3B). In these cases, the neutral loss of isocyanic acid was accompanied by highly abundant b_x-y_z products originated from a preferential cleavage site that is *C*-terminal to Cit residues (Figure 3B, C, D). Preferential cleavage of the Cit-Aaa bond of peptides in gas phase is described here for the first time and termed citrulline effect. In our opinion, cleavage of the Cit-Aaa bond is facilitated by the formation of ornithine (Orn) residues during the neutral loss of HNCO from citrulline. The following mechanism could explain the process: the peptide containing citrulline(s) is first protonated on one of its basic sites. After collision induced excitation, the proton migrates to the δ-NH-group initiating the dissociation process. The reaction leads to a protonated peptidyl-Orn residue (Figure 2A)[13]. In the second step, a favourable, six-membered lactam ring is formed (Figure 2B) from Orn. Facile cleavage of residues *C*-terminal to Orn within peptides in gas phase has been previously described by McGee and McLuckey[22].

Enhanced collision energy results in the appearance of other informative sequence fragments, as well as their 'ornithinated' variants by HNCO loss while the peaks corresponding to the citrulline effect remain very intensive. We also found (Figure S3) that proline effect was inferior to citrulline effect, similarly to previously described results for ornithine containing peptides[22]. The only exception was peptide GYXARPAK, in which a more preferred cleavage at YX peptide bond was observed leading to the intensive peak pair of b_2 and a_2 fragment ions (Figure S3).

If Cit is in an (*n*-1) position relative to the *C*-terminus, intensity of an y_1 ion will be also increased. The pentapeptide model set based on the TRGRS peptide sequence is shown on Figure S4 as an example. Y_1 ions are characteristic to tryptic peptides and confirm the identity of *C*-terminal Arg or Lys. These important fragment ions are also present in the spectra of peptides containing *C*-terminal Cit with high intensities and are generally accompanied by NH_3 loss (Figure 3B, D).

Peptide charge states having mobile protons also display the loss of HNCO, however backbone fragmentation becomes a competitive pathway. The former process is therefore less pronounced. Fragment ions show losses of HNCO as well. In these cases, complementary b/y fragment ions corresponding to citrulline effect may not have significantly higher intensities than other ones. Sequence fragments which are not present in the tandem mass spectra of charge states possessing partially mobile protons could also appear for alternative charge states having mobile protons.

Most intensive fragment ions of the peptides and ions with HNCO loss are summarised in Table 2.

Discussion

Although several methods have been developed for the analysis of citrullination, a comprehensive study of fragmentation characteristics at the peptide level is still not available. In this work, we identified basic principles connected to the MSMS behaviour of peptides containing citrulline(s) to improve mass spectrometry based detection of deimination in terms of specificity, reliability and speed in the future.

Model peptides GER, TRGRS, VERHQS, GPRVVER, GYRARPAK and all their possible deiminated variants were synthesised by solid phase peptide synthesis (Table 1). Control peptides were also synthesised by systematic replacement of Cit residues by Ala. The peptides selected are corresponding to epitope regions of proteins (collagen, filaggrin, fibrin) which are targets of immunorecognition in rheumatoid arthritic joint tissues. Some of the peptides mimic cleavage results by trypsin or LysC (Arg or Lys residues at the *C*-termini).

Our results were in good agreement with the mobile proton hypothesis, described previously for general interpretation of peptide fragmentation by tandem mass spectrometry[20].

We observed that peptide charge states having partially mobile protons provide a pronounced intensity of fragments corresponding to the Cit-Aaa cleavage at low energy CID leading to b and/or y type ions. This feature is described here for the first time and it may also be observed for parent ions with fully mobile protons. A similar phenomenon was described previously[22] for Orn-Aaa amide bond in Orn-containing peptides. We suppose that the isocyanic acid loss from Cit residues, resulting in a newly generated Orn residue in the sequence using CID, explains the facile Cit-Aaa bond cleavage (Figure 2).

Loss of isocyanic acid is also a very selective reaction of citrulline containing peptides, which can be observed from parent ions and for fragment ions as well. Sequence coverage could also be increased – to a limited extent – by raising collision energies without losing valuable peaks corresponding to citrulline effect. In our experiments, peptides were also analysed on an ion trap instrument and the compounds showed essentially the same fragmentation profiles indicating the robustness of the method. An example for low energy CID tandem mass spectra acquired on ion trap is shown on Figure S5.

Our results indicated that y_1 ions of Cit are abundantly present due to the cleavage at Aaa-Cit, if Cit was located at the *C*-terminus (Figure 3B, D). This is also an important feature, especially given the fact that the first MS-grade mutant enzyme capable of cleaving at the *C*-terminus of has been developed recently[23].

Based on our experiments, the major factors effecting favoured fragmentation routes for deiminated peptides could be outlined. These pathways, such as the neutral loss of isocyanic acid, conventional sequential fragmentation and specific sequential fragmentation pathways (e.g. citrulline effect) could be elucidated by the mobile proton theory. Increasing proton mobility decreases the intensities of peaks corresponding to the loss of HNCO as in case of other types of neutral losses. On the other hand, citrulline effect may remain intensive in case of higher proton mobilities or at elevated collision energies. Thus, utilisation of citrulline effect along with the loss of HNCO could greatly contribute to a more reliable identification and sequencing of deiminated peptides.

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Table 1 Sequence and analytical data of the peptides

Sequence	**Origin**	**Calculated Monoisotopic Molecular Mass**	**Measured Monoisotopic Molecular Mass**	**Mass accuracy /ppm**
GER	**collagen (multiple repetition)**	360,1757	360,1755	-0,6
GEX	Cit-analogue	361,1597	361,1598	0,3
GEA	Ala-analogue	275,1117	275,1118	0,4
TRGRS	**filaggrin(311-315)**	575,3140	575,3148	1,4
TRGXS	Cit-analogue	576,2980	576,2978	-0,3
TXGRS	Cit-analogue	576,2980	576,2981	0,2
TXGXS	Cit-analogue	577,2820	577,2813	-1,2
TAGAS	Ala-analogue	405,1860	405,1863	0,7
VERHQS	**fibrin α40-45**	754,3722	754,3738	2,1
VEXHQS	Cit-analogue	755,3562	755,3563	0,1
VEAHQS	Ala-analogue	669,3082	669,3073	-1,3
GPRVVER	**fibrin α36-42**	811,4664	811,4693	3,6
GPRVVEX	Cit-analogue	812,4504	812,4526	2,7
GPXVVER	Cit-analogue	812,4504	812,4489	-1,8
GPXVVEX	Cit-analogue	813,4345	813,4353	1,0
GPAVVEA	Ala-analogue	641,3384	641,3383	-0,2
GYRARPAK	**fibrin β70-77**	917,5195	917,5220	2,7
GYRAXPAK	Cit-analogue	918,5035	918,5075	4,4
GYXARPAK	Cit-analogue	918,5035	918,5067	3,5
GYXAXPAK	Cit-analogue	919,4876	919,4907	3,4
GYAAAPAK	Ala-analogue	747,3915	747,3922	0,9

Table 2 Fragmentation of the peptides. Fragments are generated from partially mobile parent ions. Fragments between 1-5 % of the base peak intensity are in brackets. Peptides containing two citrulline residues show a more intensive fragmentation due to the citrulline effect

Sequence	**Detected *N*-terminal sequential fragments**	**Detected *C*-terminal sequential fragments**	**Loss of HNCO detected**
GER	b_2	y_1, y_2	-
GEX	b_2	y_1, (y_2)	MH^+, y_1
GEA	b_2	y_1, (y_2)	-
TRGRS	a_1, b_2, b_3	y_1, y_2, y_3	-
TRGXS	a_1, b_2, b_3, b_4	y_1	(b_4)
TXGRS	a_1, b_2	y_1, y_3, y_4	b_2, (y_4)
TXGXS	b_2, b_3, b_4	(y_1), y_2, y_3, (y_4)	MH^+ (2x), b_2, b_4 (2x), y_3
TAGAS	b_2, b_3, b_4	y_1, y_2, y_3, y_4	-
VERHQS	a_1, b_2, b_3, b_4, (b_5)	y_1, y_2, y_3, y_4, y_5	-
VEXHQS	a_1, b_2, b_3, b_4, b_5	y_2, y_3, y_4, (y_5)	MH^+, b_3, b_4, b_5, y_4
VEAHQS	a_1, b_2, (b_3), (b_4), (b_5)	(y_1), y_2, (y_3), y_4, y_5	-
GPRVVER	b_2, b_3, b_4, (b_5)	y_1, y_2, y_3, y_4	-
GPRVVEX	(b_2), b_3, b_4, b_5, b_6	y_1, y_2, (y_3)	MH^{2+}
GPXVVER	b_2, b_3, b_4	y_1, y_2, y_3, y_4, y_5	b_3, b_4, y_5
GPXVVEX	(b_2), b_3, b_4, b_5, b_6	y_1, y_2, y_3, y_4	MH^{2+}, MH^{2+}(2x), (b_4), b_5, b_6, (y_3)
GPAVVEA	(b_2), b_3, b_4, b_5, b_6	y_2, y_3, y_4	-
GYRARPAK	a_2, b_3, (b_4), (b_5), (b_6)	y_1, y_2, y_3, (y_5)	-
GYRAXPAK	a_2, (b_3), (b_4), b_5, b_7	y_1, y_2, y_3	MH^{2+}, b_5, b_7
GYXARPAK	a_2, b_3, (b_4), b_5, b_7	y_1, (y_2), y_3, y_4, y_5, y_6, y_7^{2+}	MH^+, (b_3), (b_4), (b_5), (b_7), y_6
GYXAXPAK	a_2, b_3, b_4, b_5, (b_7)	y_1, (y_2), y_3, y_4, y_5, y_6	MH^{2+} (2x), (b_3), (b_4), (b_5), (y_4), y_5, (y_6), (b_7)
GYAAAPAK	b_2, b_3, b_4, (b_5), (b_6)	y_1, y_2, y_3, y_4, y_5, (y_6)	-

Figure legends:

Figure 1 Protein deimination (citrullination) by the peptidyl arginine deiminase (PAD) enzymes altering physico-chemical properties of the substrates.

Figure 2 Proposed mechanism for citrulline effect leading to facile cleavage *C*-terminal to citrulline residues for gas phase protonated peptides. First step: loss of isocyanic acid (HNCO) characteristic to citrulline residues, leading to an ornithine residue (**A**)[8]. Second step: cleavage of the Orn-Aaa bond (**B**)[12].

Figure 3 Tandem mass spectra of the heptapeptide model series. Triply protonated GPRVVER fragmented at a collision energy of 7.5 eV (**A**), doubly protonated GPRVVEX at 10.0 eV (**B**), doubly protonated GPXVVER at 10.0 eV (**C**) and doubly protonated GPXVVEX at 7.5 eV (**D**). HNCO (@), NH_3 (*), H_2O (°) and CO_2 (#) side chain losses are also indicated. Charge states and collision energies were tuned that the protonated molecules, the sequence ions and isocyanic acid loss could be detected with a reasonable yield. It should be noted that the +2 ion of peptide GPRVVER at a collision energy of 10.0 eV remained intact due to the proton sequestration of Arg. Cit-containing peptides produce abundant peaks corresponding to Cit-Aaa cleavages due to the citrulline effect with charge retention on the fragment with the larger proton affinity.

Supplementary material:

Figure S1 Ion trap single stage mass spectra demonstrating the charge shift of Cit-containing peptide ions to lower values compared to their native variants'. Note that peptide ion $[M+3H]^{3+}$ is completely missing from the spectrum of VEXHQS. Spectra were acquired on a Bruker Esquire 3000+ mass spectrometer.

Figure S2 Tandem mass spectrum of the singly protonated TXGRS peptide, a typical parent ion lacking mobile protons. Loss of HNCO is indicated by an 'at' sign (@). In the absence of other available fragmentation pathways, the protonated molecule displays abundant loss of HNCO besides other minor non-sequential neutral losses.

Figure S3 Tandem mass spectra of the octapeptide series. Triply protonated GYRARPAK at a collision energy of 10.0 eV (**A**), doubly protonated GYRAXPAK at 15.0 eV (**B**), doubly protonated GYXARPAK at 15.0 eV (**C**) and doubly protonated GYXAXPAK at 10.0 eV (**D**). Neutral losses of HNCO (@), NH_3 (*), H_2O (°) and CO_2 (#) are indicated. Note that fragments corresponding to a Cit-Ala and Cit-Pro cleavage are usually highly intensive. Comparing **B** and **C** implies that the so-called Pro effect is inferior to Cit effect. However, in case of **C**, the cleavage of Tyr-Cit amide bond is preferred.

Figure S4 Representation of citrulline effect for peptides TRGXS (**A**) and TXGRS (**B**). The Cit-Ser cleavage yields extraordinarily intensive b_4-y_1 ion pairs (**A**), while in case of TXGRS only the intensity of y_3 is enhanced significantly due to the Cit-Gly cleavage (**B**).

Figure S5 Ion trap tandem mass spectra of the heptapeptides. Triply protonated GPRVVER (**A**), doubly protonated GPRVVEX (**B**), doubly protonated GPXVVER (**C**) and doubly protonated GPXVVEX (**D**) were all fragmented at a collisional amplitude of 1.0 V. Spectra were acquired on a Bruker Esquire 3000+ mass spectrometer.

Figure 1.

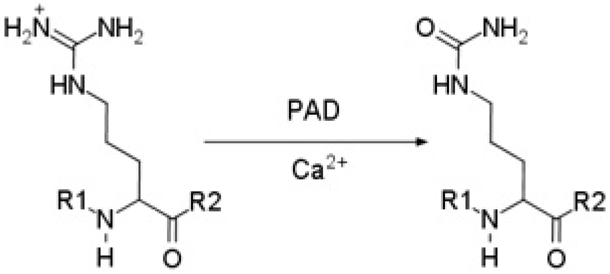


Figure 2.

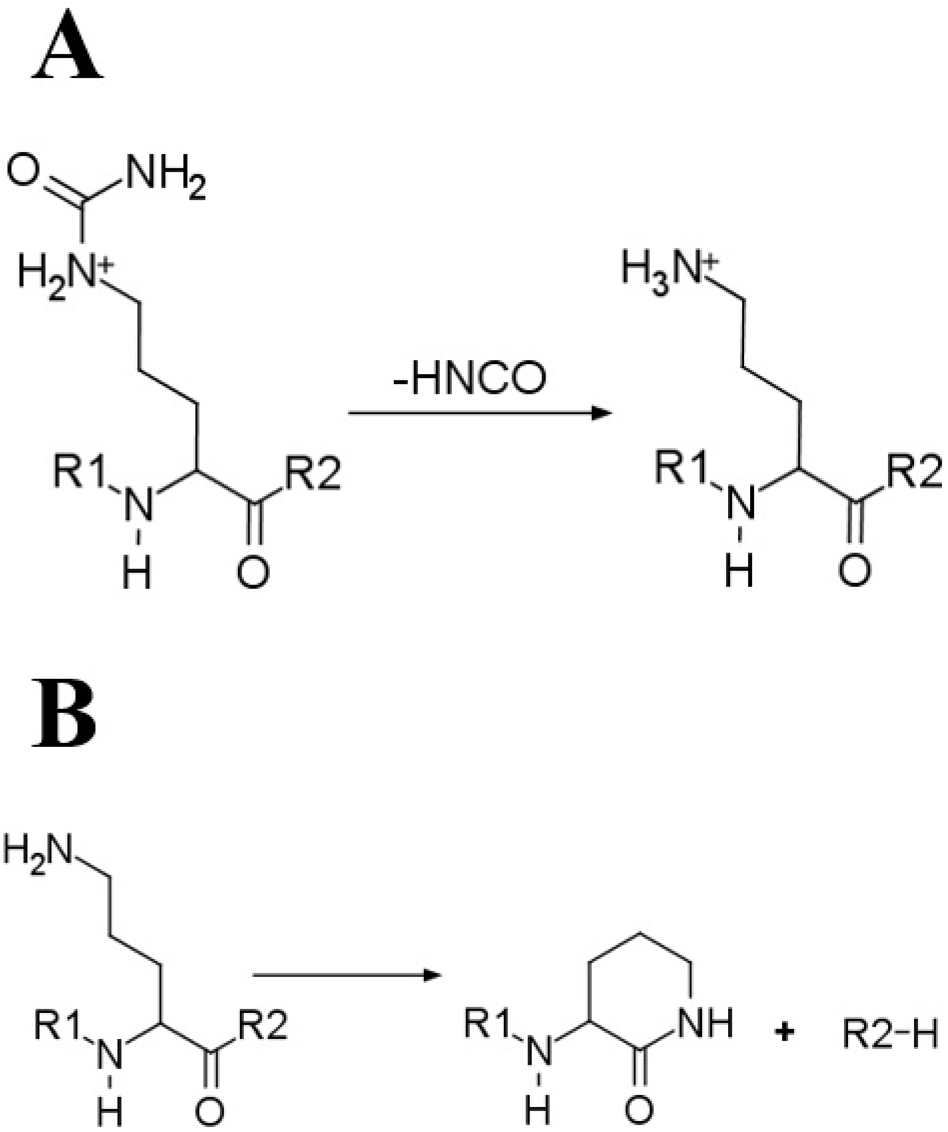

A
O
NH2
H2N+
R1
N
H
R2
O
-HNCO
H3N+
R1
N
H
R2
O
B
H2N
R1
N
H
R2
O
R1
N
H
NH
O
+
R2-H

Figure 3.

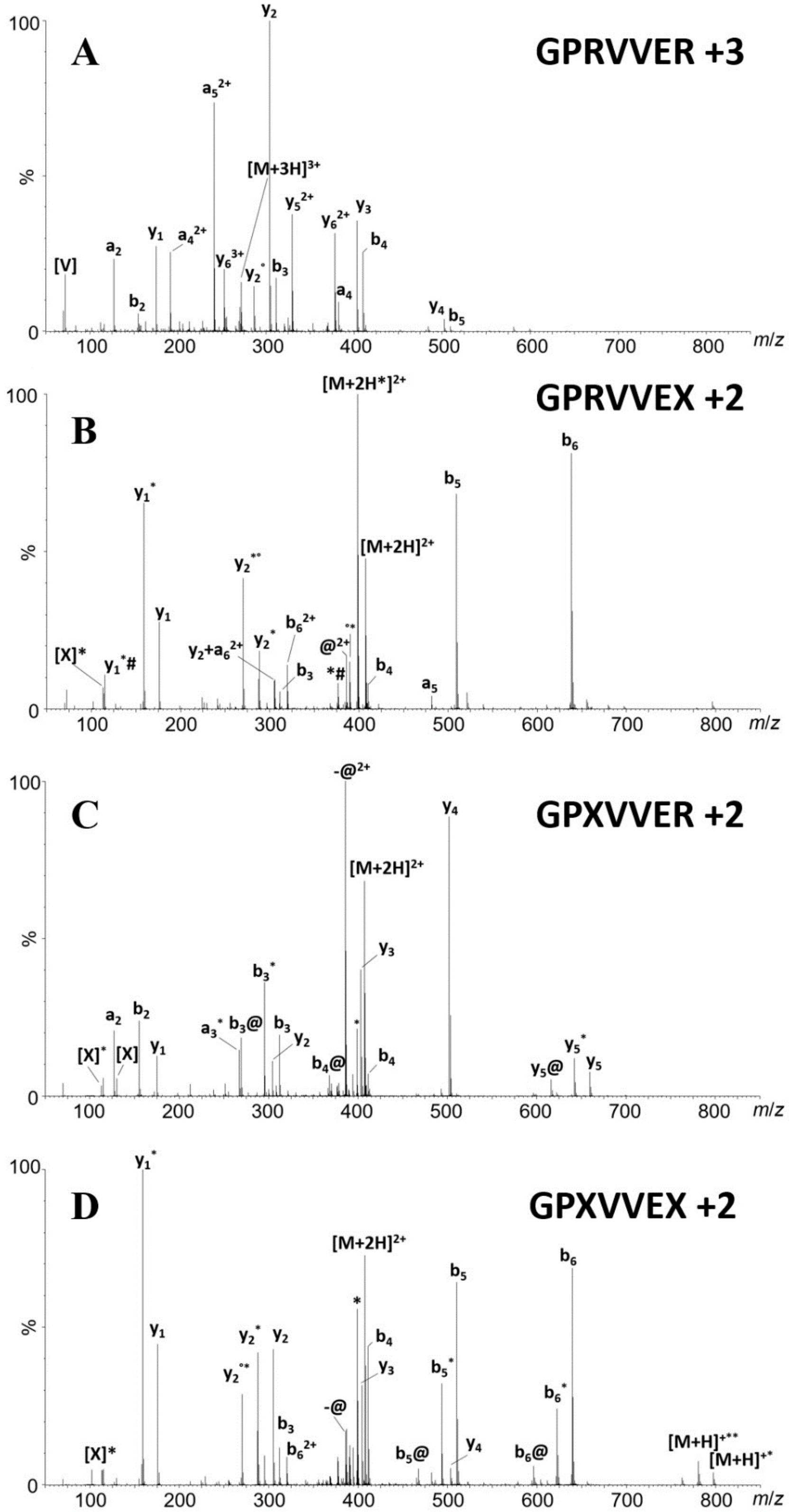
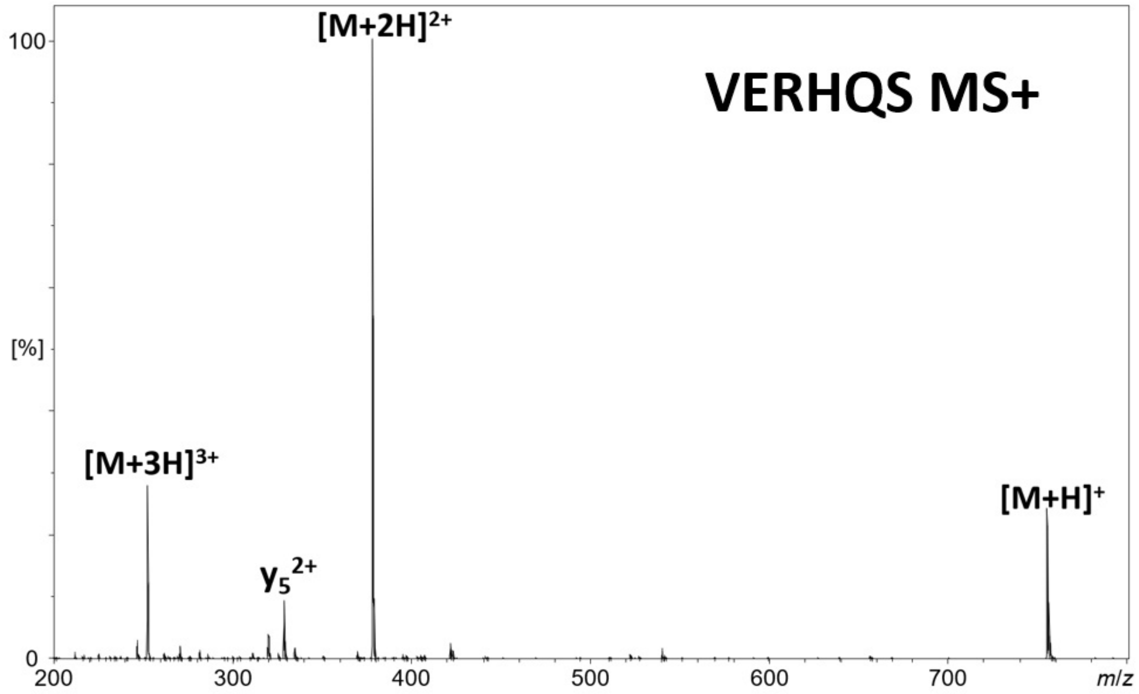

A
GPRVVER +3
y_2
a_5^{2+}
[M+3H]$^{3+}$
y_5^{2+}
y_6^{2+}
y_3
b_4
[V]
a_2
b_2
y_1
a_4^{2+}
y_6^{3+}
y_2^{*}
b_3
a_4
y_4
b_5
B
GPRVVEX +2
[M+2H*]$^{2+}$
b_6
b_5
y_1^{*}
[M+2H]$^{2+}$
$y_2^{*\circ}$
y_1
b_6^{2+}
y_2^{*}
@$^{2+}$
$^{\circ *}$
[X]*
y_1^{*}#
y_2+a_6^{2+}
b_3
*#
b_4
a_5
C
GPXVVER +2
-@$^{2+}$
y_4
[M+2H]$^{2+}$
y_3
b_3^{*}
a_2
b_2
a_3^{*}
b_3@
b_3
y_2
*
[X]*
[X]
y_1
b_4@
b_4
y_5^{*}
y_5@
y_5
D
GPXVVEX +2
y_1^{*}
[M+2H]$^{2+}$
b_5
b_6
*
y_1
y_2^{*}
y_2
b_4
y_3
$y_2^{\circ *}$
b_5^{*}
b_6^{*}
-@
b_3
b_6^{2+}
[X]*
b_5@
y_4
b_6@
[M+H]$^{+**}$
[M+H]$^{+*}$
100
%
0
100
200
300
400
500
600
700
800
m/z

Figure S1


[M+2H]$^{2+}$
VERHQS MS+
100
[%]
0
[M+3H]$^{3+}$
y_5^{2+}
[M+H]$^{+}$
200
300
400
500
600
700
m/z

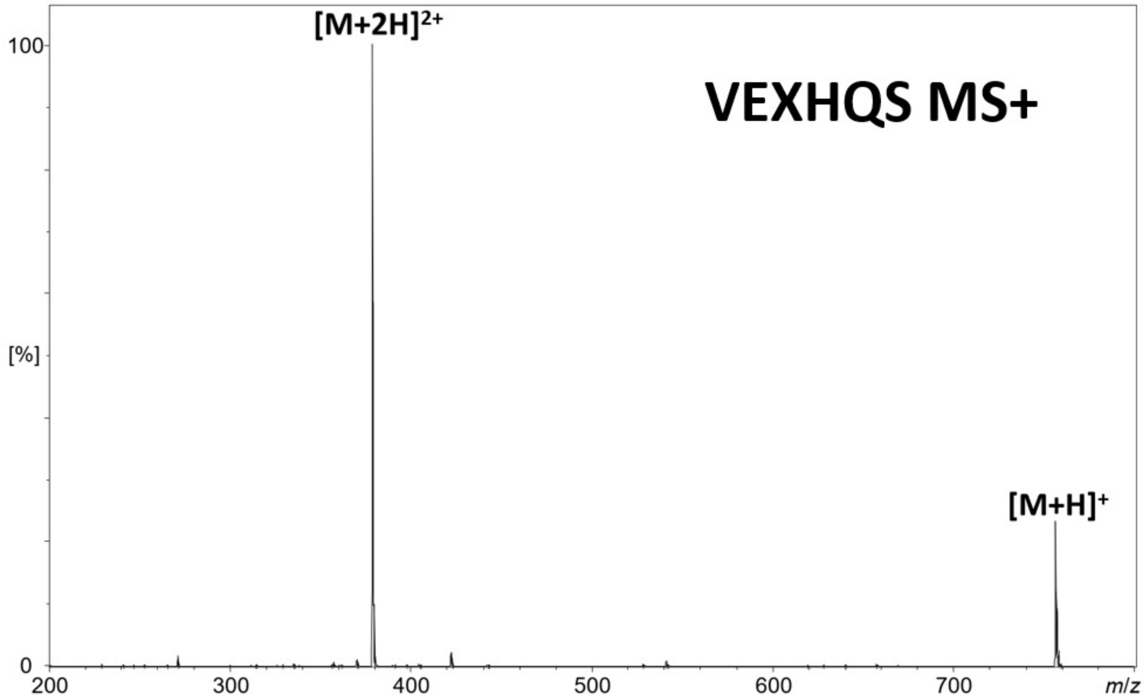

[M+2H]$^{2+}$
VEXHQS MS+
100
[%]
0
[M+H]$^{+}$
200
300
400
500
600
700
m/z

Figure S2

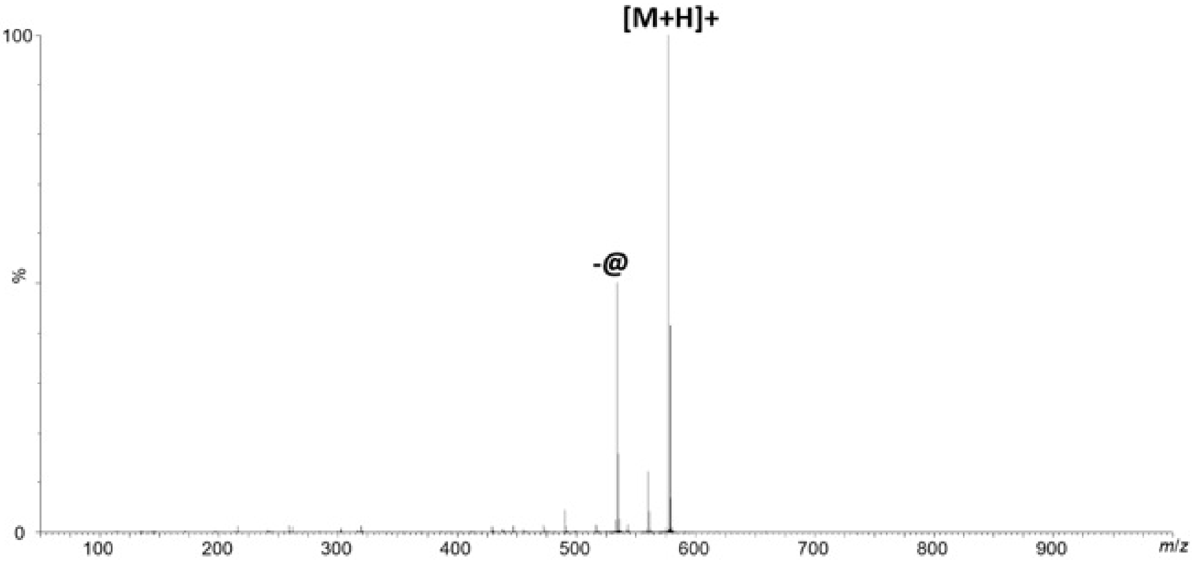


Figure S3

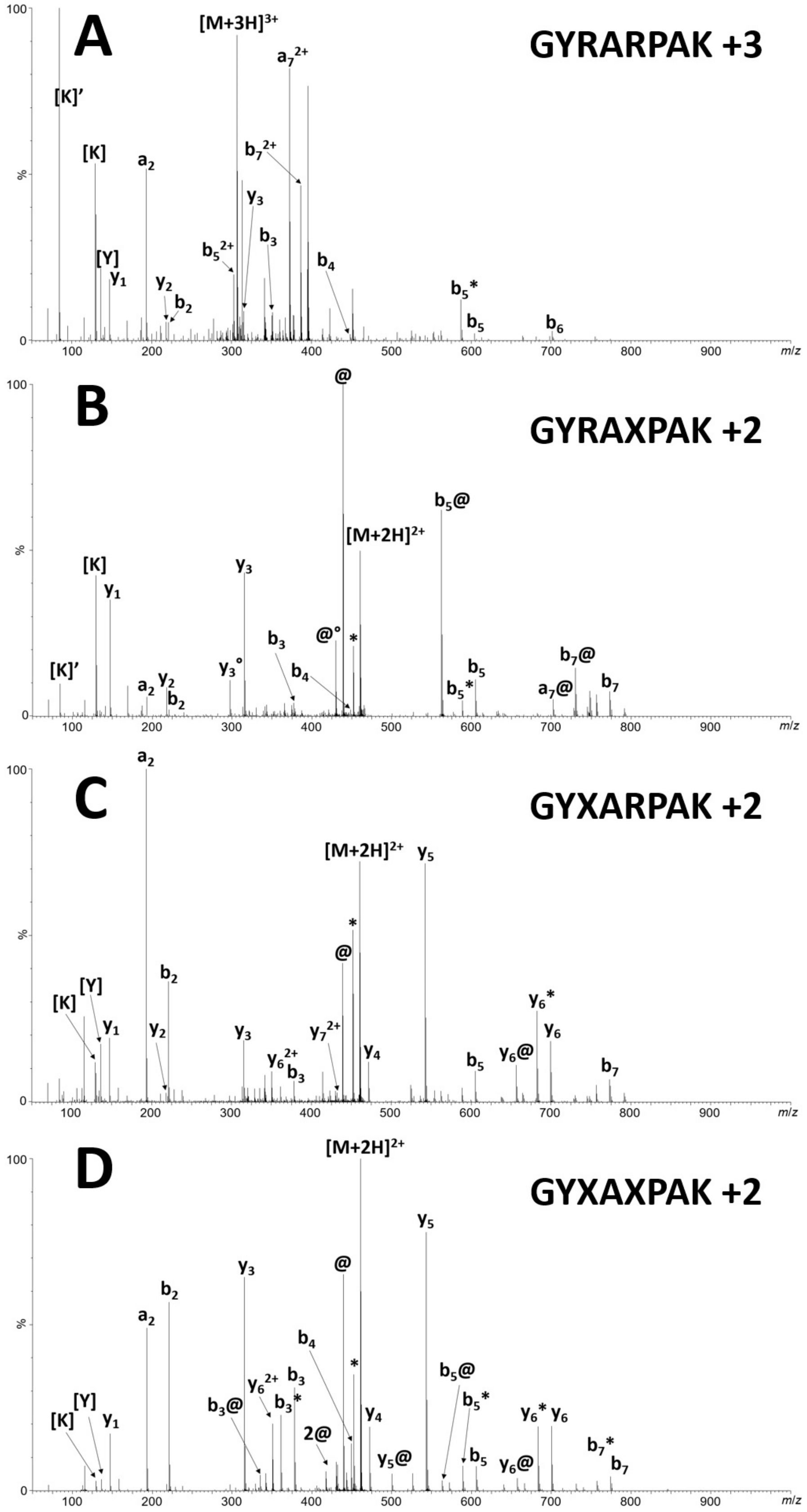

A
GYRARPAK +3
$[M+3H]^{3+}$
a_7^{2+}
[K]'
[K]
a_2
b_7^{2+}
y_3
b_3
b_5^{2+}
[Y]
y_1
b_4
y_2
b_2
b_5*
b_5
b_6
B
GYRAXPAK +2
@
b_5@
$[M+2H]^{2+}$
[K]
y_3
y_1
b_3
@°
*
[K]'
y_3°
b_4
a_2
y_2
b_2
b_5
b_7@
b_5*
a_7@
b_7
C
GYXARPAK +2
a_2
$[M+2H]^{2+}$
y_5
*
@
b_2
[Y]
[K]
y_1
y_2
y_3
y_7^{2+}
y_6^{2+}
b_3
y_4
b_5
y_6*
y_6@
y_6
b_7
D
GYXAXPAK +2
$[M+2H]^{2+}$
y_5
y_3
@
b_2
a_2
b_4
*
b_5@
y_6^{2+}
b_3
[Y]
b_3@
b_3*
b_5*
[K]
y_1
2@
y_4
y_6*
y_6
y_5@
b_5
y_6@
b_7*
b_7
100
%
0
100
200
300
400
500
600
700
800
900
m/z

Figure S4

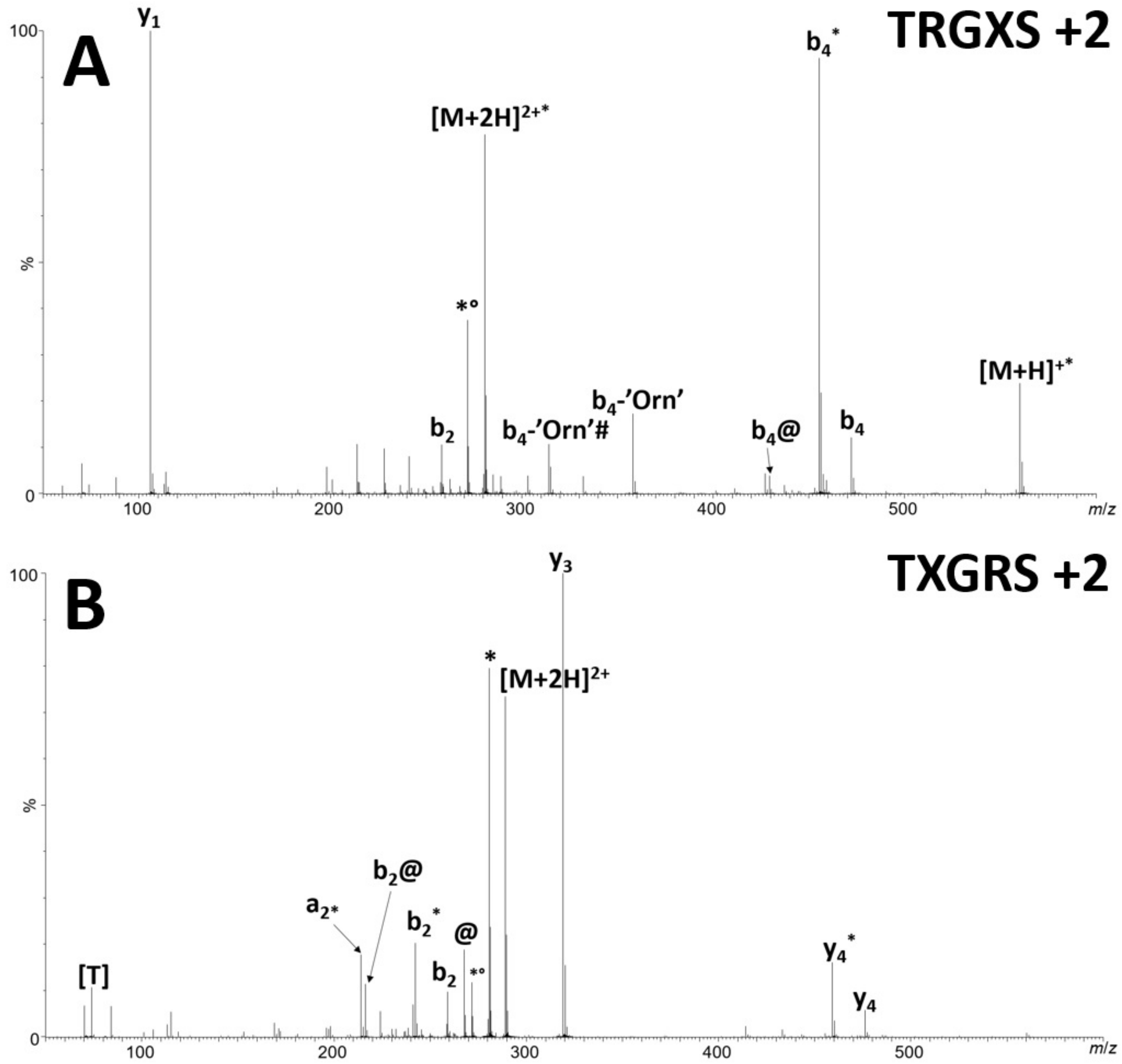
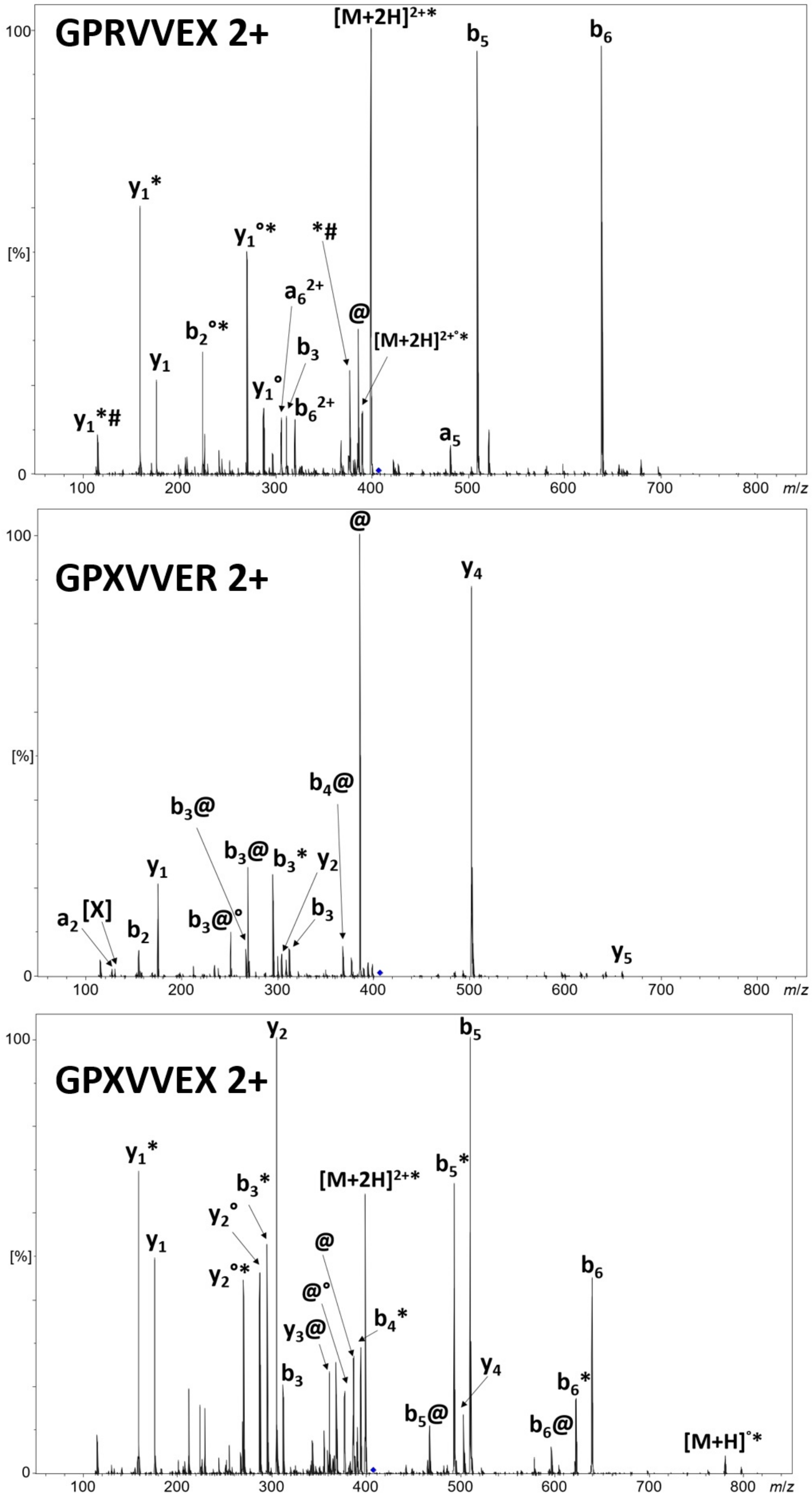

A
TRGXS +2
100
%
0
y1
b4*
[M+2H]2+*
*°
[M+H]+*
b4-'Orn'
b2
b4-'Orn'#
b4@
b4
100
200
300
400
500
m/z
B
TXGRS +2
100
%
0
y3
*
[M+2H]2+
b2@
a2*
b2*
@
[T]
b2
*°
y4*
y4
100
200
300
400
500
m/z

Figure S5


GPRVVEX 2+
[M+2H]2+*
b5
b6
y1*
y1°*
*#
a6 2+
@
[M+2H]2+°*
b2°*
b3
y1
y1°
b6 2+
y1*#
a5
100
[%]
0
100
200
300
400
500
600
700
800
m/z
GPXVVER 2+
@
y4
b4@
b3@
b3@
b3*
y2
y1
a2
[X]
b2
b3@°
b3
y5
100
[%]
0
100
200
300
400
500
600
700
800
m/z
GPXVVEX 2+
y2
b5
y1*
b3*
[M+2H]2+*
b5*
y2°
y1
@
b6
y2°*
@°
b4*
y3@
b3
y4
b6*
b5@
b6@
[M+H]°*
100
[%]
0
100
200
300
400
500
600
700
800
m/z