Supplementary materials

Sensitive and specific identification of carboxymethylated and carboxyethylated peptides in tryptic digests of proteins and human plasma

Uta Greifenhagen, Viet Duc Nguyen, Johann Moschner, Athanassios Giannis, Andrej Frolov, and Ralf Hoffmann

Table of contents

Protocol S1: Synthesis of ^{t}Bu glyoxal and ^{t}Bu pyruvate

Esterification of fumaryl chloride.

To a solution of ^{t}BuOH (27 mL, 284 mmol, 1.9 eq.) *n*-butyl lithium (99 mL, 1.6 mol/L in hexane, 158 mmol, 1.1 eq.) was added over a period of 50 min at 0 °C and stirred for another 30 min. The ice-water bath was removed and a mixture of fumaryl chloride (8 mL, 74 mmol) in absolute diethyl ether (36 mL) was added at room temperature over 1.5 h. After 6.5 h the reaction mixture was diluted with ethyl acetate (3 mL) and water (30 mL). The organic phase was washed with saturated aqueous solutions of $NaHCO_3$ (100 mL) and NaCl (100 mL) and dried over Na_2SO_4. Solvents were removed by distillation under reduced pressure. The resulting brown material was dissolved in *n*-hexane and purified on a silicagel column (SiO_2, 20 cm length, 5 cm inner diameter) using a mixture of hexane and ethyl acetate (95/5, v/v). Di-^{t}Bu fumarate was obtained as colorless needles (14.6 g, 63.7 mmol, 86%, Lit: 68%[1]).

TLC (silicagel) R_f (hexane/ethyl acetate 3/1 (v/v)) = 0.8

R_f (hexane/ethyl acetate 95/5 (v/v)) = 0.3

^{1}H-NMR (300 MHz, $CDCl_3$) δ = 6.60 (s, 2H, C<u>H</u>=C<u>H</u>), 1.43 (s, 18 H, 2* OC(C<u>H</u>$_3$)$_3$).

Ozonolysis of di-^{t}Bu fumarate. Di-^{t}Bu fumarate (14.6 g, 63.7 mmol) dissolved in dry CH_2Cl_2 (74 mL) was cooled to -78 °C before a mixture of O_2/O_3 was introduced into the stirred solution until its blue color indicated the reaction's completion (1.5 h). Excess O_3 was removed by purging the reaction mixture with nitrogen. The secondary ozonide was opened by slowly adding $S(CH_3)_2$ (20 mL, 274 mmol, 2.6 eq.) to the cooled reaction solution which was then stirred for 16 h while being allowed to warm to room temperature. CH_2Cl_2 and excess $S(CH_3)_2$ were removed by distillation under reduced pressure. ^{t}Bu glyoxal was obtained in a slightly yellow, viscose dimethyl sulfoxide (DMSO) solution (17.6 g, 3.84 mol/L, 68%). The concentration was calculated using the relative peak areas in ^{1}H-NMR spectrum.

^{1}H-NMR (300 MHz, $CDCl_3$) δ = 9.23 (s, 1H, C<u>H</u>O), 2.57 (s, 6H, OS(C<u>H</u>$_3$)$_2$), 1.50 (s, 9H, OC(C<u>H</u>$_3$)$_3$).

^{13}C-NMR (75 MHz, $CDCl_3$) δ = 185.0 (<u>C</u>HO), 158.4 (<u>C</u>OO), 84.2 (O<u>C</u>(CH$_3$)$_3$), 40.6 (OS(<u>C</u>H$_3$)$_2$), 27.4 (OC(<u>C</u>H$_3$)$_3$).

Ozonolysis of ^{t}*Bu methacrylate*. ^{t}Bu methacrylate (47 mL, 289 mmol) was dissolved in dried CH_2Cl_2 (340 mL), cooled, and ozonolyzed for 5 h as described above. Opening of the secondary ozonide was performed via addition of $S(CH_3)_2$ (56 mL, 766 mmol, 2.6 eq.) to the cooled, stirred solution which was then allowed to warm to room temperature. After 48 h CH_2Cl_2, excess $S(CH_3)_2$, and HCHO were removed by distillation under reduced pressure. The reaction mixture was filtered and the formed white precipitate was washed with ethyl acetate. Concentration of the filtrate yielded ^{t}Bu pyruvate as a colorless solution in DMSO (24.0 g, 2.9 mol/L, 17%). The concentration was calculated using the relative peak areas in ^{1}H-NMR spectrum.

^{1}H-NMR (300 MHz, $CDCl_3$) δ = 2.57 (s, 12H, 2 eq. OS(C<u>H</u>$_3$)$_2$), 2.34 (s, 3H, C(O)C<u>H</u>$_3$), 1.48 (s, 9H, OC(C<u>H</u>$_3$)$_3$).

^{13}C-NMR (75 MHz, $CDCl_3$) δ = 192.8 (<u>C</u>(O)CH$_3$), 159.9 (<u>C</u>OO), 83.6 (O<u>C</u>(CH$_3$)$_3$), 40.4 (OS(<u>C</u>H$_3$)$_2$), 27.4 (OC(<u>C</u>H$_3$)$_3$), 26.2 (C(O)<u>C</u>H$_3$).

Thin layer chromatography. Reactions were monitored by TLC using Merck silica gel 60 F_{254} TLC aluminium sheets and visualized under a UV lamp and/or with Seebach staining solution.

NMR spectroscopy. NMR spectra were recorded in $CDCl_3$ with a Fourier 300 (Bruker). Raw data were processed with MestReNova Lite 1.6 software. An exponential window function with 0.2 Hz peak width was used and spectra size was increased by zero filling (^{1}H: 16→32 k,

^{13}C: 32→64 k). Referencing was done to tetramethyl silane (^{1}H, $\delta = 0.00$) and $CDCl_3$ (^{13}C, $\delta =$ 77.02), respectively. Phases were corrected manually, while the base line automatically.

Protocol S2: Peptide purification

Peptides were purified by ion pair reversed phase high performance liquid chromatography (IP-RP-HPLC) on an Äkta Purifier 10 system (GE Healthcare Amersham Biosciences Europe GmbH, Freiburg, Germany) using aqueous acetonitrile gradients on Jupiter C18 columns (15 or 10 µm particle size, 300 Å pore size, Phenomenex Ltd, Aschaffenburg, Germany). Ion pair reagents were trifluoroacetic acid (TFA, 0.1% (v/v)) for peptide *1* and heptafluorobutyric acid (HFBA, 0.1% (v/v)) peptides *2* - *9*.

Eluent A: 0.1% (v/v) aqueous TFA,

Eluent B: 60% (v/v) aqueous acetonitrile containing 0.1% (v/v) TFA,

Eluent C: 0.1% (v/v) aqueous HFBA, and

Eluent D: acetonitrile containing 0.1% (v/v) HFBA.

Table S1: ESI-source parameters and mass analyzer settings applied on the 4000 Q TRAP™.

Parameter \ Experiment	Q1-MS scan	MS/MS scans		
		Product ion	Precursor ion	Enhanced product ion (EPI)
ESI-source				
IS [V]	5500	5500	5500	5500
Temperature	0	0	0	0
Nebulizer gas (Gas 1, psig)	10	10	15	10
Drying gas (Gas 2, psig)	0	0	0	0
Curtain gas (CUR, psig)	15	15	10	15
Mass analyzer				
Declustering potential (DP, V)	10-250[a]	60-205	25	70
Collision cell entrance potential (EP, V)	10	10	10	-
Collision cell exit potential (CXP, V)	-	10	10	-
Interface heating	on	on	on	on
Q_1 resolution	unit	unit	high	high
Q_3 resolution	-	unit	high	-
Q_3 entry barrier (V)	-	-	-	8
Collision (CAD) gas	-	medium	medium	medium
Collision potential (CE, V)	-	5-130[b]	40, 50[c]	35-55[d]
***m/z* range**	350-1400[d]	50 - 1000	300-900	50-1500
Scan rate (*m/z* s^{-1})	10	10	0.5	250
Step size (*m/z*)	0.1	0.1	0.1	0.03

[a] DP ramping was performed in 5 V steps; [b] CE ramping was performed in 5 V steps;

[c] depended on the reporter ion; [d] depended on peptide sequence and charge state.

Table S2: ESI-source parameters and mass analyzer settings applied on the ESI-LTQ-Orbitrap XL ETD.

Experiment / Parameter	Survey scan (Orbitrap - MS)	Dependent scan (LTQ - MS/MS)
ESI-source		
Spray voltage (kV)	1.70	
Capillary temperature (°C)	200	
Capillary voltage (V)	36	
Tube lens (V)	110	
Mass analyzer		
***m/z* range**	400-2000	adjusted automatically
Resolution	60,000	
Activation type	-	collision-induced dissociation (CID)
Normalized collision energy	-	35%
Activation frequency	-	0.250
Activation time (ms)	-	30,000
Isolation width (Da)	-	2.00
Data-dependent acquisition (DDA) parameters		
Max. no. MS/MS per MS scan		6
Minimum signal intensity (MS)	500.0	
Exclusion list size (no. *m/z* values)		500
Exclusion duration (s)		60
Database search parameters		
Modification	**Mass increment [Da]**	**Amino acid(s)**
Carbamidomethyl (cam)	+57.021	C
Oxidation (ox)	+15.995	C, D, F, H, K, M, N, P, R, W, Y
Dioxidation (diox)	+31.990	C, F, H, K, M, P, R, W, Y
Trioxidation (triox)	+47.985	C, W
Deamidation (deam)	+0.984	N, Q, R
Amadori (Am)	+162.053	K

CML/CMA (CM)	+58.056	K, R
CEL/CEA (CE)	+72.021	K, R
Glarg (Glarg)	+39.995	R
MG-H 1,2,3 (MG-H)	+54.011	R

Table S3 Instrumental (LOD_is) and method detection limits (LOD_ms) for precursor ion scans with *m/z* 187.1 and 201.1 acquired at the 4000 QTRAP operated in QqQ mode. Peptides were infused with a syringe pump (5 µL/min).

Peptide	**Sensitivity**			
	LOD_i (nmol/L)[a]	**Ion**	**LOD_m (nmol/L)[b]**	**Ion**
2	5	$[M + H]^+$	20	$[M + H]^+$
3	2.5	$[M + 2H]^{2+}$	50	$[M + H]^+$

[a] Samples were dissolved in 60% (v/v) aq. acetonitrile with 0.1% (v/v) formic acid.

[b] Samples were spiked into a tryptic digest of HSA (500 nmol/L) dissolved in 60% (v/v) aq. acetonitrile containing 0.1% (v/v) formic acid.

Table S4: Singly charged fragment ions that could interfere with CML- and CEL-specific reporter ions.

Modification	**Reporter ion (*m/z*)**	**b_2 and internal ions**
CML	142.1	-
	187.1	(E,G), (D,A), (S,V)
CEL	156.1	-
	201.1	(E,A), (L/I,S), (C,P), (T,V)

Table S5: Peptides identified in a tryptic digest of glycated HSA that may have interfered with the precursor ion scan signals.

No[a]	Precursor ion scan					nanoLC-LIT-Orbitrap-MS DDA					
	m/z	Reporter ion rel. int. [%]		Detected at *m/z*		t_R (min)	*m/z*	*z*	X_{Corr}[d]	Prob.	Sequence
		187.1	142.1	Original[b]	lower *z*[c]						
1	440.7	6	-	X		19.9	440.721	2	2.34	2.4	AEFAEVSK
2	476.4	11	8	X		23.7	476.221	2	2.61	2.0	DLGEENFK
3	547.4	7	-	X		28.5	547.315	3	4.70	25.5	KVPQVSTPTLVEVSR

[a] Peptides are numbered by increasing *m/z* values.

[b] Originally detected in the modification-specific precursor ion scan.

[c] *m/z* recalculated to lower charge state.

[d] Only peptides annotated with $X_{corr} \geq 2.20$ for doubly- and ≥ 3.75 for triply charged ions are listed.

Table S6 Peptides identified in analyses of diabetic plasma whose sequences may have caused false positive precursor ion scan signals.

No[a]	Precursor ion scan					nanoLC-LIT-Orbitrap-MS DDA							
	m/z	reporter ion rel. int. [%]		found by *m/z*		t_R (min)	*m/z*	*z*	XCorr[d]	Prob.	Annotated sequence[e]	Protein	
		187.1	142.1	PIS[b]	lower *z*[c]							Accession number	Protein description
1	409.6	11	-	X		32.6	409.5403	3	3.97	1.0	FKDLGEENFK	P02768	Serum albumin precursor [ALBU_HUMAN]
2	440.8	18	-	X		26.0	440.7241	2	2.46	1.0	AEFAEVSK	P02768	Serum albumin precursor [ALBU_HUMAN]
3	476.3	14	8	X		25.9	476.2242	2	2.74	7.2	DLGEENFK	P02768	Serum albumin precursor [ALBU_HUMAN]
4	480.8	-	49	X		26.2	480.7590	2	3.58	3.8	ADLSGITGAR	P01011	Alpha-1-antichymotrypsin [AACT_HUMAN]
5	500.7	11	-	X		20.5	500.5480	3	3.93	20.8	ADDKETC$_{CAM}$F-AEEGK	P02768	Serum albumin precursor [ALBU_HUMAN]
				X		29.1	500.7533	2	2.31	1.0	YLGEEYVK	P02787	Serrotransferrin [TRFE_HUMAN]
6	508.4	8	-	X		34.4	508.3105	2	2.23	1.0	SVLGQLGITK	P01009	Alpha-1-antitrypsin [A1AT_HUMAN]
7	547.4	15	4	X		31.3	547.3181	3	4.63	1.0	KVPQVSTPTL-VEVSR	P02768	Serum albumin precursor [ALBU_HUMAN]

8	581.4	15	5	X		32.4	581.3190	2	2.54	1.0	NQVSLT-C_{CAM}LVK	P01859	Ig gamma-2 chain C region [IGHG2_HUMAN]
9	644.7	4	-	X		33.8	644.3272	2	3.06	32.9	GPSVFPLA-PC_{CAM}SR	P01859	Ig gamma-2 chain C region [IGHG2_HUMAN]
10	708.3	5	-	X		28.8	708.3645	2	2.61	29.3	SVIPSDGPSV-AC_{CAM}VK	P02787	Serrotransferrin [TRFE_HUMAN]

[a] Peptides are numbered by increasing *m/z* values.

[b] Originally detected in the modification-specific precursor ion scan.

[c] *m/z* recalculated to lower charge state.

[d] Only peptides annotated with $X_{corr} \geq 2.20$ for doubly- and ≥ 3.75 for triply charged ions are listed.

[e] $_{CAM}$, carbamidomethylation.

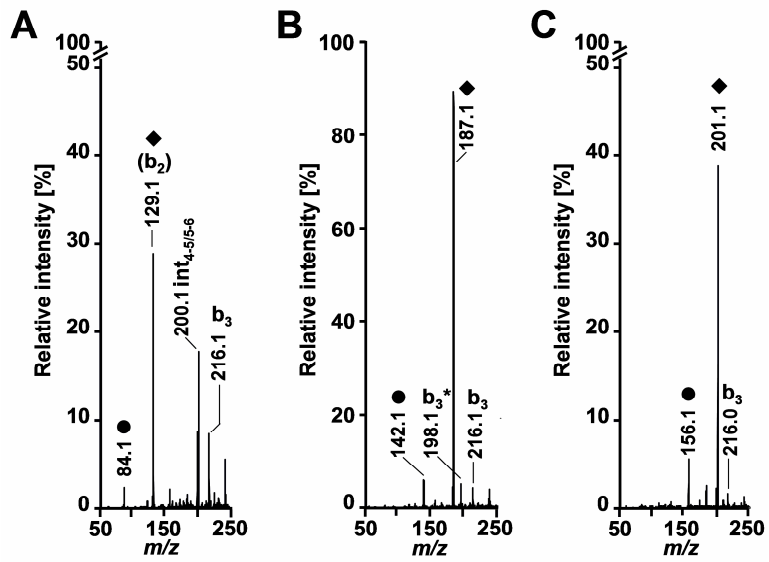

Figure S1 Sections of the tandem mass spectra of *m/z* 865.5 (A), 923.5 (B), and 937.5 (C) corresponding to $[M+H]^+$ ions of peptides *1*, *2*, and *3*, respectively, recorded with a QqLIT mass spectrometer operating in positive ion mode (collision potential 50 V). Signals indicating neutral losses of water are marked with asterisks (*) and α-amino-ε-caprolactam- and tetrahydropyridine-related lysine-derived ions are labeled with full diamonds (♦) and circles (●), respectively. Signals indicating internal fragment ions are denoted as int_{a-b} (a and b as terminal residues).

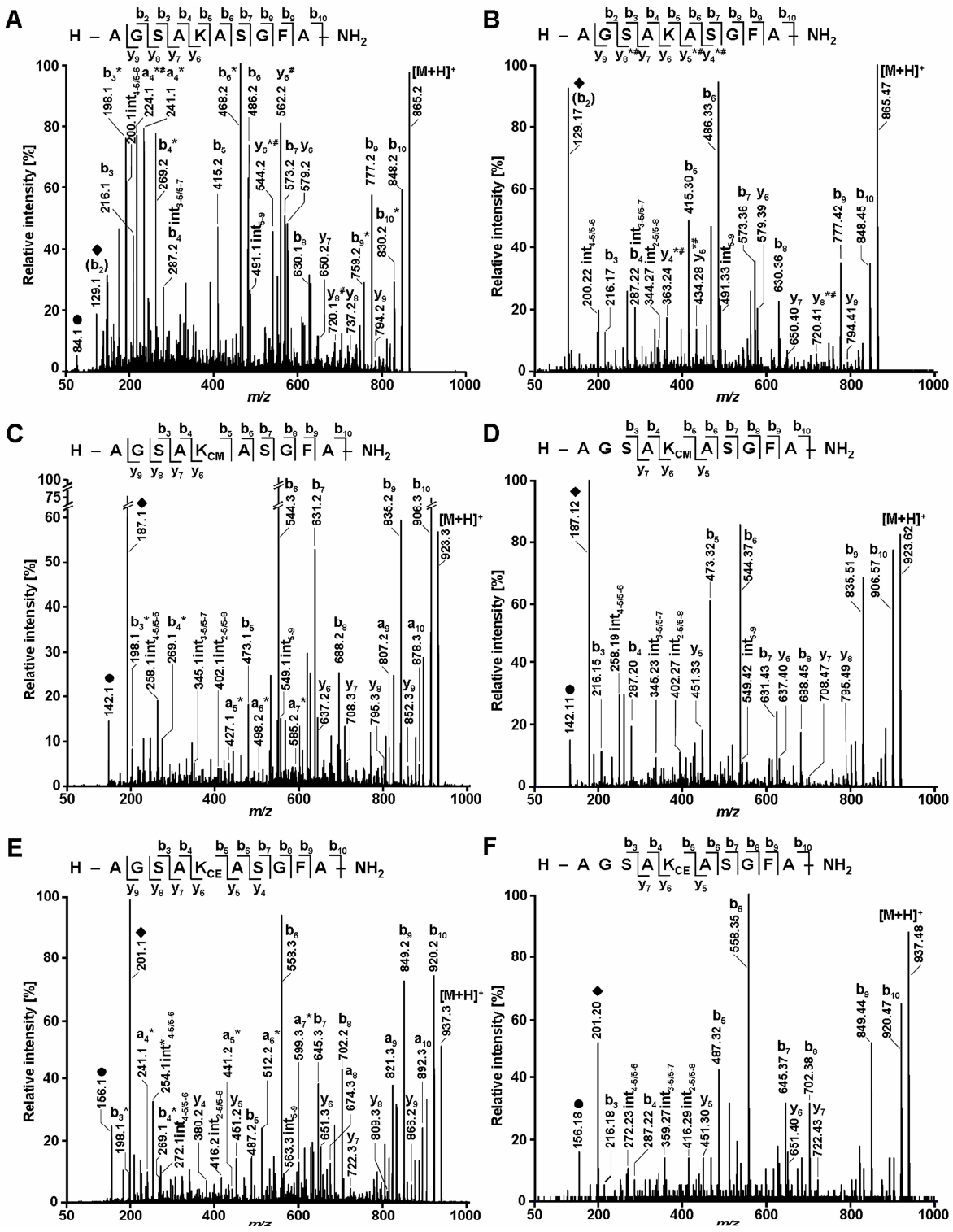

Figure S2: Tandem mass spectra of *m/z* 865.3, 923.3, and 937.3 corresponding to singly protonated peptides *1* (A, B), *2* (C, D), and *3* (E, F), respectively, recorded on a QqLIT-MS in enhanced product ion mode (A, C, and E) or a QqTOF-MS (B, D, and F) using a collision potential of 50 V (55 V in A). Signals indicating water and ammonia losses are marked with asterisks (*) or hashtags (#), respectively. Signals indicating internal fragment ions are denoted as int_{a-b} (a and b as terminal residues). ◆ and ● denote signals indicating α-amino-ε-caprolactam and tetrahydropyridine reporter ions, respectively.

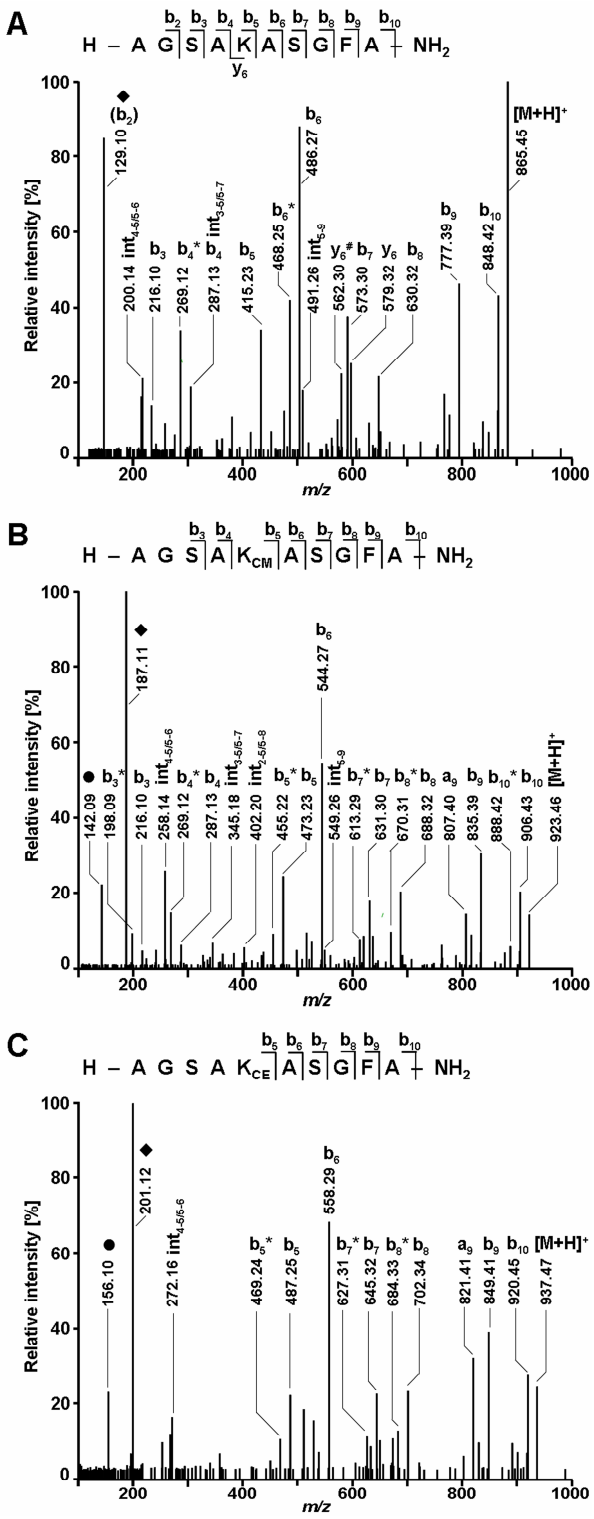

Figure S3 Tandem mass spectra of *m/z* 865.5 (A), 923.5 (B), and 937.5 (C) corresponding to $[M+H]^+$ ions of peptides *1*, *2*, and *3*, respectively, recorded on a LTQ-Orbitrap mass spectrometer using HCD fragmentation (normalized collision potential 30 V, activation time 30 ms). Signals indicating neutral losses of water and ammonia are marked with asterisks (*) and hashtags (#), respectively; α-amino-ε-caprolactam- and tetrahydropyridine-related lysine-derived ions are labeled with full diamonds (♦) and circles (●), respectively. Signals indicating internal fragment ions are denoted as int_{a-b} (a and b as terminal residues).

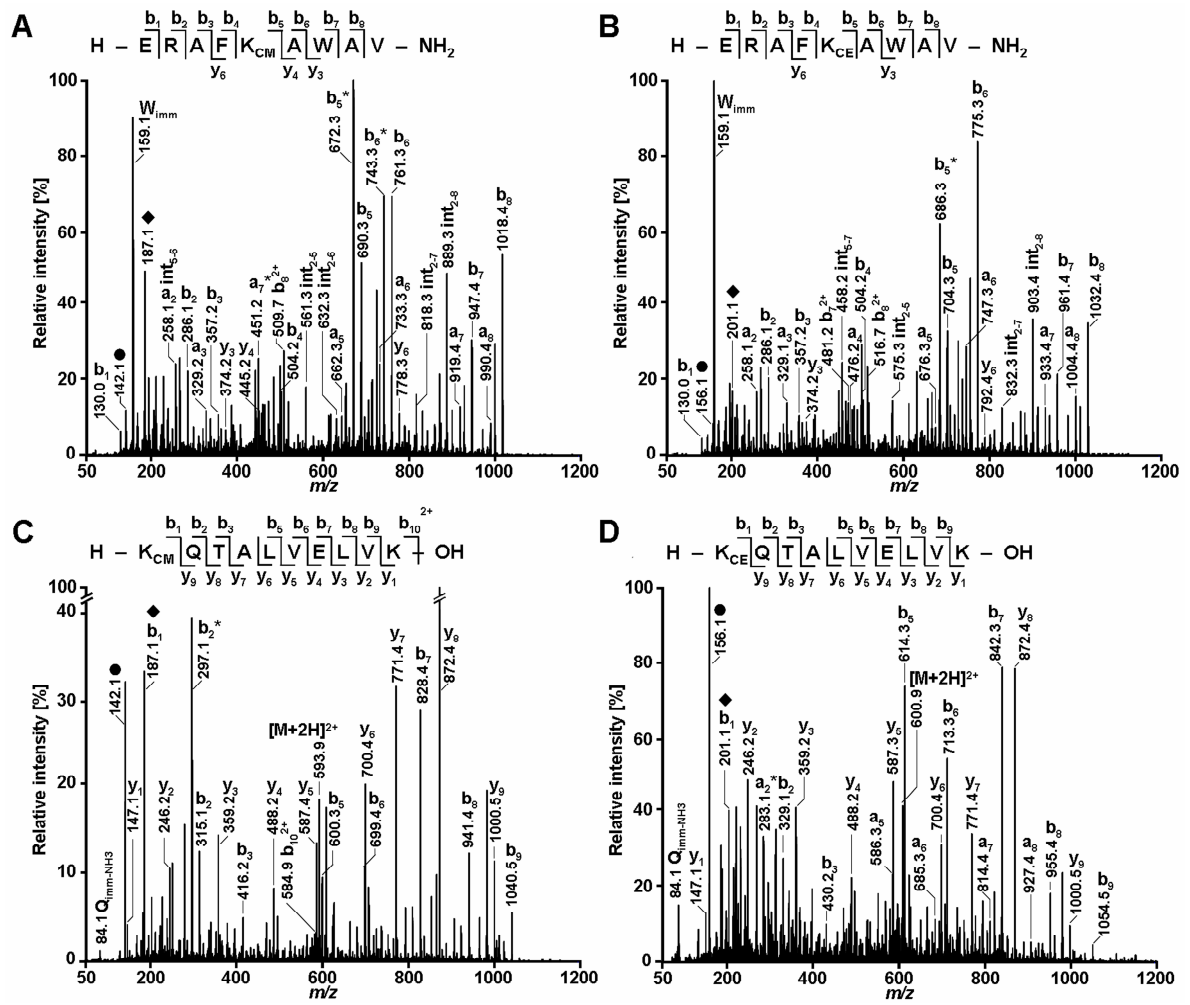

Figure S4: Tandem mass spectra of *m/z* 567.8, 574.8, 593.9, and 600.9 corresponding to doubly protonated peptides *4* (A), *5* (B), *6* (C), and *7* (D). Mass spectra were recorded on a QqLIT-MS in enhanced mode applying a collision potential of 35 V (40 V for *4*). Signals indicating internal fragment ions are denoted as int_{a-b} (a and b as terminal residues). ◆ and ● denote signals indicating α-amino-ε-caprolactam and tetrahydropyridine reporter ions, respectively.

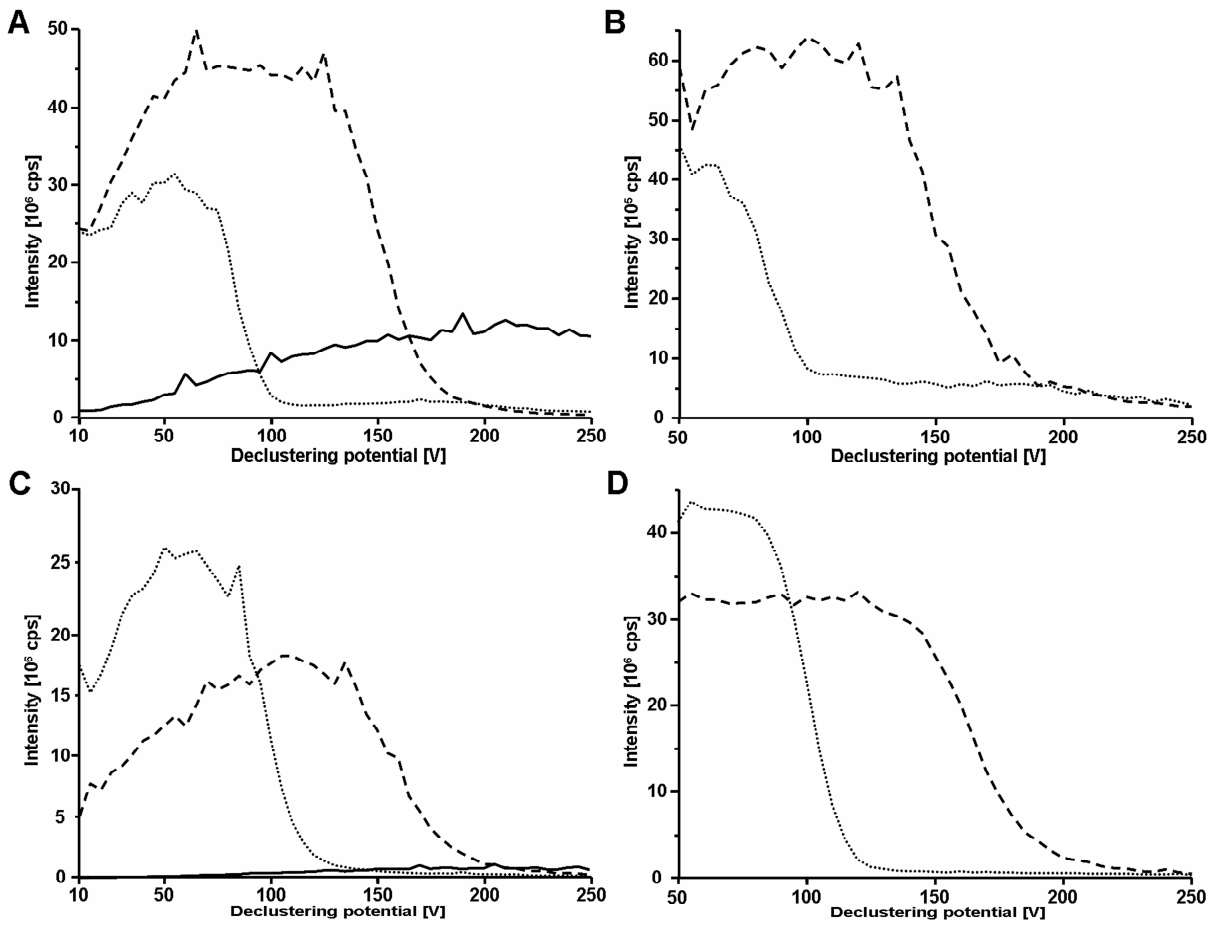

Figure S5: Signal intensities (*m/z* ± 0.5 Da) of peptides *6* (A), *7* (B), *8* (C) and *9* (D) in Q1 scans using different declustering potentials (DP). Peptides were dissolved (2.5 µmol/L) in aqueous acetonitrile (60%, v/v) containing formic acid (0.1%, v/v) and infused (5 µL/min) into a QqLIT mass spectrometer operating in a positive ion mode. DP was ramped in increments of 5 V. Signal intensities of $[M+H]^+$, $[M+2H]^{2+}$, and $[M+3H]^{3+}$ are shown as full, dashed, and dotted lines, respectively.

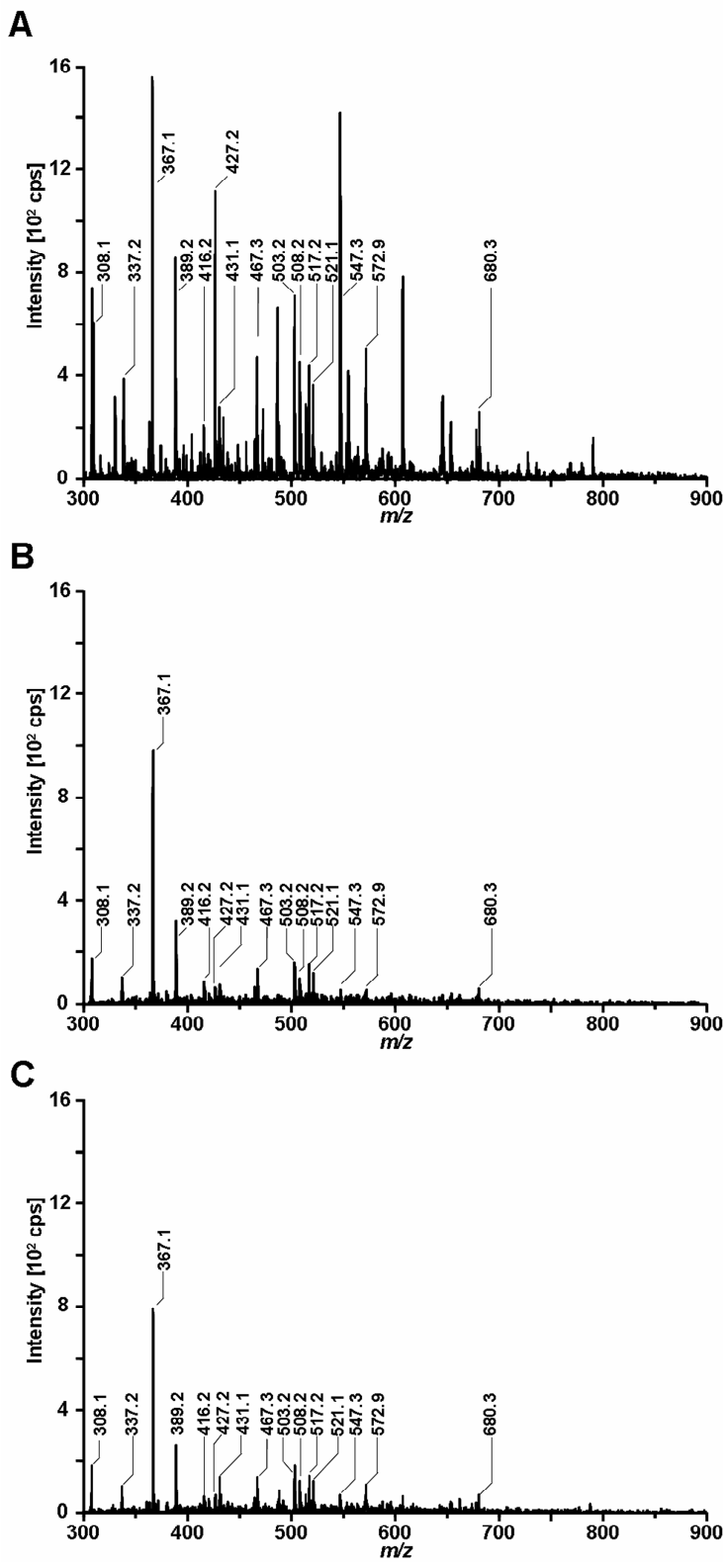

Figure S6: Precursor ion scans for *m/z* 187.1 of a tryptic digest of glycated HSA (10 µmol/L) recorded with declustering potentials of 70 (A), 25 (B), and 10 V (C). The sample was dissolved in aqueous acetonitrile (60%, v/v) containing formic acid (0.1%, v/v) and infused via a syringe pump at 5 µL/min into a QqLIT mass spectrometer operating in QqQ positive ion mode.

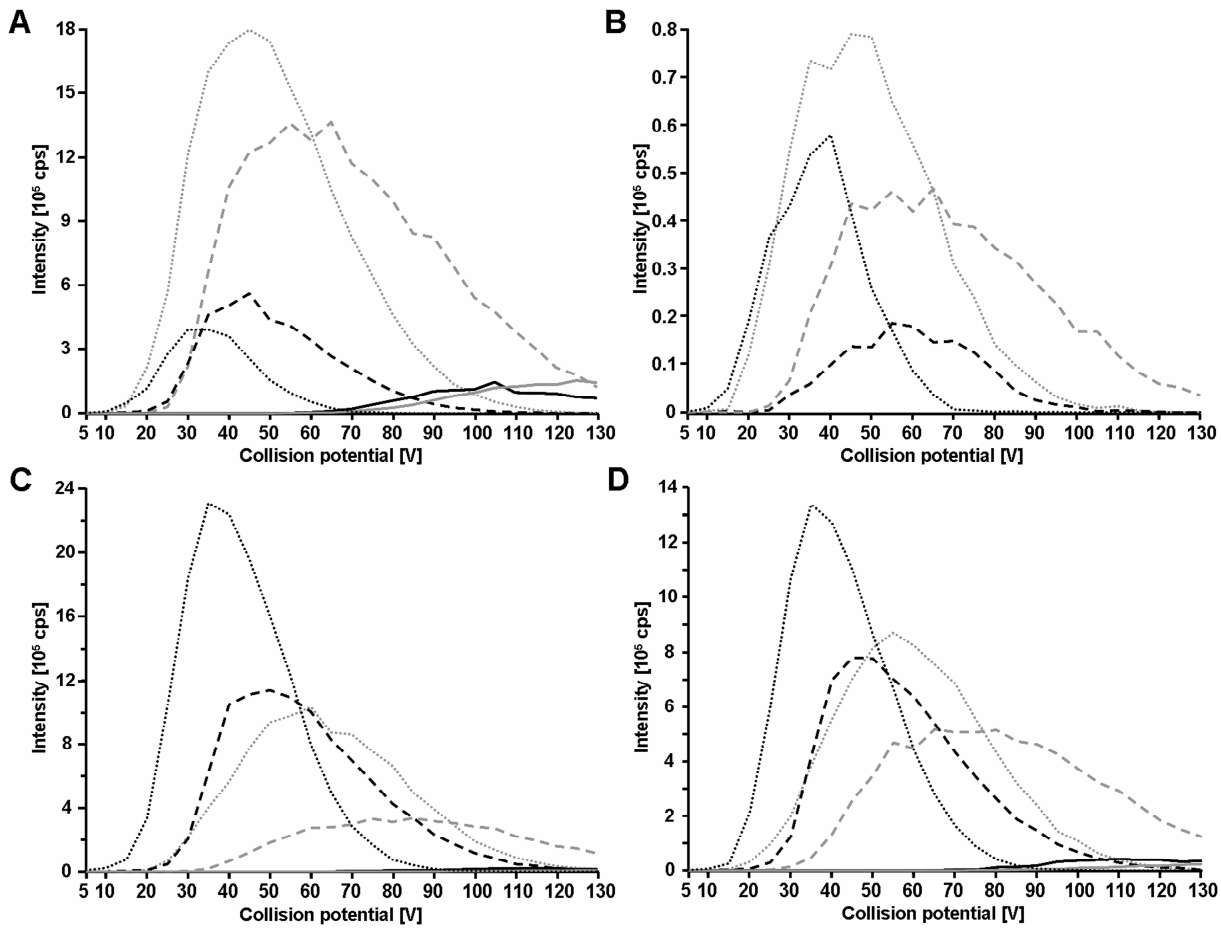

Figure S7 Intensity of the reporter ion signals for different collision potentials applied in a product ion scan of peptides *6* (A), *7* (B), *8* (C), and *9* (D). Shown are signal intensities corresponding to α-amino-ε-caprolactam-derived ions (black) for CML (*m/z* 187.1) and CEL (*m/z* 201.1) and tetrahydropyridine-derived ions (grey) for CML (*m/z* 142.1) and CEL (*m/z* 156.1) obtained by fragmentation of singly (full), doubly (dashed), or triply charged (dotted) precursor ions. Peptides were dissolved (2.5 µmol/L) aqueous acetonitrile (60%, v/v) containing formic acid (0.1%, v/v) and infused at a flow rate of 5 µL/min into a QqLIT mass spectrometer operated in QqQ mode.

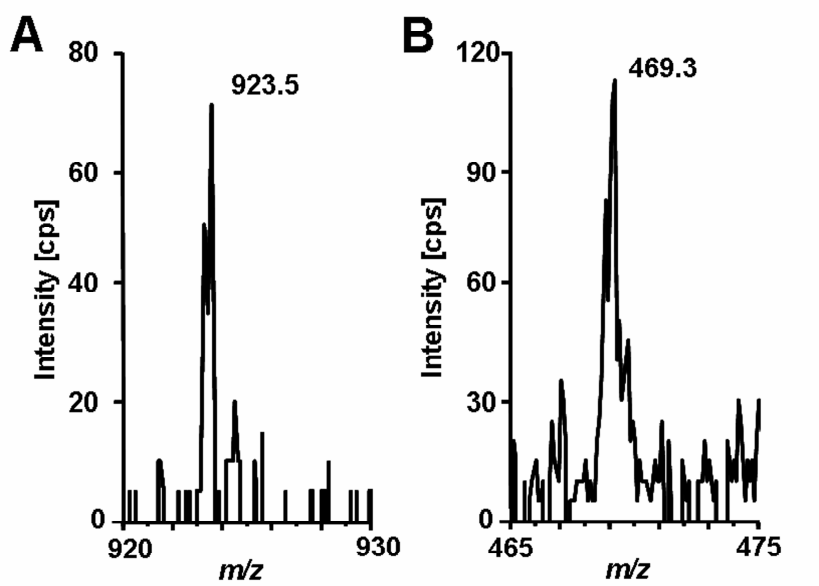

Figure S8: Precursor ion scans acquired for *m/z* 187.1 (A) and 201.1 (B) with peptides *2* (A, $[M+H]^+$) and *3* (B, $[M+2H]^{2+}$) at concentrations corresponding to their LOD_is (i.e. 2.5 and 5 nmol/L, respectively). Peptides were dissolved in aqueous acetonitrile (60%, v/v) containing formic acid (0.1%, v/v) and infused at a flow rate of 5 µL/min into a QqLIT mass spectrometer operated in QqQ mode.

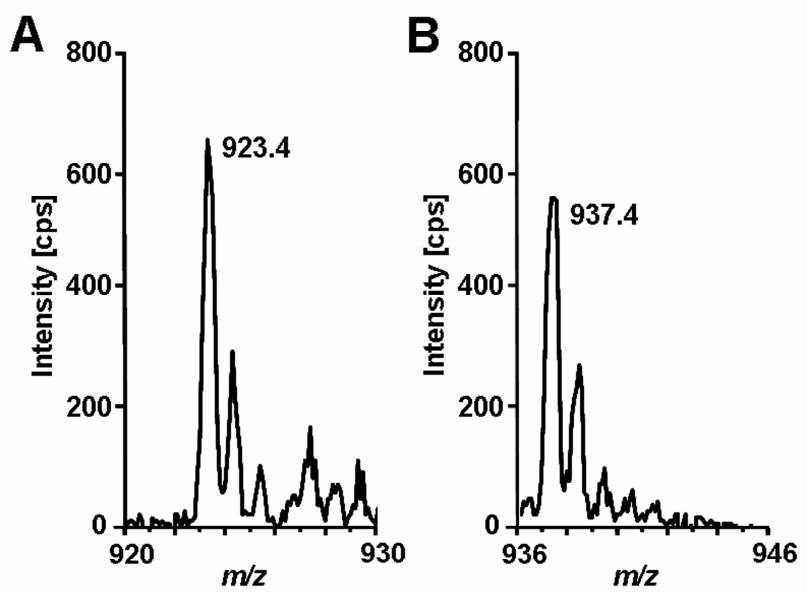

Figure S9: Precursor ion scans acquired for *m/z* 187.1 (A) and 201.1 (B) with peptides *2* (A, $[M+H]^+$) and *3* (B, $[M+2H]^{2+}$) at concentrations corresponding to their LOD_ms (i.e. 20 and 50 nmol/L, respectively). Peptides were dissolved in aqueous acetonitrile (60%, v/v) containing formic acid (0.1%, v/v) and infused at a flow rate of 5 µL/min into a QqLIT mass spectrometer operated in QqQ mode.

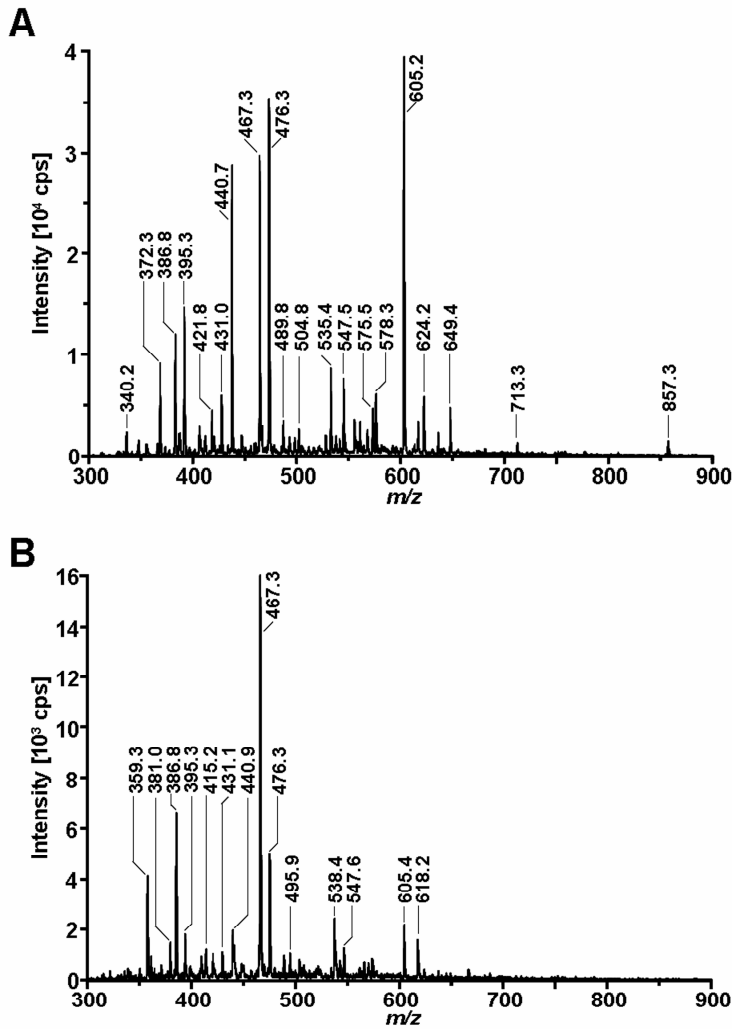

Figure S10 Precursor ion scans of a tryptic digest of glycated HSA (10 µmol/L) acquired for *m/z* 201.1 (A) and 156.1 (B). The digest was dissolved in aqueous acetonitrile (60%, v/v) containing formic acid (0.1%, v/v) and infused at a flow rate of 5 µL/min into a QqLIT mass spectrometer operated in QqQ mode.

References

1. Yamago, S.; Nakamura, M.; Wang, X. Q.; Yanagawa, M.; Tokumitzu, S.; Nakamura, E. Thermal hetero [3+2] cycloaddition of dipolar trimethylmethane to O-akyloximes. Straightforward synthetic routes to substituted pyrrolidines and prolines. *J. Org. Chem.* 1998, *63*, 1694-1703.