Application note

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Application of Z-sinapinic matrix in peptide MALDI-MS analysis

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Since introduction of sinapinic acid (SA) and α-cyano-4-hydroxycinnamic acid as matrices, successful application of matrix-assisted laser desorption/ionization mass spectrometry started for protein/polypeptides. Both show some limitations in short peptide analysis because matrix clusters are quite abundant. Cinnamics currently used are E-cinnamics. Here, Z-SA as matrix for peptides is studied and compared with E-SA and α-cyano-4-hydroxycinnamic acid. Minor number of clusters is always observed in the low *m/z* region allowing the detection of short peptides. The results here described show that this novel matrix is a tool of choice for direct, rapid and sensitive detection of hydrophilic and hydrophobic peptides. Copyright © 2017 John Wiley & Sons, Ltd.

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Keywords: MALDI-MS; peptides; CHCA clusters; Z-sinapinic acid

Introduction

The use of short peptides in the health industry increases day by day. They are applied as therapeutic drugs especially due to their antimicrobial, antiproliferative or antimetastasic activity[1,2]. Also, they are utilized as economic and selective ligands in affinity chromatography for recombinant proteins purification from crude bioreactor broth [3,4]. Key peptides may be found in nature by analyzing extracts from living organism. Screening of biological and chemical synthesized peptide libraries is a valuable tool for the optimization of natural or the discovery of novel peptides [5,6]. Peptide chemical modifications and the use of non-natural amino acids allow improving their performance as biopharmaceuticals and as ligands in affinity chromatography [7,8].

Today, mass spectrometry is the technique of choice for natural, synthetic and chemically modified short peptide analysis. While both electrospray ionization (ESI) and matrix-assisted laser-desorption ionization (MALDI) are widely used, in some cases MALDI has some advantage over ESI. As MALDI is a solid state technique, it allows the analysis of solid samples such as tissues [9] or solid supports with immobilized peptides. Also, MALDI-MS is ideal for peptide screening processes in which multiple samples are analyzed, because less time is needed to acquire each spectrum. Furthermore, unlike ESI, in MALDI analysis, each sample, which is spotted to a target plate, can be reanalyzed, and mixtures can be analyzed in straight way. However, clusters from the matrix used in MALDI analysis frequently interfere with MS spectrum. In the analyses of short peptides, usually α-cyano-4-hydroxycinnamic acid (CHCA) [10] is used instead of E-4-hydroxy-3,5-dimethoxycinnamic acid (sinapinic acid, E-SA) [10], because peptide ionization is efficient enough to obtain spectra with higher intensity, peptide signal and fewer matrix clusters. However, CHCA matrix–matrix and analyte–matrix clusters sometimes interfere with peptide mass spectra interpretation. When using other matrices such as 2,5-dihydroxybenzoic acid (DHBA) [10], matrix clusters are reduced but also peptide ionization and thus the sensitivity of the MS analysis [11]. As an attempt to minimize CHCA cluster interference, the use of additives (co-matrix) enhancing the peptide ion formation improving the signal/noise (S/N) ratio and favoring the suppression of matrix clusters and peptide-sodiated adducts has been described (i.e. ammonium sulfate [12], ammonium monobasic phosphate or ammonium dibasic citrate [13], serine or threonine [14], protonated weak bases [15], glycerol [15], etc.). Recently, the synthesis of Z-SA was described [16] as well as its application as matrix in the analysis of carbohydrates, demonstrating its outstanding performance in contrast with the commercial available E-SA [17]. In the present work, we evaluated the performance of the Z-SA in short peptide MS analysis in comparison with the E-SA and CHCA. Besides, in some examples, DHBA was also tested as matrix.

Results and discussion

For the study, several peptides were used as analytes: commercial peptides (bradykinin (1–7), bradykinin (1–8), angiotensin I, angiotensin II) and model peptides with different amino acid side chains, synthesized by solid phase peptide synthesis using Fmoc strategy and HMBA-ChemMatrix resin beads as previously described [3]

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(Electronic Supplementary Information, Table S1). Single peptide-beads located into separate micro tubes were placed in a drying chamber together with a flask containing ammonia. Released peptides were eluted from each bead with 10 μl of acetic acid (AcOH)/acetonitrile (MeCN)/H_2O 0.1% trifluoroacetic acid (TFA) (3:4:3) overnight, and 1 μl of sample was loaded onto the sample plate and air dried [11,18]. Then, 1 μl of MALDI matrix solution was added on the sample dry layer [18]. MALDI-TOF mass spectra were recorded in an Ultraflex II TOF/TOF (Bruker Daltonics GmbH, Leipzig, Germany) and acquired in the reflector positive-ion mode.

As an example, Fig. 1 shows the MALDI-MS mass spectra obtained for peptide KSG (H-Lys-Ser-Gly-NH_2; Table S1). When using CHCA (Fig. 1a), E-SA (Fig. 1c) and DHBA (Fig. 1d), peptide signals ($[M + H]^+$, $[M + Na]^+$ and $[M + K]^+$) were obtained with lower intensity than matrix clusters signals, making difficult the analysis of novel peptides with unknown molecular weight. The best results were obtained when using Z-SA (Fig. 1b); the spectrum showed high peptide signals without significant interference of matrix cluster signals. Similar results were obtained with the hydrophobic peptide FVG (H-Phe-Val-Gly-NH_2; Fig. S1, Electronic Supplementary Information).

As an extreme example, for the hydrophobic peptide FVFALIFVG (H-Phe-Val-Phe-Ala-Leu-Ile-Phe-Val-Gly-NH_2; Table S1, Fig. S2), only Z-SA (Fig. S2B) showed the corresponding molecular ion as the sodiated species $[M + Na]^+$. A similar completed suppression of a peptide signal using CHCA and E-SA was observed after chymotrypsin treatment of the peptide FKFRYTAHSGASG (H-Phe-Lys-Phe-Arg-Tyr-Thr-Ala-His-Ser-Gly-Ala-Ser-Gly-NH_2; Fig. 2a and c). In the mixture obtained, the peptide TAHSGASG (H-Thr-Ala-His-Ser-Gly-Ala-Ser-Gly-NH_2) as the $[M + H]^+$ species could be only observed when using Z-SA (Fig. 2b). DHBA as matrix sowed the lowest intensity peptide signal (Fig. S3a–d). Additional examples included in electronic supplementary material show that Z-SA is a good matrix of choice for short peptide analysis (Electronic Supplementary Information, Figs. S4–S7) because always the peptide signal is observed. Similar advantage was observed when after bovine serum albumin (BSA) trypsin digestion, Z-SA and E-SA were used as matrix. The number of peptides detected with Z-SA was higher than those detected with E-SA (Fig. S8 and Table S2, Electronic Supplementary Information).

Dynamic range, linearity and limit of detection (LOD) studies for some peptides were conducted in positive ion mode with the examined matrices: Z-SA, E-SA and CHCA (Table S3, Electronic Supplementary Information). The dynamic ranges in which peptide signals were detected with good S/N ratios (S/N ≥ 4) are listed in Table S3. Bradykinin (1–7) analysis with Z-SA showed satisfactory LOD and dynamic range, and the intensity of the signals was similar to those obtained with CHCA in all the range studied (1–1320 picomol/μl, Table S3, Fig. S9A; Electronic Supplementary Information). E-Sinapinic acid was not an efficient matrix in the range of concentrations studied (Table S3, Fig. S9B) because molecular ion signals were very low or not detected. For FVG and KSG, we conducted similar experiments. Those analyses performed with Z-SA showed similar LOD and dynamic range than CHCA, although the absolute signal intensity was lower (Figs. S10–S11; Table S3). However, when using CHCA, abundant satellite matrix clusters near the analyte signal were always observed (Fig. 1; Electronic Supplementary Information, Fig. S1). E-Sinapinic acid showed a poor performance as matrix yielding peptide signals of low intensity together with abundant satellite matrix clusters. Additional experiments were conducted with commercial BDK(1–8), ANG II and ANG I sold for calibration (Table S1, Figs. S12–S14, Electronic Supplementary Information). For these peptides, CHCA sowed higher signal intensity and LOD; E-SA and Z-SA showed quite similar intensity signals, but CHCA and E-SA always showed abundant and high intensity satellite cluster signals. Serine is often used as additive to CHCA matrix to decrease cluster signals [14]. However, when using E-SA and Z-SA, samples were not doped with serine because higher intensity and minor number of cluster signals were observed in the experimental conditions evaluated. Doping E-SA and Z-SA with serine did not significantly improve the LOD (results not shown) and did not diminish the presence of cluster signals at $m/z < 500$ (Electronic Supplementary Information). Z-Sinapinic acid showed better LOD and dynamic ranges for the hydrophobic (FVG) and hydrophilic polar-cationic (KSG) polypeptides specially designed, and they were superior to E-SA, and sometimes to CHCA, taking into account the abundant satellite clusters produced by both matrices.

Molecular modeling suggests that the superiority of the matrix Z-SA and its similarity to CHCA in some examples are probably originated from Z-SA capability to participate in a special analyte–matrix interaction at molecular level because of its no flat

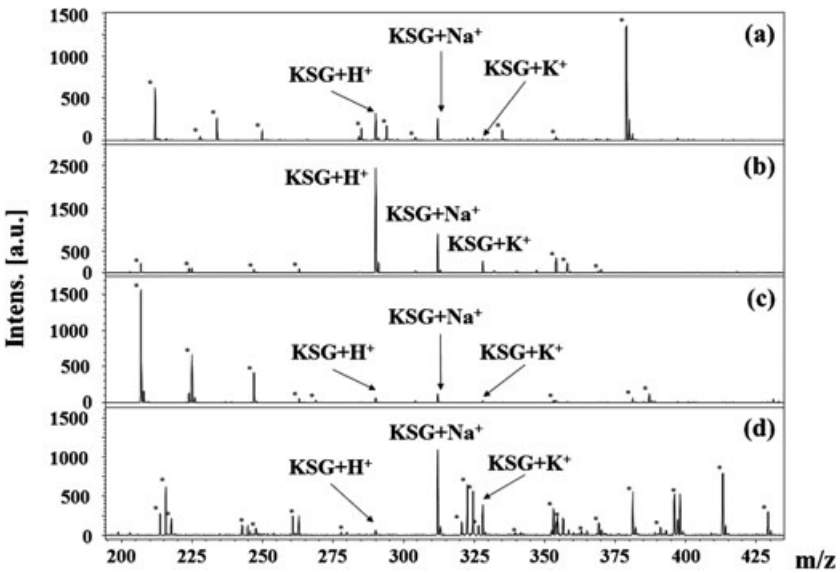


Figure 1. MALDI mass spectra of peptide H-Lys-Ser-Gly-NH_2 (KSG, MW 289.3). Matrix: (a) CHCA, (b) Z-SA, (c) E-SA and (d) DHBA. (*) Matrix clusters.

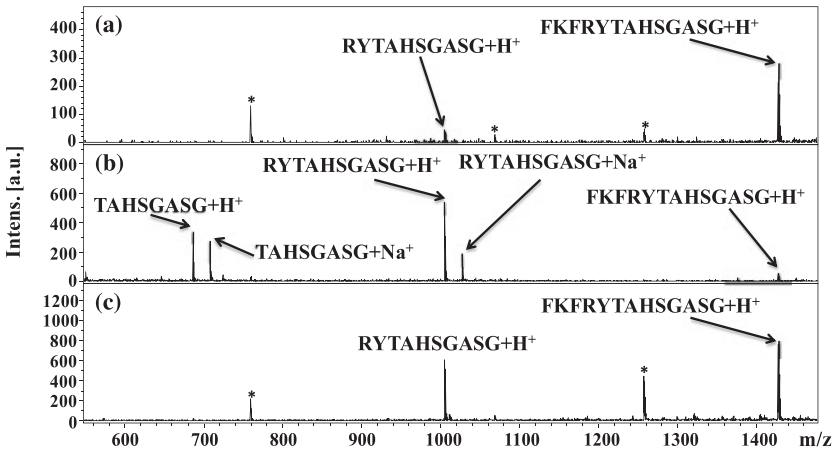
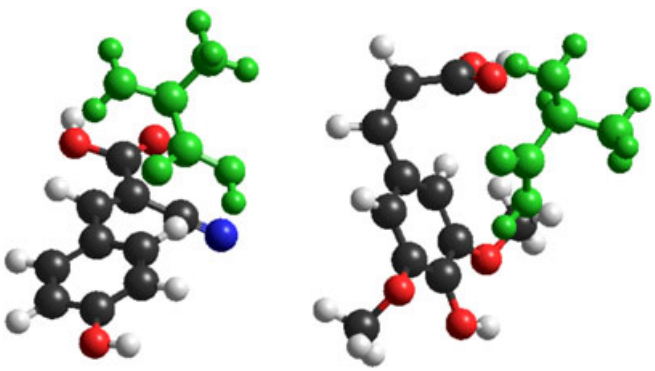


Figure 2. MALDI mass spectra of peptide H-Phe-Lys-Phe-Arg-Tyr-Thr-Ala-His-Ser-Gly-Ala-Ser-Gly-NH_2 (FKFRYTAHSGASG, MW 1427.6) after treatment with chymotrypsin. Expected fragments: RYTAHSGASG (MW 1005.1) and TAHSGASG (MW 685.7). Matrix: (a) CHCA, (b) Z-SA and (c) E-SA. (*) Matrix clusters.

structure (Scheme S1A, E-SA and Z-SA, Scheme S1B, CHCA rotamers, see details included in Electronic Supplementary Information).

We hypothesized that the carboxylic group of the CHCA would approach the cyanno moiety (α-cyanno group; NC) of the cinnamic acid structure in a synclinal overlapping like-fashion to provide the required distance among functional groups to generate stabilizing intermolecular (CHCA-(α-amino acid; aa) moiety) interactions such as hydrogen bridge (aa-COOH----NC-CHCA; aa-NH----HOOC-CHCA) interactions (Scheme 1; structure on the left; aa in green). As is shown in Scheme 1, similar interactions can take place between Z-SA and the α-amino acid moiety (aa-COOH----MeO-Z-SA; aa-NH----HOOC-Z-SA) (Scheme 1; structure on the right; aa in green). This approach can easily occur, independently of the rotamers formed by the peptide molecula (peptide secondary structure) and of its size (MW). This can be the first level of control of matrix–peptide interaction (in the solid sample). This level of control is imparted through the structure of the particular analyte matrix pair. A second level of control of the process can be imparted through the melting point and releasing of enery by the MALDI matrix. As a conclusion, the presence of the α-CN group in the CHCA structure induces deformation and the molecule cannot be as flat as E-SA is. CHCA can interact better with the α-amino acid moiety by two hydrogen bridges as Z-SA does (Scheme 1). Complementary morphological inspection of matrix solid samples deposited on the probe and LDI mass spectra were conducted. Digital images and LDI spectra are included in Figs. S15–S20 (Electronic Supplementary Information).



SCHEME 1. Molecular interaction (left) CHCA (color) – -amino acid moiety (green); (right) Z-SA (color) -amino acid moiety (green). -Amino acid moiety, serine (green). See details of molecular modeling in Molecular modeling section. [Colour figure can be viewed at wileyonlinelibrary.com]

Experimental

Chemicals and material

9 *H*-Pyrido[3,4-*b*]indole (*nor*-harmane, nHo), DHBA, E-3,5-dimethoxy-4-hydroxycinnamic acid (E-SA), E-CHCA and aliphatic organic amines (ethanolamine, butylamine) were purchased from Adrich Chemical Co. Commercial protein, BSA and peptides: BDK(1–7), bradykinin (1–7); commercial kit for calibration 4700 Proteomics Analyzer Mass of Applied Biosystems. Applied Biosystems: BDK (1–8), bradykinin (1–8); ANG II, angiotensin II; ANG I, angiotensin I; stock solution of each peptide: BDK(1–7) 1.32 μmol/ml, BDK(1–8) 1.00 μmol/ml, ANG I 2.0 μmol/ml and ANG II 2.0 μmol/ml, in H_2O 0.1% TFA were obtained from Sigma-Aldrich. Peptides FVG, KSG, NSG, TSG, LIFVG, NENSG, NYTSG, NHKSG, QTNQNYTSG, FVFALIFVG, DTSENENSG, KHNRNHKSG and FKFRYTAHSGASG were prepared by solid phase peptide synthesis (for details see Ref. [3]). Peptides RYTAHSGASG and TAHSGASG were obtained by treatment of FKFRYTAHSGASG with chymotrypsin [19]. Representative molecule structure of analytes used is shown in Table S1 (Electronic Supplementary Information). Stock solution of each peptide: 6.3 μmol/ml in AcOH/MeCN/H_2O 0.1% TFA (3:4:3 *v/v/v*) were used for MALDI-MS experiments. All the solvents (Sigma-Aldrich HPLC grade) were used as purchased without further purification. Water of very low conductivity (Milli-Q grade) was used. Z-3,5-Dimethoxy-4-hydroxycinnamic acid (Z-SA) was synthetized as was described elsewhere[16]. It was full characterized (m.p., ^{1}H and ^{13}C–NMR, UV–vis absorption spectroscopy; EI-HRMS) by comparison with the authentic sample previously described[16] (^{1}H-NMR data for Z- and E-acids are included in Electronic Supplementary Information).

Sample preparation

Matrix stock solutions were made by dissolving 5 mg of the selected compound in 1 ml MeCN/H_2O 0.1% TFA (1:1 *v/v*). Peptide solutions were freshly prepared as follows: Stock solution of each commercial peptide: bradykinin(1–7) (BDK(1–7)) 1.32 μmol/ml, bradykinin(1–8) (BDK(1–8)) 1.00 μmol/ml, angiotensin I (ANG I) 2.0 μmol/ml and angiotensin II (ANG II) 2.0 μmol/ml, in H_2O 0.1% TFA. Stock solution of each prepared peptide: 6.3 μmol/ml in AcOH/MeCN/H_2O 0.1% TFA (3:4:3 *v/v/v*). To prepare the analyte–matrix sample, the thin-film layer method [18] (dry layers) was used. Typically, 1.0 μl of the peptide solution was placed on the sample probe electrode and air-dried at room

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temperature. Then, 1.0 μl of the matrix solution was placed on the sample probe covering the peptide and partially dissolving it and air-dried. The matrix to analyte ratio was 1:1 (*v/v*), and the matrix and analyte solution loading sequence was: (i) analyte, (ii) matrix [18].

Protein (BSA) digestion was as follows [20,21]: proteins were reduced and alkylated using 10 mM DTT and 100 mM iodoacetamide, and digested with 100 ng of trypsin in 25 mM ammonium bicarbonate overnight at 37 °C. Peptides desalted using C18 zip tips (Merck Millipore) and eluted in 10 μl of MeCN/H_20 o.1% TFA (40:60 *v/v*).

MALDI mass spectrometry experiments

Spectra were recorded on a BrukerUltraflex II TOF/TOF, controlled by the FlexControl 3.0 software (BrukerDaltonics, Bremen, Germany). Desorption/ionization was performed using a frequency tripled Nd:YAG laser emitting at 355 nm with a 100-Hz shot frequency. All mass spectra were taken in the positive ion modes in the linear and reflectron mode. Experiments were performed using first the full range setting for laser firing position in order to select the optimal position for data collection, and second fixing the laser firing position in the sample sweet spots. The laser power was adjusted to obtain high S/N ratio while ensuring minimal fragmentation of the parent ions, and each mass spectrum was generated by averaging 100 laser pulses per spot. Spectra were obtained and analyzed with the programs FlexControl and FlexAnalysis, respectively. MTP 384 target plate steel TF was used (Part No.: 209519; target frame (# 74115); 384 circular spots, 3.5-mm diameter; S/N 03630).

Molecular modeling

The ground state geometry of CHCA, Z- and E-cinnamic acids were fully optimized without imposing any symmetry constraints by *ab initio* and semiempirical methods (Scheme S1A, (a) E-SA, (b) Z-SA and Scheme S1B, CHCA rotamers, in Electronic Supplementary Information). For *ab initio* density functional theory calculations, we used the hybrid gradient-corrected exchange functional combined with the gradient-corrected correlation functional, commonly known as B3LYP which has been shown to be quite reliable for geometries. For geometry optimization, the standardized 6-311G(d,p) basis set was used. We denote our B3LYP calculations by B3LYP/6-311G(d,p). For single point calculations, the standarized 6–311++G(d,p) basis set was used at B3LYP theory level (B3LYP/6-311++G(d,p)//B3LYP/6-311G(d,p). All the *ab initio* calculations were carried out, using Gaussian 98W program [22,23].

Conclusions

Taking into account that the MALDI-MS analysis of low molecular weight peptides and peptides in general faces some limitations because matrix clusters are quite abundant, the results here described show that Z-SA acid as MALDI matrix is a new tool of choice for direct, rapid and sensitive detection of polypeptides including hydrophobic and hydrophilic species.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web site.

ELECTRONIC SUPPLEMENTARY INFORMATION

APPLICATION OF Z-SINAPINIC MATRIX IN PEPTIDE MALDI-MS ANALYSIS

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Table S1- Polypeptides studied.

Abbreviation	**Peptide amino acid composition**	**MW**	**pI**	**Hydrophobicity**
BDK(1-7) [a]	H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-OH	756.8	9.75	Hydrophilic
BDK(1-8) [a]	H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-OH	904.0	9.75	Hydrophilic
ANG I [a]	H-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-OH	1296.4	6.92	Hydrophilic
ANG II [a]	H-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-OH	1046.1	6.74	Hydrophilic
FVG [b]	H-Phe-Val-Gly-NH_2	320.3	7.81	Hydrophobic
KSG [b]	H-Lys-Ser-Gly-NH_2	289.3	9.69	Hydrophilic
NSG [b]	H-Asn-Ser-Gly-NH_2	275.3	7,81	Hydrophilic
TSG [b]	H-Thr-Ser-Gly-NH_2	262.3	7.81	Hydrophilic
LIFVG [b]	H-Leu-Ile-Phe-Val-Gly-NH_2	546.7	7.81	Hydrophobic
NENSG [b]	H-Asn-Glu-Asn-Ser-Gly-NH_2	518.5	5.55	Hydrophilic
NYTSG [b]	H-Asn-Tyr-Thr-Ser-Gly-NH_2	539.5	7.79	Hydrophilic
NHKSG [b]	H-Asn-His-Lys-Ser-Gly-NH_2	540.6	9.72	Hydrophilic
QTNQNYTSG [b]	H-Gln-Thr-Asn-Gln-Asn-Tyr-Thr-Ser-Gly-NH_2	1011.5	7.79	Hydrophilic
FVFALIFVG [b]	H-Phe-Val-Phe-Ala-Leu-Ile-Phe-Val-Gly-NH_2	1011.3	7.81	Hydrophobic
DTSENENSG [b]	H-Asp-Thr-Ser-Glu-Asn-Glu-Asn-Ser-Gly-NH_2	950.9	3.73	Hydrophilic
KHNRNHKSG [b]	H-Lys-His-Asn-Arg-Asn-His-Lys-Ser-Gly-NH_2	1076.2	11.82	Hydrophilic
FKFRYTAHSGASG [b]	H-Phe-Lys-Phe-Arg-Tyr-Thr-Ala-His-Ser-Gly-Ala-Ser-Gly-NH_2	1427.6	10.45	Hydrophilic
RYTAHSGASG [c]	H-Arg-Tyr-Thr-Ala-His-Ser-Gly-Ala-Ser-Gly-NH_2	1005.1	9.36	Hydrophilic
TAHSGASG [c]	H-Thr-Ala-His-Ser-Gly-Ala-Ser-Gly-NH_2	685.7	7.87	Hydrophilic

[a] Commercial product from Sigma-Aldrich.: BDK(1-7), bradykinin (1-7); commercial kit for calibration 4700 Proteomics Analyzer Mass of Applied Biosystems. Applied Biosystems: BDK(1-8), bradykinin (1-8); ANG II, angiotensin II; ANG I, angiotensin I; stock solution of each peptide: BDK(1-7) 1.32 μmol/mL, BDK(1-8) 1.00 μmol/mL, ANG I 2.0 μmol/mL and ANG II 2.0 μmol/mL, in H_2O 0.1% TFA. [b] Peptides prepared by solid phase peptide synthesis. For details see experimental details in Ref [1]; Stock solution of each peptide: 6.3 μmol/mL in AcOH)/MeCN/H_2O 0.1% TFA (3:4:3 v/v/v). [c] Obtained by treatment of FKFRYTAHSGASG with chymotrypsin [2].

Synthesis and characterization of the Z-SA

Physical properties for characterization of *E*-SA and *Z*-SA, and *Z*-SA synthesis have been described elsewhere [3, 4, 5]. Here it is important to note that the m.p. of the *E*-isomer is higher than that of the *Z*-isomer: *E*-SA 185 ^{O}C and *Z*-SA 116-118 ^{O}C [3, 4, 5].

NMR Analysis. The ^{1}H-NMR spectra were recorded at 200 MHz on a Bruker AC-200 spectrometer in DMSO solution. ^{1}H spectra were carried out on a Bruker AVANCE II 500 NMR spectrometer operating at 500.14 and 125.76 MHz for ^{1}H and ^{13}C, using DMSO-d6 ($(CD_3)_2SO$) as solvent. Tetramethylsilane was used as the internal standard. Chemical shift values are reported in ppm and the coupling constants are given in Hertz.

Z- (cis-) and E (trans)-3-phenyl-2-propenoic (cinnamic) acid (C_6H_5CH=$CHCOOH$) have different $J_{H,H}$ values for the coupling between the protons attached to the double bond carbons. The coupling constant of *Z* protons is generally smaller (7-12 Hz) than that of *E* protons (13-30 Hz).

***Z*-SA (Z-4-Hydroxy-3,5-dimethoxycinnamic acid; Z-sinapinic acid)** [3].

m.p.; ^{13}C NMR; UV-vis absorption spectrum; EI MS (70 eV); HR-ESI-MS negative ion mode; HRESI-MS MS/MS negative ion mode were described elsewhere [3, 4, 5].

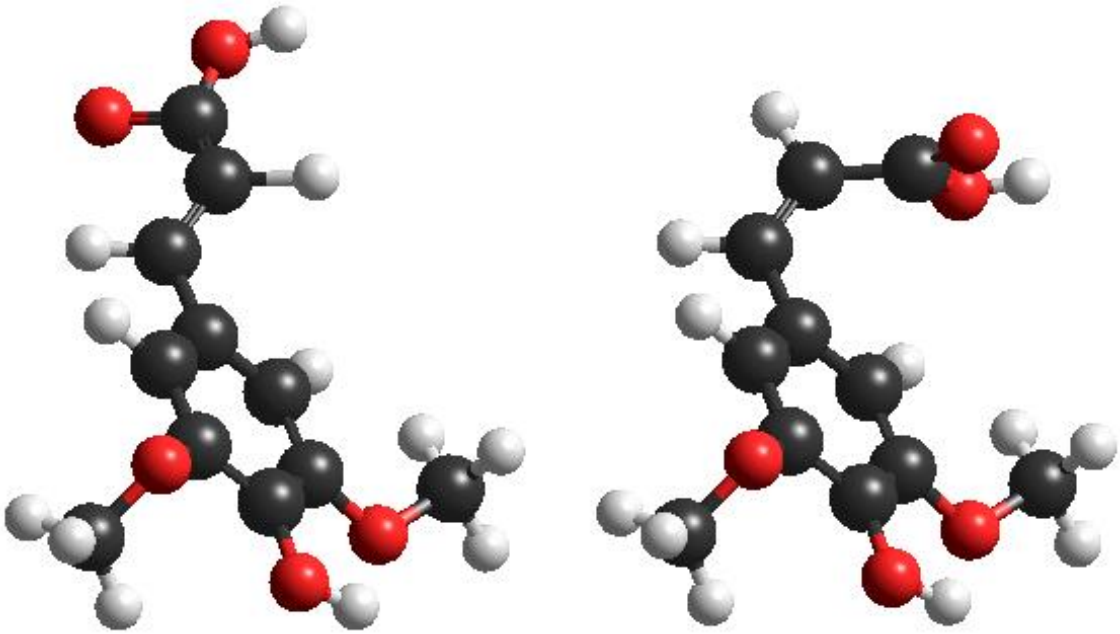
^{1}H NMR (200 MHz, $(CD_3)_2SO$) δ 3.75 (s, 6H, OCH_3), 5.76 (d, J = 12.8 Hz, 1H, Hα), 6.76 (d, J = 12.8 Hz, 1H, Hβ), 7.23 (s, $HC_{2\text{-}6}$), 8.88 (s, 1H, OH).

***E*-SA (E-4-Hydroxy-3,5-dimethoxycinnamic acid; E-sinapinic acid)** [Sigama-Aldrich].

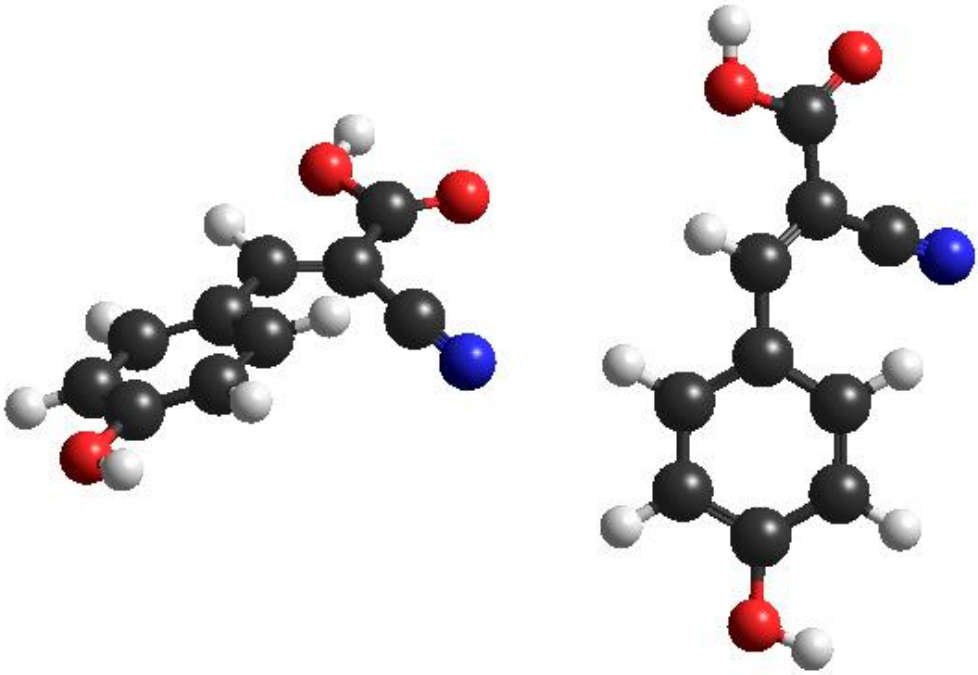
m.p.; ^{13}C-NMR; UV-vis absorption spectrum; EI-MS (70 eV); HRESI-MS negative ion mode; HRESI-MS MS/MS negative ion mode were described elsewhere [3, 4, 5].

^{1}H NMR (200 MHz, $(CD_3)_2SO$) δ: 3.80 (s, 6H, OCH_3), 6.42 (d, J = 15.8 Hz, 1H, Hα), 6.99 (s, 2H, HC(2) and HC(6)), 7.50 (d, J = 16 Hz, 1H, Hβ).

Samples for MALDI mass spectrometry analysis were prepared according to method previously described [6].



Scheme S1A. Molecular structure of E-SA (left) and Z-SA (right). See details of molecular modeling in Molecular Modeling section [7, 8].



Scheme 1B. Molecular structure of CHCA. Two views of the same rotamer. See details of molecular modeling in Molecular Modeling section [7, 8].

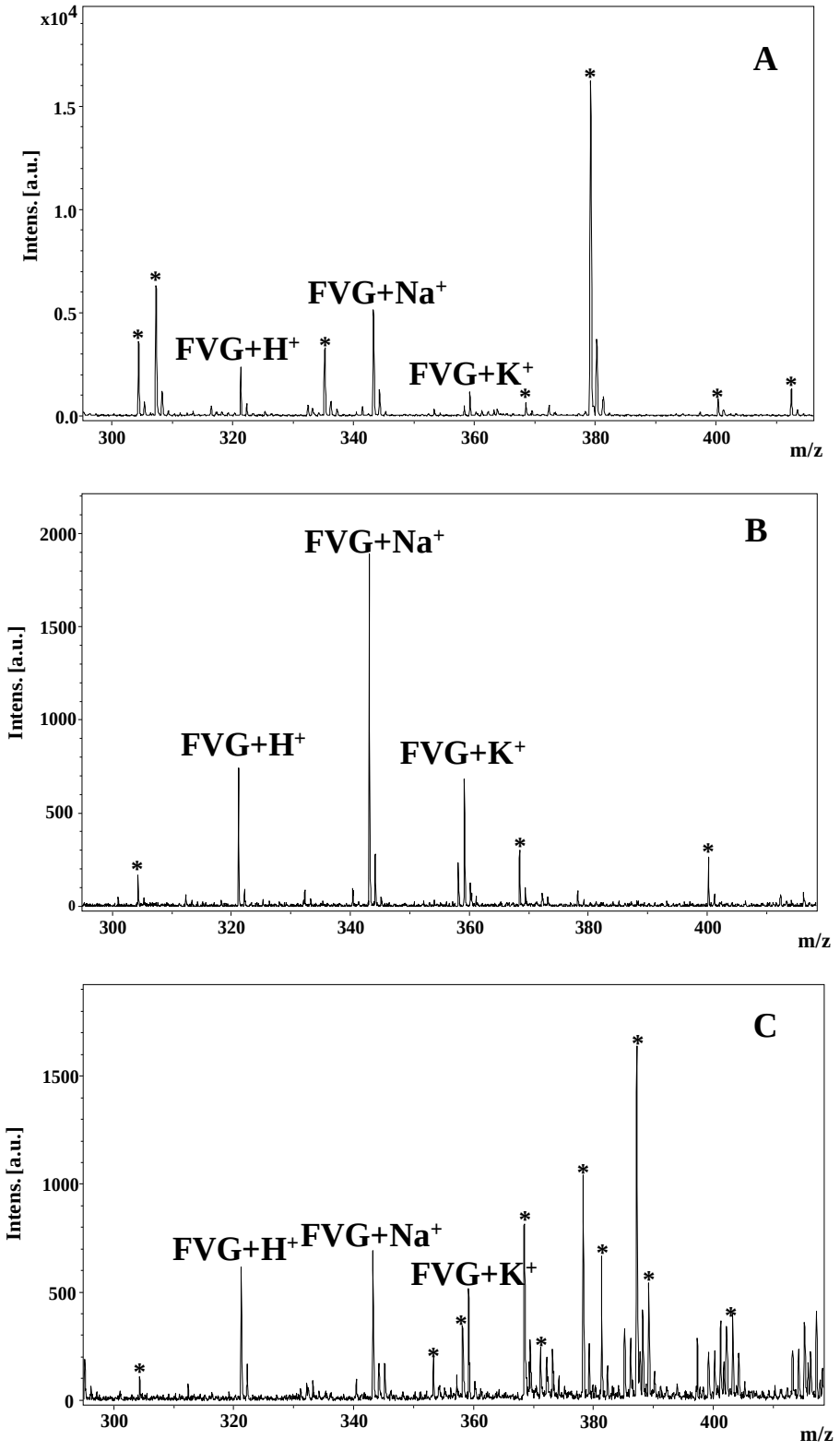


Figure S1. UV-MALDI mass spectra of peptide FVG (H-Phe-Val-Gly-NH_2, MW 320.3), Positive ion mode. Matrix: A) CHCA, B) Z-SA and C) E-SA. (*) matrix clusters.

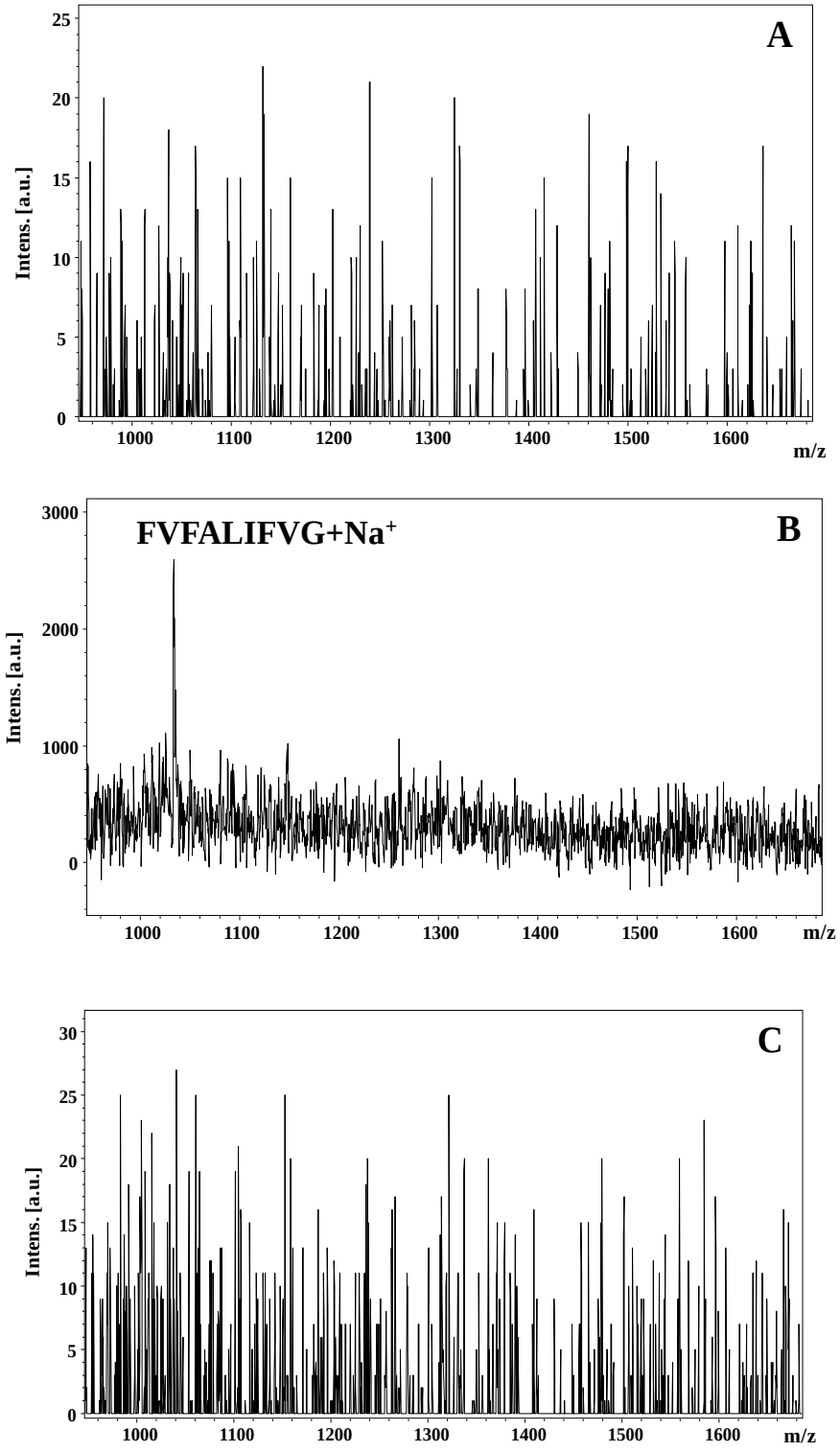


Figure S2. UV-MALDI mass spectra of peptide FVFALIFVG (H-Phe-Val-Phe-Ala-Leu-Ile-Phe-Val-Gly-NH_2, MW 1011.3), Positive ion mode. Matrix: A) CHCA, B) Z-SA and C) E-SA. (*) matrix clusters.

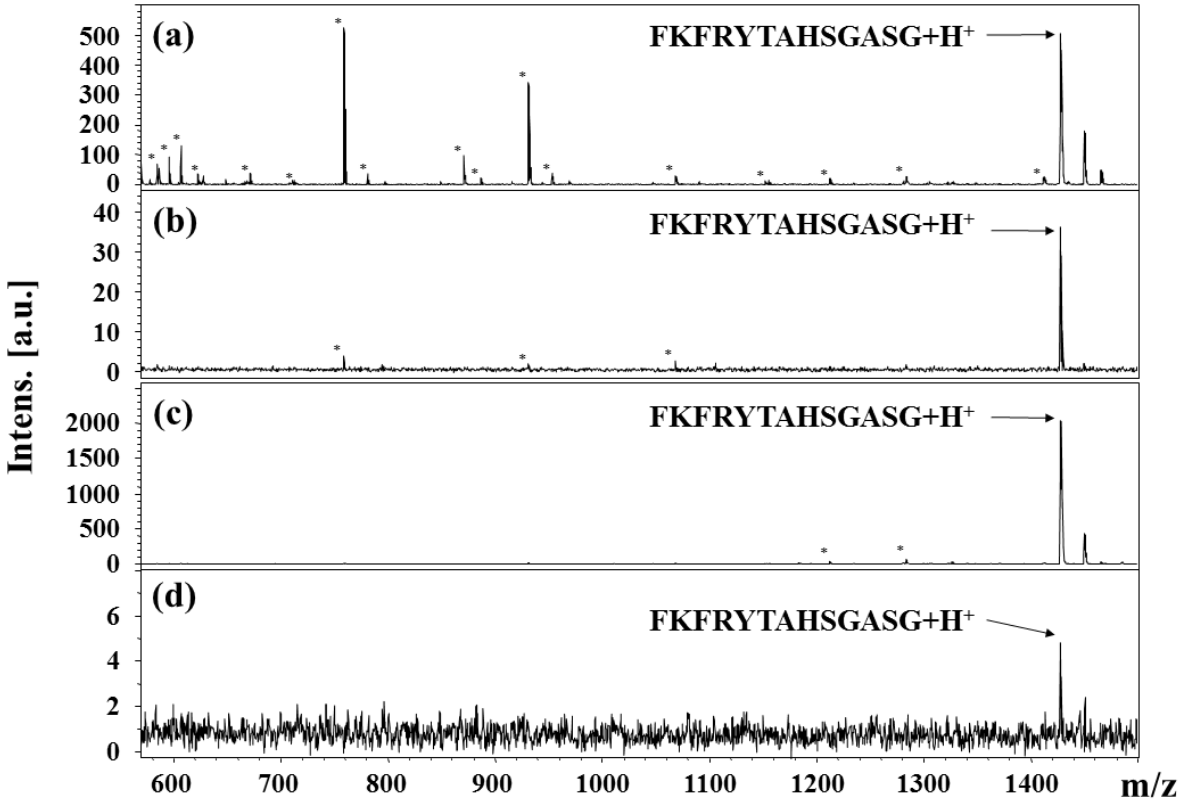


Figure S3. MALDI mass spectra of peptide FKFRYTAHSGASG (H-Phe-Lys-Phe-Arg-Tyr-Thr-Ala-His-Ser-Gly-Ala-Ser-Gly-NH_2, MW 1427.6). Positive ion mode. Matrix: (a) CHCA, (b) Z-SA, (c) E-SA and (d) DHBA. (*) Matrix clusters.

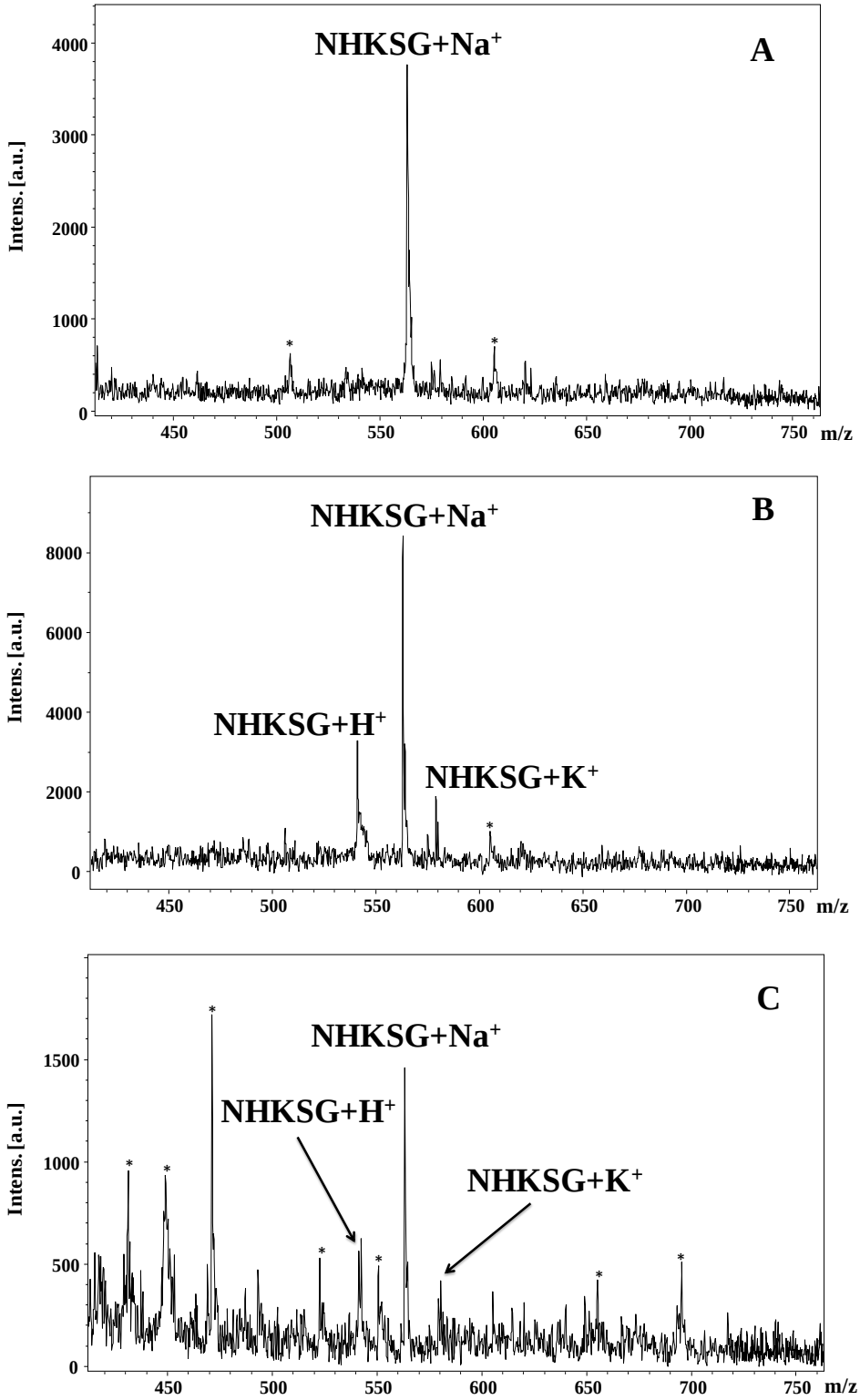


Figure S4. MALDI mass spectra of peptide NHKSG (H-Asn-His-Lys-Ser-Gly-NH_2, MW 540.6).Positive ion mode. Matrix: A) CHCA, B) Z-SA and C) E-SA. (*) matrix clusters.

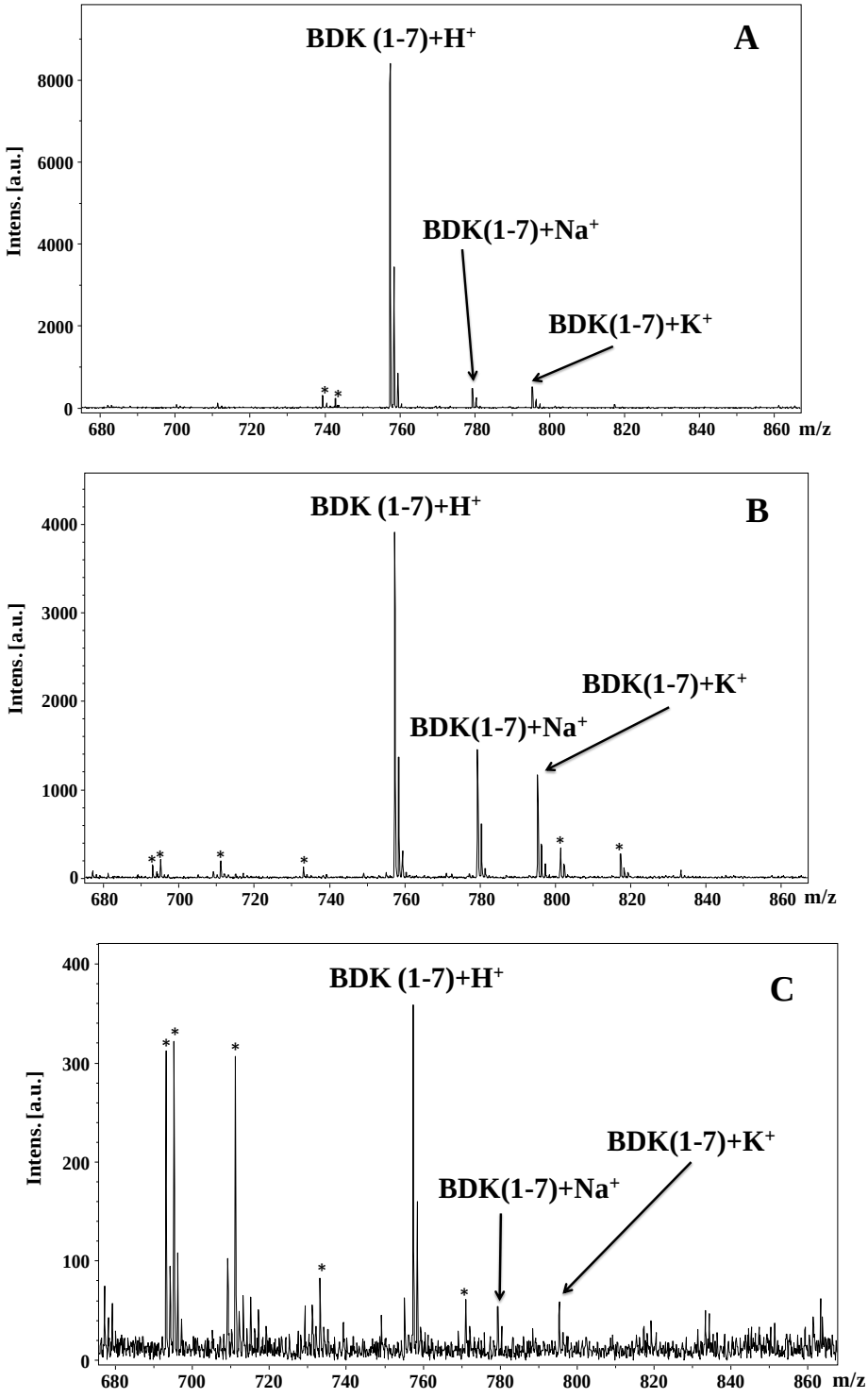


Figure S5. MALDI mass spectra of BDK(1-7) (H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-OH, MW 756.8) 5 pmol/μL in H_2O 0.1% TFA, monitored as $[M+H]^+$, $[M+K]^+$ and $[M+Na]^+$. Matrix (5 mg/mL in MeCN/H_2O 0.1% TFA (1:1)): (A) CHCA; (B) Z-SA and (C) E-SA.

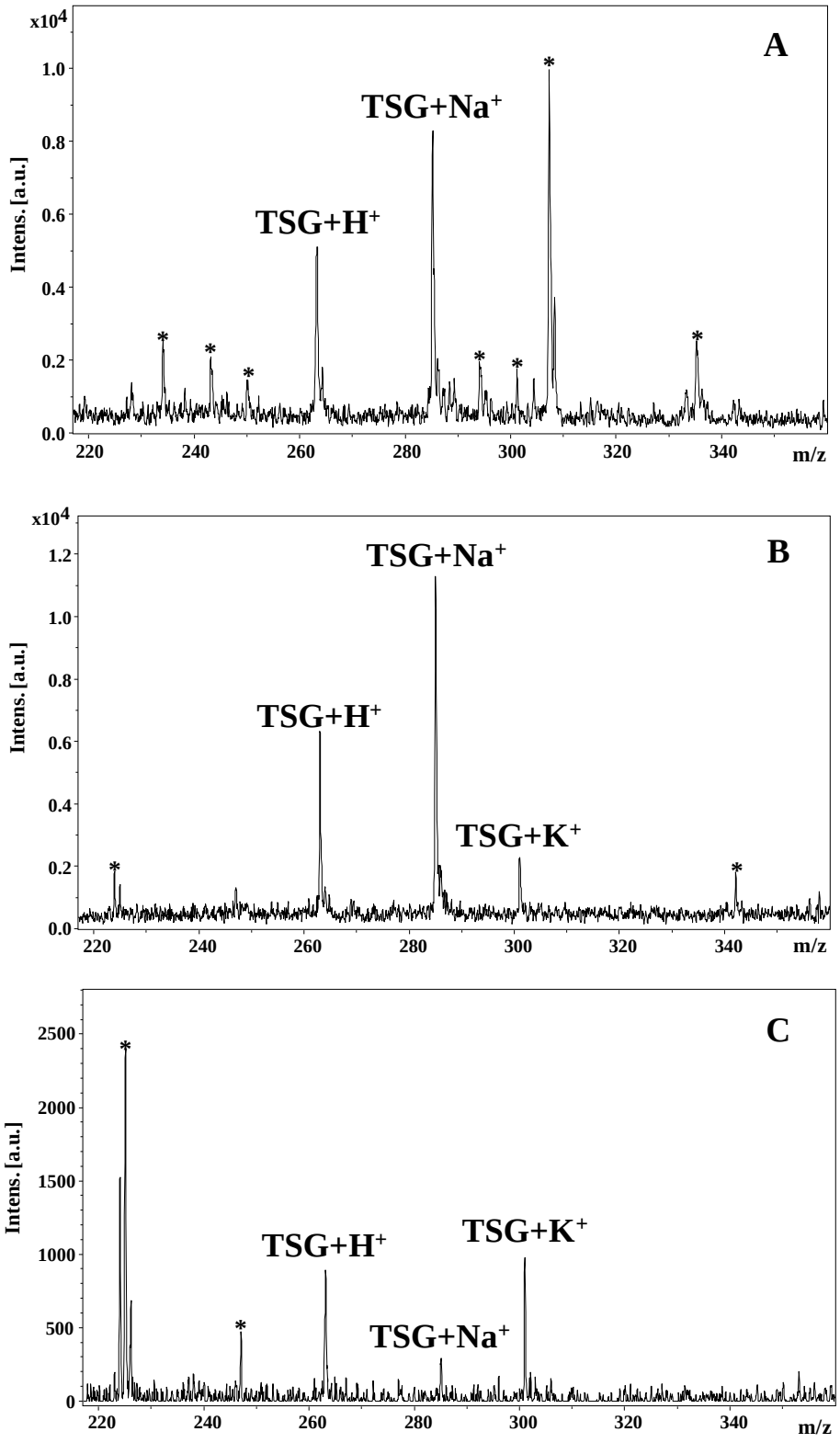


Figure S6. UV-MALDI mass spectra of peptide TGG (H-Thr-Ser-Gly-NH_2, MW 262.3). Positive ion mode. Matrix: A) CHCA, B) Z-SA and C) E-SA. (*) matrix clusters.

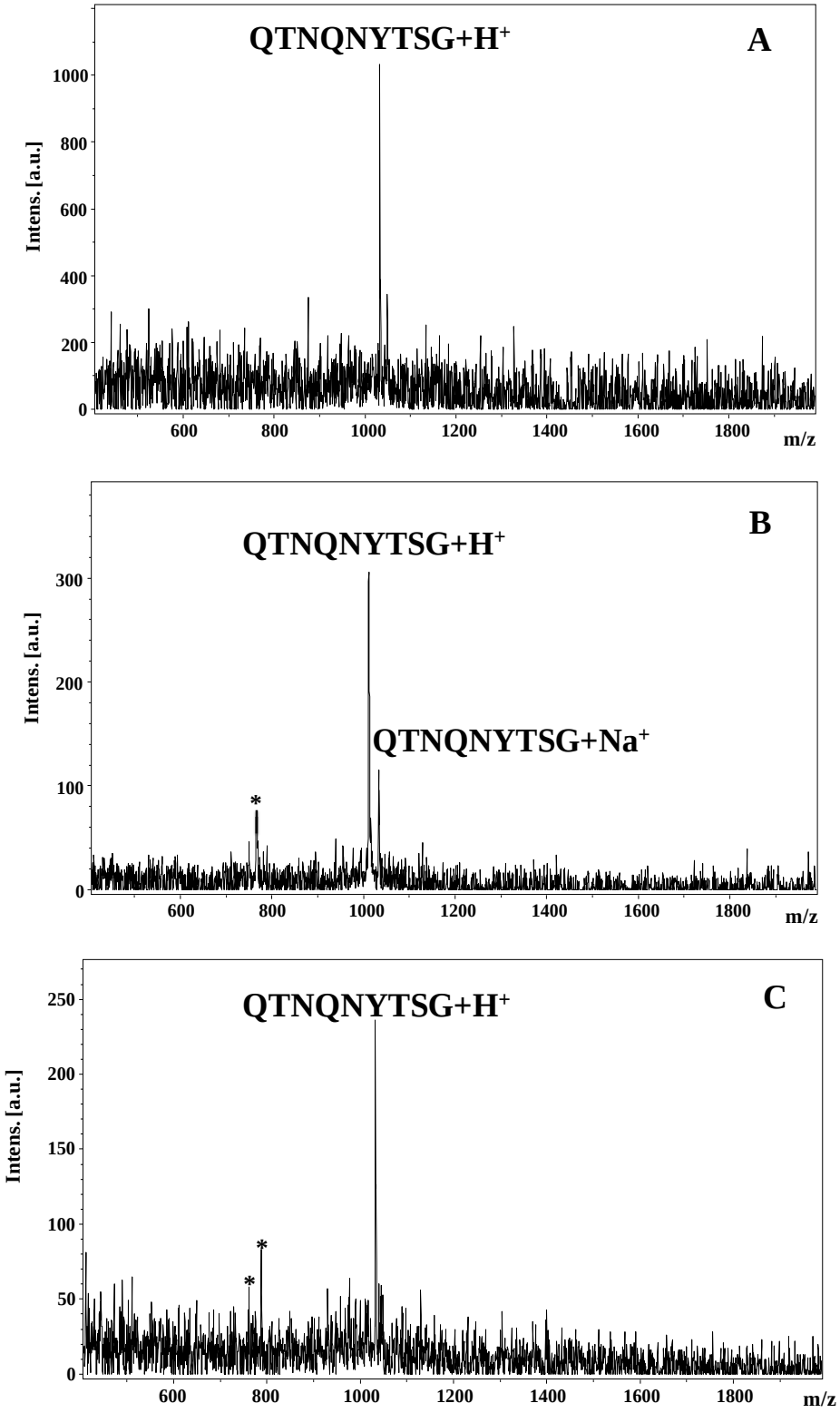


Figura S7. UV-MALDI mass spectra of peptide (QTNQNYTSG (H-Gln-Thr-Asn-Gln-Asn-Tyr-Thr-Ser-Gly-NH_2, **MW 1011.5)**. Positive ion mode. Matrix: A) CHCA, B) Z-SA and C) E-SA. (*) matrix clusters.

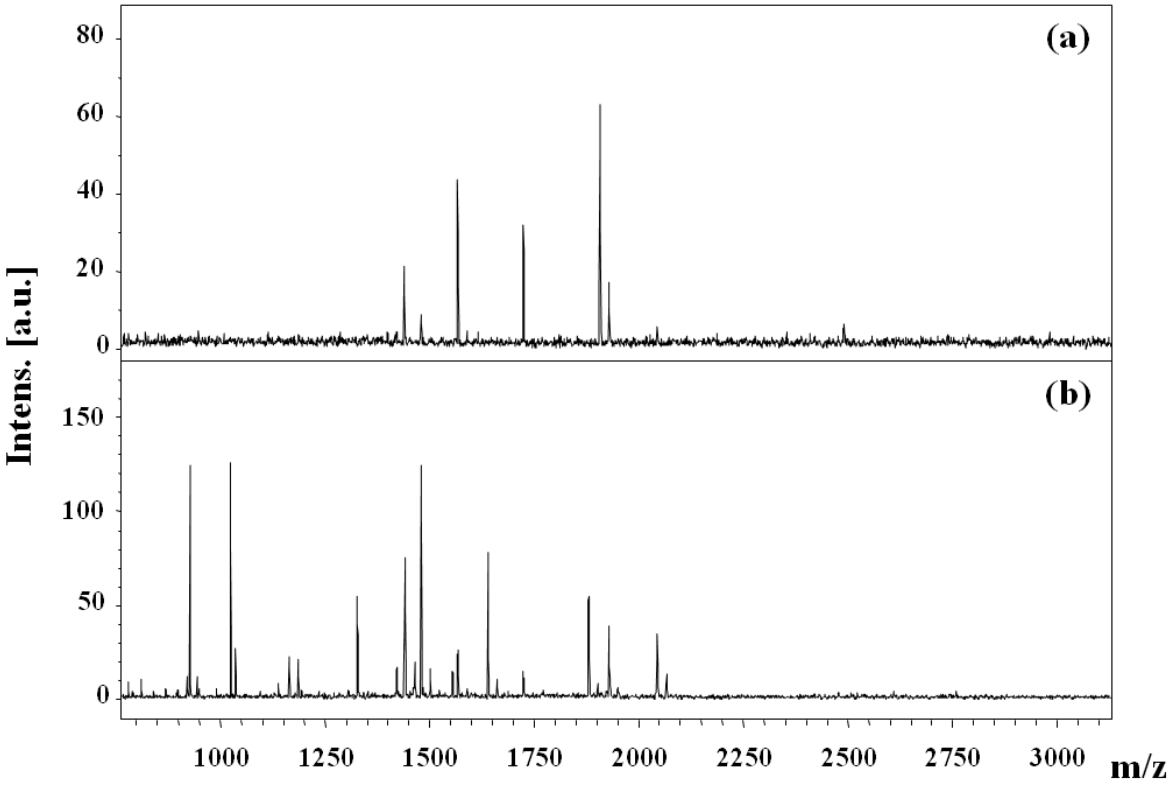


Figura S8. UV-MALDI mass spectra of BSA trypsin digested. Positive ion mode. Matrix: (a) E-SA and (b) E-SA. See m/z values and fragments structure assignment in Table S2

Table S2. Z-SA and E-SA as MALDI matrix for BSA trypsin digested. Peptides observed in the m/z 830 to 2610 region.

m/z $[M+H]^+$ (calculated)	Z-SA	E-SA	Peptide structure assigned [a]
839.3022	+		(K)C(Carbamidomethyl)C(Carbamidomethyl)AADDK(E)
927.4934			(K)YLYEIAR(R)
1001.5891	+ [b]		(R)ALKAWSVAR(L)
1138.4980	+		(K)C(Carbamidomethyl)C(Carbamidomethyl)TESLVNR(R)
1163.6307	+		(K)LVNELTEFAK(T)
1193.6022			(R)DTHKSEIAHR(F)
1249.6212			(R)FKDLGEEHFK(G)
1308.7270			(K)HKPKATEEQLK(T)
1419.6937	+		(K)SLHTLFGDELC(Carbamidomethyl)K(V)
1439.8118	+	+	(R)RHPEYAVSVLLR(L)
1443.6420			(K)YIC(Carbamidomethyl)DNQDTISSK(L)
1466.7090	+		(K)VTKC(Carbamidomethyl)C(Carbamidomethyl)TESLVNR(R)
1479.7954	+	+	(K)LGEYGFQNALIVR(Y)
1502.6138	+		(K)EYEATLEEC(Carbamidomethyl)C(Carbamidomethyl)AK(D)
1554.6529	+		(K)DDPHAC(Carbamidomethyl)YSTVFDK(L)
1567.7427	+	+	(K)DAFLGSFLYEYSR(R)
1639.9377	+		(R)KVPQVSTPTLVEVSR(S)
1724.8346	+	+	(R)MPC(Carbamidomethyl)TEDYLSLILNR(L)
1747.7050		+	(K)YNGVFQEC(Carbamidomethyl)C(Carbamidomethyl)QAED K(G)
1880.9211	+		(R)RPC(Carbamidomethyl)FSALTPDETYVPK(A)
1907.9208	+ [c]	+ [c]	(K)LFTFHADIC(Carbamidomethyl)TLPDTEK(Q)
2045.0280	+	+	(R)RHPYFYAPELLYYANK(Y)
2199.1002			(K)ATEEQLKTVMENFVAFVDK(C)
2487.1101			(K)YNGVFQEC(Carbamidomethyl)C(Carbamidomethyl) QAEDKGAC(Carbamidomethyl)LLPK(I)
2492.2642		+	(K)GLVLIAFSQYLQQC(Carbamidomethyl)PFDEHVK(L)
2608.2018			(K)LVNELTEFAKTC(Carbamidomethyl)VADES HAGC(Carbamidomethyl)EK(S)

[a] Ref [9], [10] and http://www.ionsource.com/Card/protein/BovineSerumAlbumin.htm
[b] Observed as $[M+H]^+$ and $[M+Na]^+$.
[c] Observed as $[M+H]^+$ and $[M+K]^+$.

Dynamic range and limit of detection (LOD)

Table S3. Dynamic range and LOD of FVG, KSG, BDK(1-7), BDK(1-8), ANG I and ANG II in the positive ion mode using Z-SA, E-SA and CHCA as matrices.[a]

	Z-SA		E-SA		CHCA	
	Dynamic range	LOD	Dynamic range	LOD	Dynamic range	LOD
FVG[b]	31.5-6300	31.5	42.0-6300	42.0	31.5-6300	31.5[c]
KSG[b]	31.5-6300	31.5	63-6300	63.0	31.5-6300	31.5 [c]
BDK(1-7)[b]	1.9-1320	1.9	8.8-1320	8.8 [c]	1.9-1320	1.9 [c]
BDK(1-8)[b]	20-1000	20	6-1000	6	6-1000	6[c]
ANG I[b]	40-2000	40	12-2000	12	4-2000	4[c]
ANG IIc [b]	40-2000	40	20-2000	20	10-2000	10[c]

[a]indicated as pmol of peptide deposited on the probe; S/N ≥ 4; E-SA and CHCA commercial products, Z-SA prepared according to Ref [3]. [b]Positive ion mode. Monitoring ions $[M + H]^+$, $[M + Na]^+$ and $[M + K]^+$. [c]Signal detected among cluster with higher intensity signal (clusters show higher and lower m/z than the peptide).

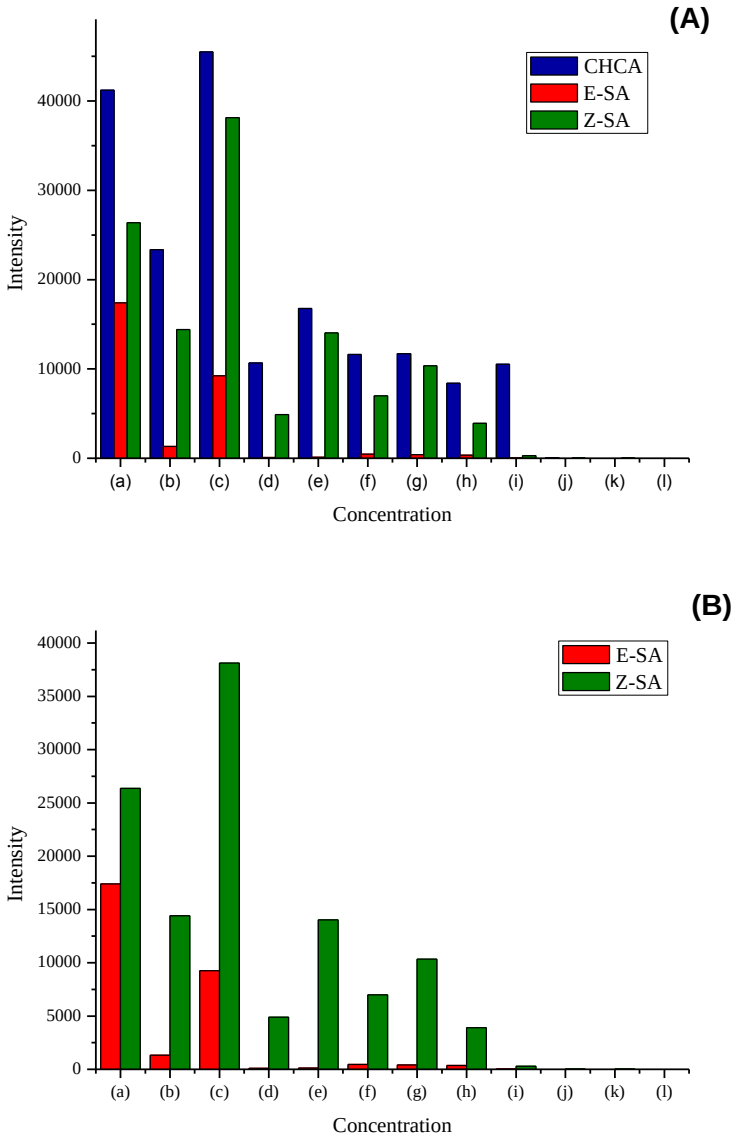


Figure S9. Signal intensity vs concentration. Analyte: BDK(1-7) in H_2O 0.1% TFA. Matrix: CHCA (blue), E-SA (red) and Z-SA (green), 5 mg/mL in MeCN:H_2O 0.1% TFA 1:1 v/v. Dilutions with H_2O 0.1% TFA. Measurements on: (a): original solution, 1.32 µmol/mL; Dilutions (v/v): (b) 1/5, (c) 1/10, (d) 1/20, (e) 1/50, (f) 1/100, (g) 1/150, (h) 1/200, (i) 1/500, (j) 1/700 and (k) 1/1000**.** (A) Including the three matrices; (B) Including only E-SA and Z-SA.

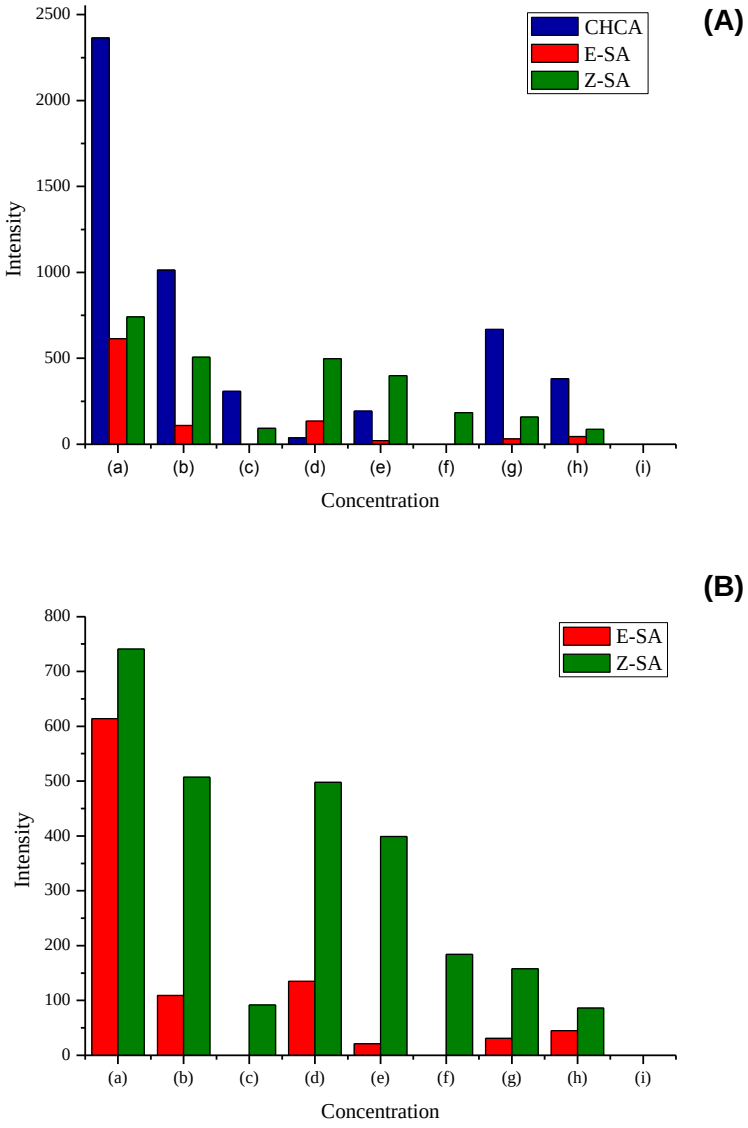


Figure S10. Signal intensity vs concentration. Analyte: FVG in AcOH:MeCN:H_2O 0.1% TFA, 3:4:3 v/v/v. Matrix: CHCA (blue), E-SA (red) and Z-SA (green), 5 mg/mL in MeCN:H_2O 0.1% TFA 1:1 v/v. Dilutions with AcOH:MeCN:H_2O 0.1% TFA, 3:4:3 v/v/v. Measurements on: (a): original solution, 6.3 μmol/mL; Dilutions (v/v): (b) 1/5, (c) 1/10, (d) 1/20, (e) 1/50, (f) 1/100, (g) 1/150, (h) 1/200 and (i) 1/500. (A) Including the three matrices; (B) Including only E-SA and Z-SA.

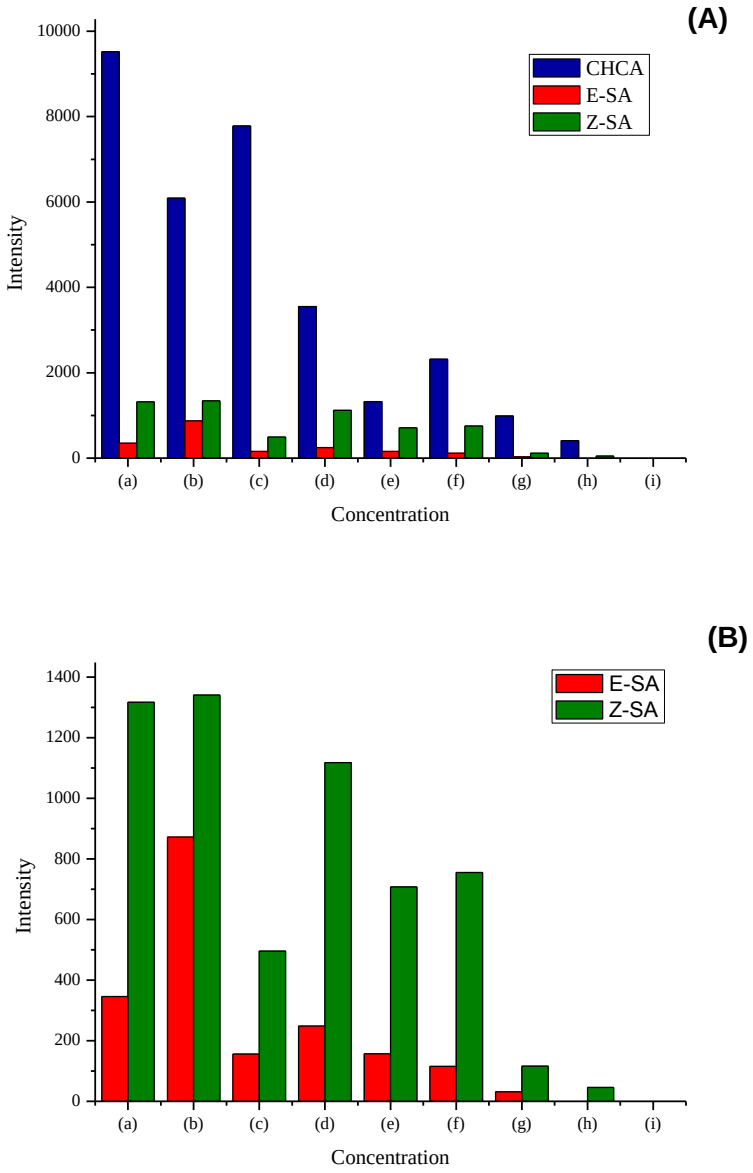


Figure S11. Signal intensity vs concentration. Analyte: KSG in AcOH:MeCN:H_2O 0.1% TFA, 3:4:3 v/v/v. Matrix: CHCA (blue), E-SA (red) and Z-SA (green), 5 mg/mL in MeCN:H_2O 0.1% TFA 1:1 v/v. Dilutions with AcOH:MeCN:H_2O 0.1% TFA, 3:4:3 v/v/v. Measurements on: (a): original solution, 6.3 μmol/mL; Dilutions (v/v): (b) 1/5, (c) 1/10, (d) 1/20, (e) 1/50, (f) 1/100, (g) 1/150, (h) 1/200 and (i) 1/500. (A) Including the three matrices; (B) Including only E-SA and Z-SA.

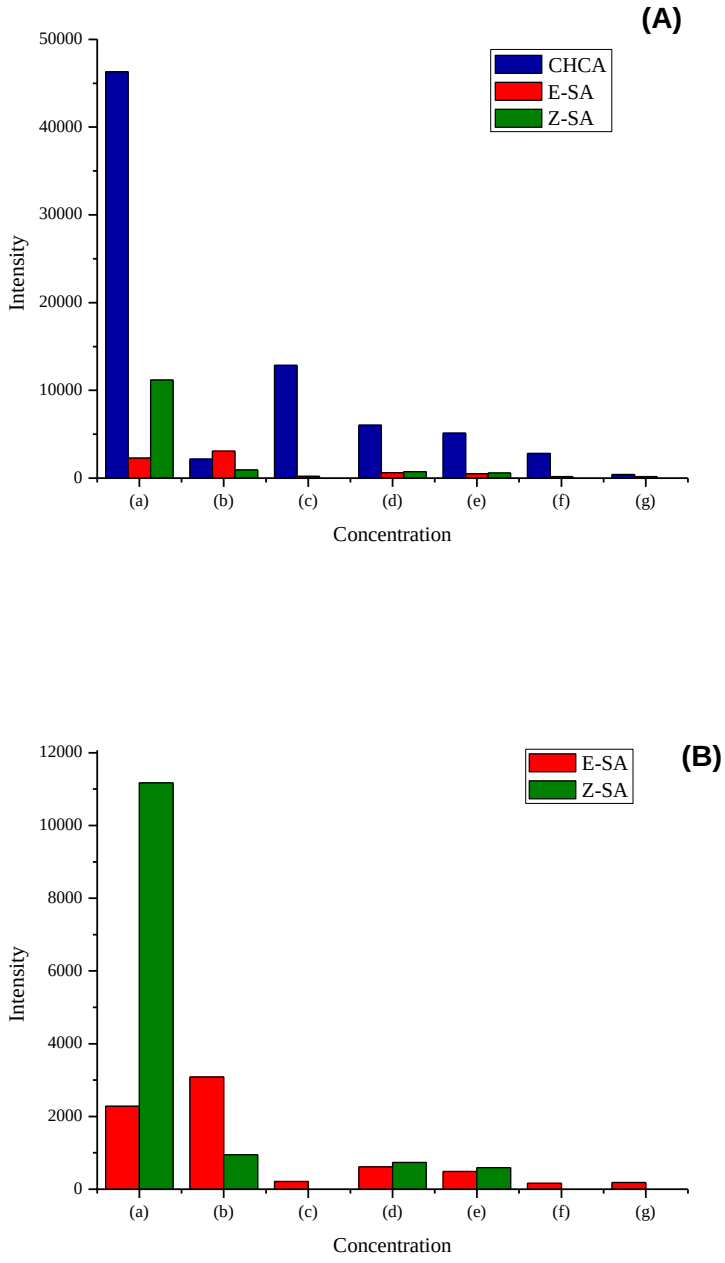


Figure S12. Signal intensity vs concentration. Analyte: BDK(1-8) in H_2O 0.1% TFA. Matrix: CHCA (blue), E-SA (red) and Z-SA (green), 5 mg/mL in MeCN:H_2O 0.1% TFA 1:1 v/v. Dilutions with H_2O 0.1% TFA. Measurements on: (a): original solution, 1 μmol/mL; Dilutions (v/v): (b) 1/5, (c) 1/10, (d) 1/20, (e) 1/50, (f) 1/100, (g) 1/150, (h) 1/200 and (i) 1/500. (A) Including the three matrices; (B) Including only E-SA and Z-SA.

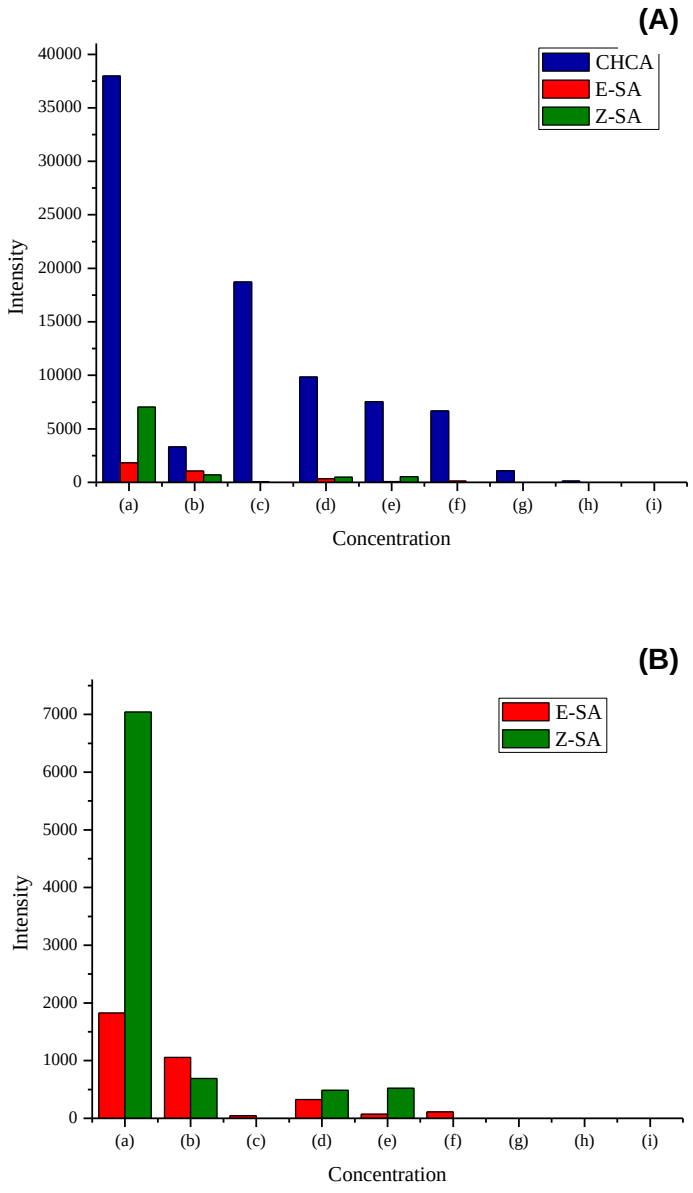


Figure S13. Signal intensity vs concentration. Analyte: ANG II in H_2O 0.1% TFA. Matrix: CHCA (blue), E-SA (red) and Z-SA (green), 5 mg/mL in MeCN:H_2O 0.1% TFA 1:1 v/v. Dilutions with H_2O 0.1% TFA. Measurements on: (a): original solution, 2 μmol/mL; Dilutions (v/v): (b) 1/5, (c) 1/10, (d) 1/20, (e) 1/50, (f) 1/100, (g) 1/150, (h) 1/200 and (i) 1/500. (A) Including the three matrices; (B) Including only E-SA and Z-SA.

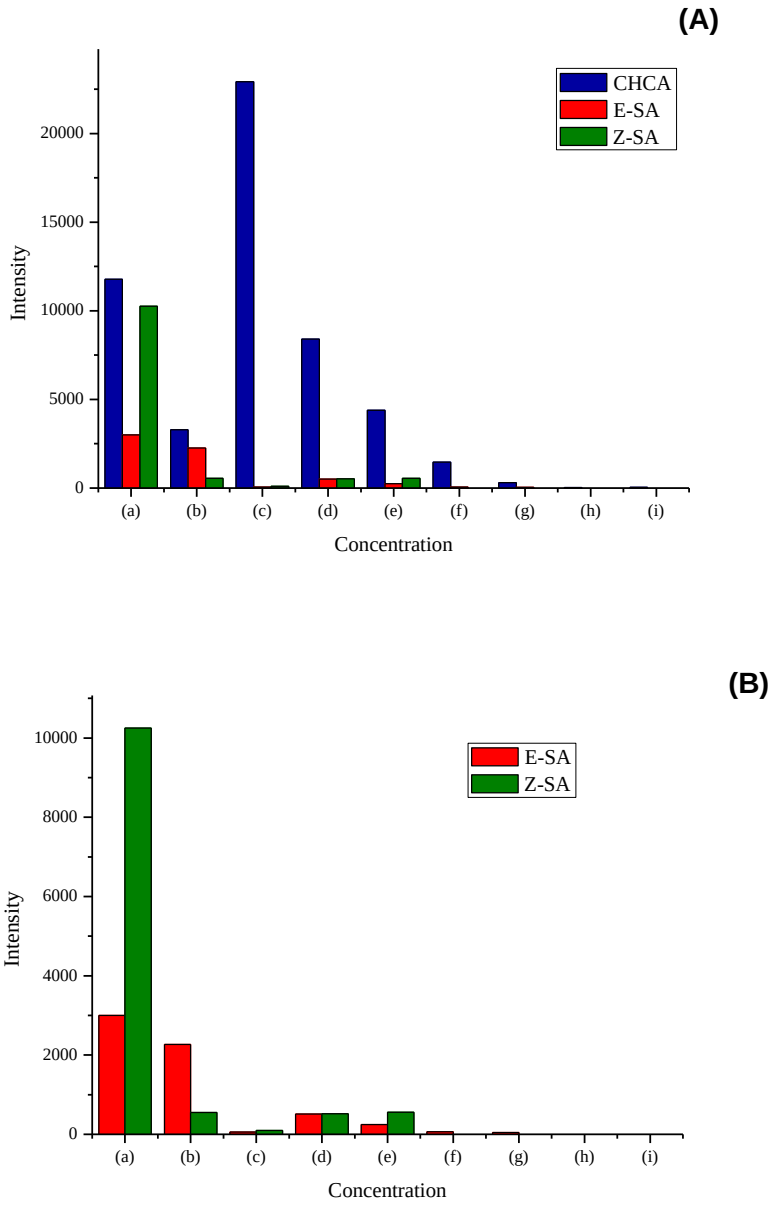


Figure S14. Signal intensity vs concentration. Analyte: ANG I in H_2O 0.1% TFA. Matrix: CHCA (blue), E-SA (red) and Z-SA (green), 5 mg/mL in MeCN:H_2O 0.1% TFA 1:1 v/v. Dilutions with H_2O 0.1% TFA. Measurements on: (a): original solution, 2 μmol/mL; Dilutions (v/v): (b) 1/5, (c) 1/10, (d) 1/20, (e) 1/50, (f) 1/100, (g) 1/150, (h) 1/200 and (i) 1/500. (A) Including the three matrices; (B) Including only E-SA and Z-SA.

Morphological inspection of solid samples

For morphological inspection of the solid matrix deposited on the probe the dried droplet method was used to prepare the sample. Fresh matrix solution (1 μL; matrix solution 5 mg/mL in MeCN:H_2O 0.1% TFA 1:1 v:v) was loaded on to the probe and air dried. As a result, CHCA and E-SA gave small crystals distributed at random, as aggregates, all over the sample surface and *Z*-SA showed the aspect of a solid solution (glass) with few tinny heterogeneous round spots; on the contrary the CHCA yielded a solid deposit with heterogeneous aspect (Figs. S15, S17 and S19 respectively). The corresponding laser desorption ionization (LDI) mass spectrum of each matrix showed, abundant and high intensity clusters signals for CHCA and E-SA (Figs. S15 and S17) while for Z-SA the intensity of cluster signals were quite lower although they were quite abundant (Fig. S19). Doping with serine (serine solution, 1 M in H_2O; matrix to serine 4:1 vol/vol) although changed dramatically the physical aspect of the solid sample deposit (Figs. S15-S20), did not significantly improve the results. For both E-SA and Z-SA did not diminish the presence of cluster signals at m/z < 500 (Figs. S16 and S18) and no significant effect was observed on Z-SA LDI mass spectrum (Fig. S20). When solid analyte-matrix sample were prepared on the probe with CHCA, *E*-SA and *Z*-SA, the morphological aspect of each sample was similar to that of the matrix alone (image not shown) and doping with serine did not showed any significant effect on the spectra quality.

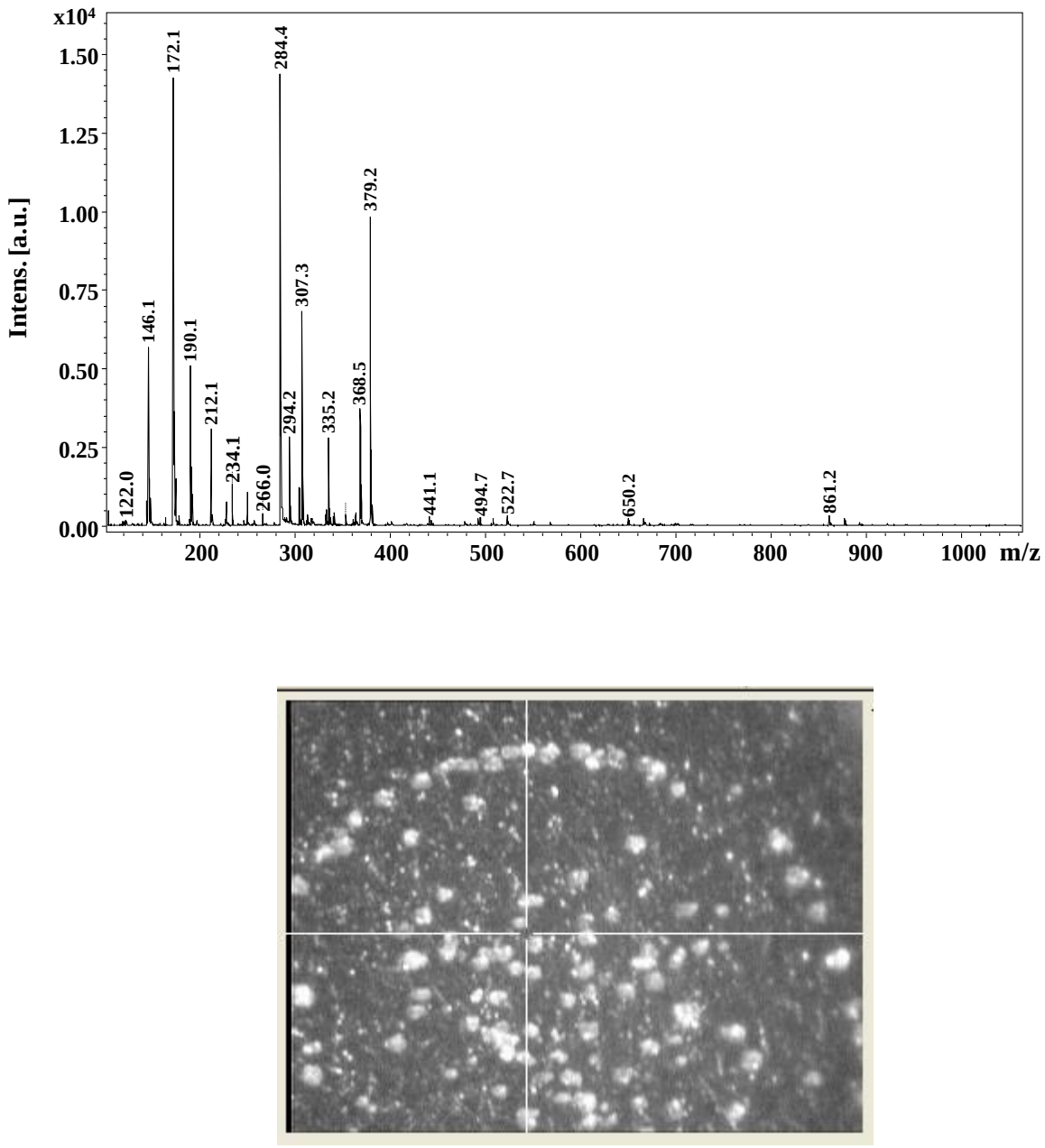


Figure S15. (top) LDI mass spectrum of CHCA (5 mg/mL in MeCN:H_2O 0.1% TFA 1:1 v:v) and (bottom) morphologycal inspection of the solid CHCA deposited. Circular spot surface, 9.62 mm^2. Digital images from Bruker Ultraflex II TOF/TOF.

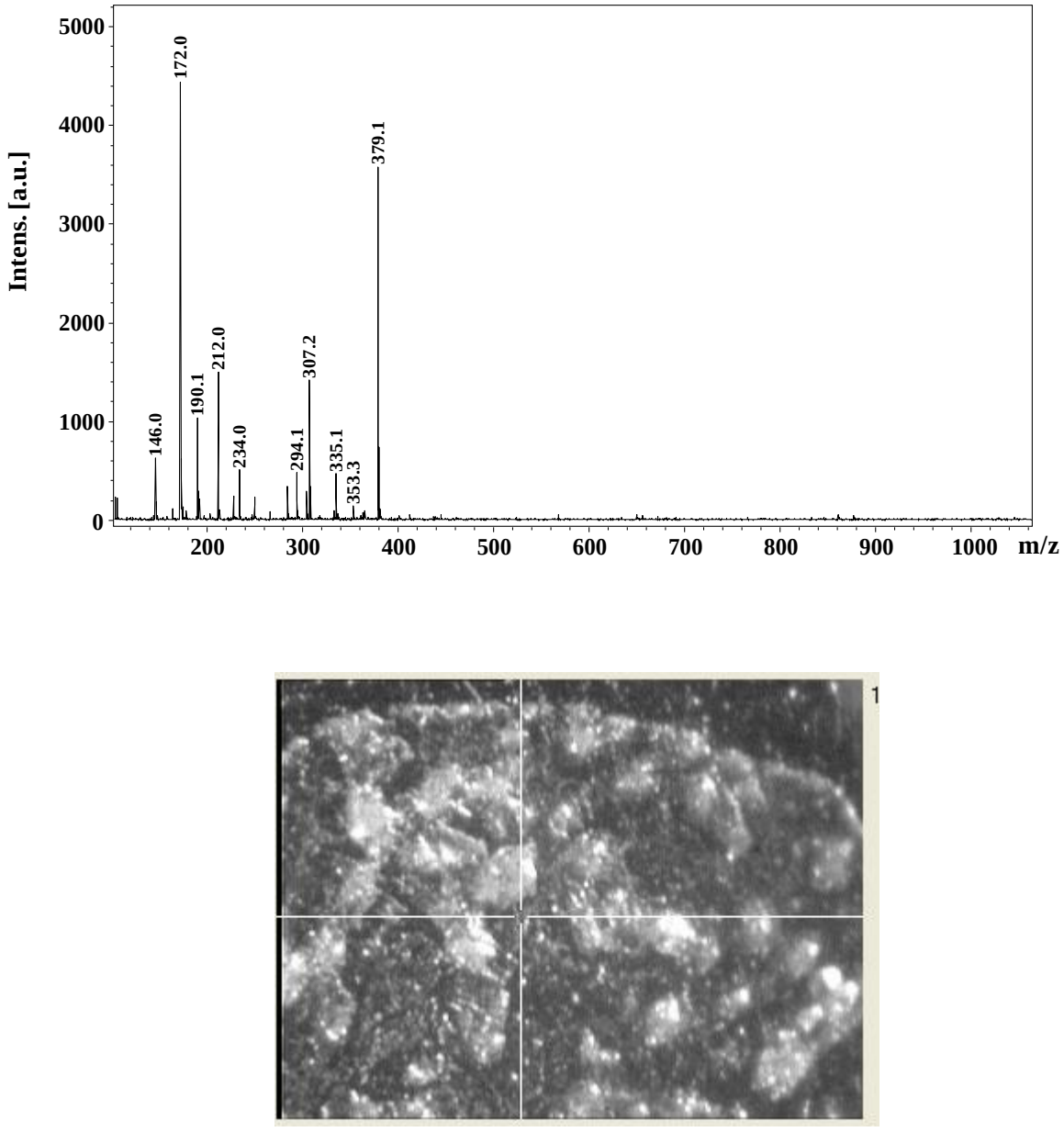


Figure S16. (top) LDI mass spectrum of CHCA + serine (4:1 vol/vol) (CHCA, 5 mg/mL in MeCN:H_2O 0.1% TFA 1:1 v:v; serine 1M in H_2O) and (bottom) morphologycal inspection of the solid CHCA + serine deposited. Circular spot surface, 9.62 mm^2. Digital images from Bruker Ultraflex II TOF/TOF.

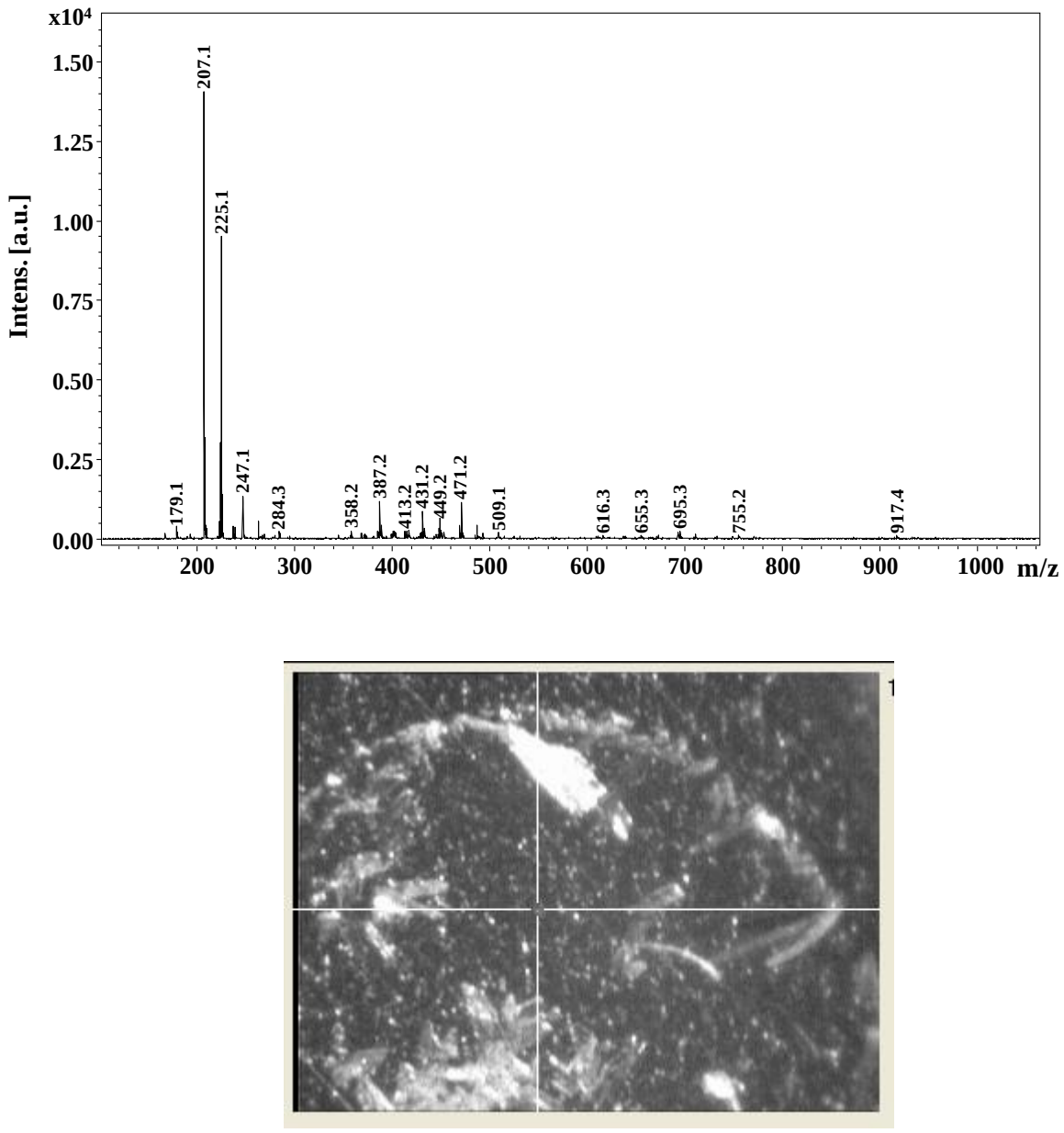


Figure S17. (top) LDI mass spectrum of E-SA (5 mg/mL in MeCN:H_2O 0.1% TFA 1:1 v:v) and (bottom) morphologycal inspection of the solid E-SA deposited. Circular spot surface, 9.62 mm^2. Digital images from Bruker Ultraflex II TOF/TOF.

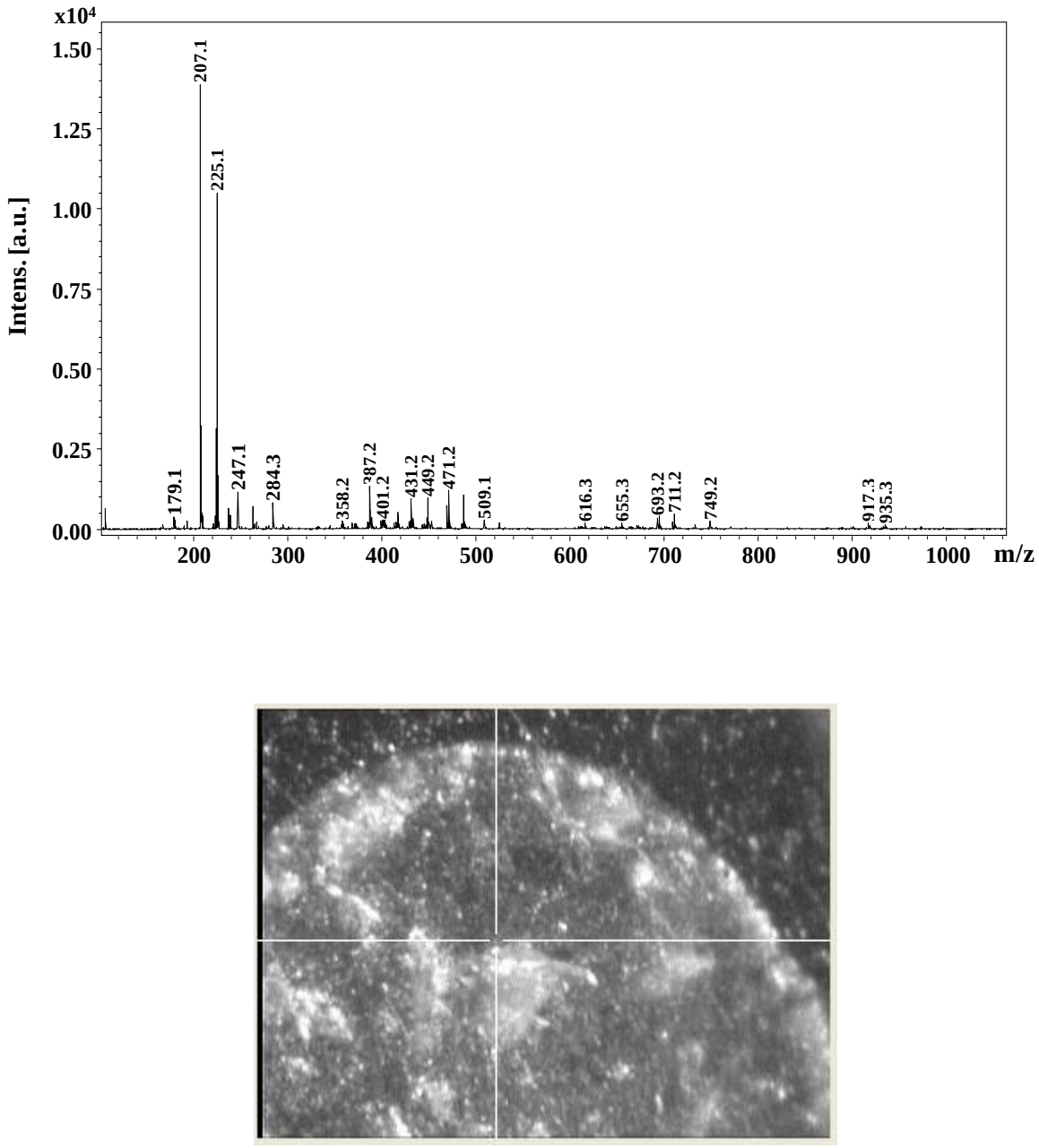


Figure S18. (top) LDI mass spectrum of E-SA + serine (4:1 vol/vol) (E-SA, 5 mg/mL in MeCN:H_2O 0.1% TFA 1:1 v:v; serine 1M in H_2O) and (bottom) morphologycal inspection of the solid E-SA + serine deposited. Circular spot surface, 9.62 mm^2. Digital images from Bruker Ultraflex II TOF/TOF.

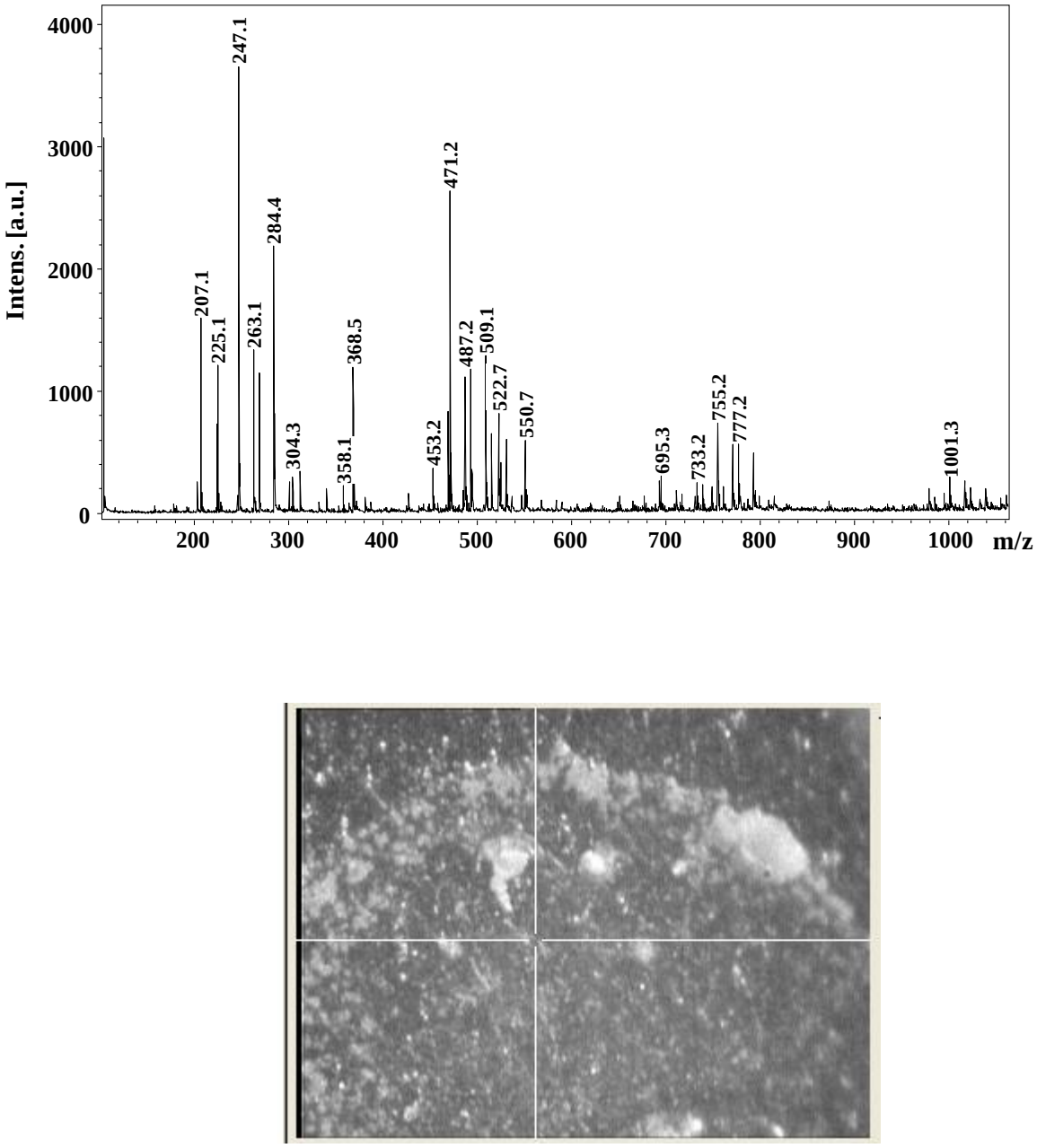


Figure S19. (top) LDI mass spectrum of Z-SA (5 mg/mL in MeCN:H_2O 0.1% TFA 1:1 v:v) and (bottom) morphologycal inspection of the solid Z-SA deposited. Circular spot surface, 9.62 mm^2. Digital images from Bruker Ultraflex II TOF/TOF.

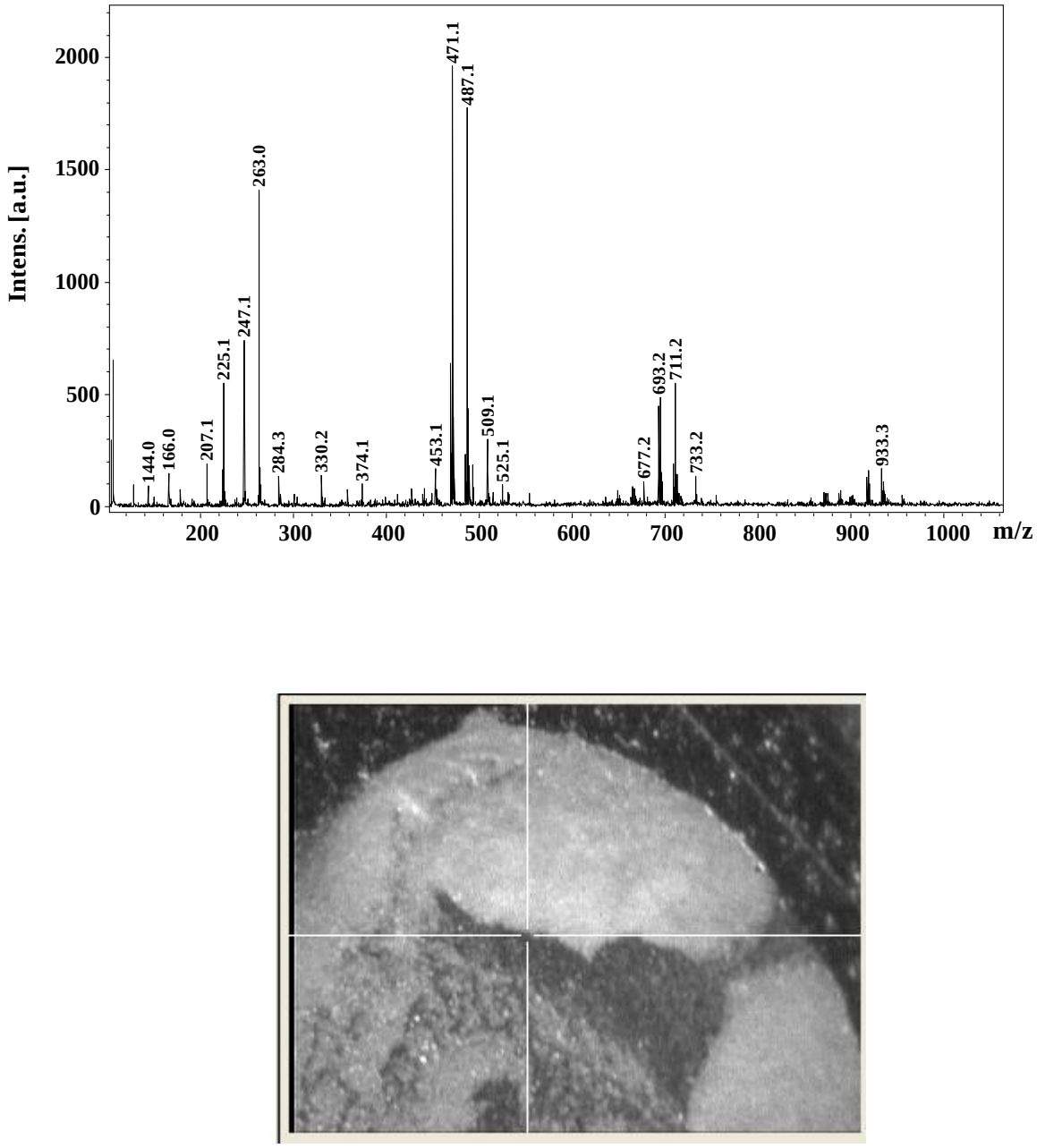


Figure S20. (top) LDI mass spectrum of Z-SA + serine (3:1 vol/vol) (Z-SA, 5 mg/mL in MeCN:H_2O 0.1% TFA 1:1 v:v; serine 1M in H_2O) and (bottom) morphologycal inspection of the solid Z-SA + serine deposited. Circular spot surface, 9.62 mm^2. Digital images from Bruker Ultraflex II TOF/TOF.

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