**Matrix-assisted ionization vacuum for protein detection, fragmentation and
PTM analysis on a high resolution linear ion trap-orbitrap platform**

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ABSTRACT

Matrix-assisted ionization vacuum (MAIV) is a novel ionization technique that generates multiply charged ions in vacuum without the use of laser ablation or high voltage. MAIV can be achieved in intermediate-vacuum and high-vacuum matrix-assisted laser desorption/ionization (MALDI) sources and electrospray ionization (ESI) source without instrument modification. Herein, we adapt MAIV onto the MALDI-LTQ-Orbitrap XL platform for biomolecule analysis. As an attractive alternative to MALDI for in solution and *in situ* analysis of biomolecules, MAIV coupling to high resolution and accurate mass (HRAM) instrument has successfully expanded the mass detection range and improved the fragmentation efficiency due to the generation of multiply charged ions. Additionally, the softness of MAIV enables potential application in labile post-translational modification (PTM) analysis. In this study, proteins as large as 18.7 kDa were detected with up to 18 charges; intact peptides with labile PTM were well preserved during the ionization process and characterized MS/MS; peptides and proteins in complex tissue samples were detected and identified both in liquid extracts and *in situ*. Moreover, we demonstrated that this method facilitates MS/MS analysis with improved fragmentation efficiency compared to MALDI-MS/MS.

Keywords: Mass spectrometry, matrix-assisted ionization vacuum, high resolution and accurate mass, post-translational modification, multiply charged ions, protein analysis

1. Introduction

In the late 1980s, the development of MALDI [1] and ESI [2] revolutionized the field of mass spectrometry (MS) application in the analysis of large biomolecules [3]. MALDI utilizes laser desorption to produce mostly singly charged ions from a solid matrix, while ESI employs high voltage to produce multiply charged ions from solution. The capability of ionizing analytes directly from solid surface makes MALDI an ideal tool for *in situ* tissue analysis. However, as MALDI predominantly produces singly charged ions, a few challenges remain for MALDI analysis, especially for HRAM protein analysis. With the commonly used ion activation methods such as collisional induced dissociation (CID) and high energy collisional induced dissociation (HCD) techniques, the singly charged ions usually have lower fragmentation efficiencies compared with that of multiply charged ions, making the identification of analyte molecules difficult. Additionally, most current commercially available high-performance Orbitrap instruments cannot detect ions larger than m/z 10000. Moreover, in-source or post-source fragmentations of molecules are usually observed in MALDI process [4, 5]. On the other hand, although ESI overcomes most of these problems mentioned above, it is impossible to preserve the spatial information on tissue sections using conventional ESI analysis since the analytes must be dissolved in a volatile solution.

In recent years, many approaches have been taken to address these challenges. “Electron-free” MALDI was observed to produce higher percentage of multiply charged ions, when the number of electrons was limited in the plume [6]. Desorption electrospray ionization (DESI) ionizes analytes from surface by electrospray generated charged droplets and solvent ions [7, 8]. Matrix-assisted laser desorption electrospray ionization (MALDESI) generates ESI-like multiply charged ions with the assistance of matrix [9, 10]. Electrospray-assisted laser desorption

ionization (ELDI) is capable of ionizing peptides and proteins in solid materials by laser desorption and post-ionization electrospray without matrix [11-15].

Laserspray ionization (LSI) and MAIV are relatively new ionization techniques that produce ESI-like multiply charged ions while being able to preserve spatial information on tissue sections. Unlike DESI, MALDESI or ELDI, LSI and MAIV can be readily achieved using commercially available MALDI sources without any instrumentation modification. These two ionization techniques were first introduced by the Trimpin lab and have been demonstrated on several MS platforms [16-19]. LSI utilizes volatile small molecule matrices with laser ablation to ionize analytes under atmosphere pressure (1.01 bar) [20], intermediate-vacuum (10^{-3} to 33 mbar) [18, 21] and high-vacuum (10^{-6} to 10^{-3} mbar) MALDI sources [22]. However, the involvement of laser in LSI could induce in-source or post-source fragmentations. In contrast, MAIV is a softer ionization method that generates multiply charged ions in a triboluminescence process, which does not involve either laser or high voltage application during the ionization process [23]. With the assistance of small volatile matrices, MAIV can generate highly charged ions from a wide variety of compounds [24]. The original MAIV study was performed on both LTQ and quadrupole time-of-flight instruments with low to medium mass resolution. The use of this ionization method was also demonstrated on high resolution Fourier transform instruments for full MS analysis [17, 19]. However, to our knowledge, no study has been performed on a MALDI-Orbitrap platform, and MAIV-MS/MS data, especially for post-translational modification (PTM) analysis, is also quite limited in previous studies.

Many biological processes are regulated by PTM of peptides and proteins [25]. Understanding PTMs is essential for understanding the biological functions of various proteins and studying cell regulations. However, some labile PTMs, such as glycosylation, can be easily

1 detached during ionization process, making them difficult to be analyzed by MS. Due to its
2 softness, we hypothesize that MAIV can reduce in-source and post-source fragmentation of
3 biomolecules, especially peptides and proteins with labile PTMs.

4 The introduction of hybrid MALDI mass spectrometers has expanded the capability of
5 MALDI MS analysis. The MALDI-LTQ-Orbitrap XL system (Thermo Fisher Scientific, Bremen,
6 Germany) incorporates linear ion trap and orbitrap mass analyzers [26], making HRAM analysis
7 as well as MSⁿ by collisional induced dissociation (CID) and high-energy collision dissociation
8 (HCD) possible on one platform. However, this instrument has a limited *m/z* range of 50-4000.
9 With singly charged ions generated in the MALDI source, this instrument cannot analyze large
10 molecules such as proteins, polysaccharides and polynucleotides.

11 The development of LSI [27, 28] and MAIV [29] prompted us to investigate the
12 possibility of employing the MALDI-LTQ-Orbitrap XL as a HRAM platform for protein
13 characterization. LSI has been adapted to MALDI-LTQ-Orbitrap XL hybrid system and the
14 application has been expanded to *in situ* MS/MS analysis and *de novo* sequencing [18]. In this
15 study, we adapted the recently developed MAIV technique to our hybrid MALDI-LTQ-Orbitrap
16 XL system for peptide and protein analysis. Multiply charged ions were detected with this
17 HRAM MS platform in the analysis of protein and peptide standards, tissue extracts and *in situ*
18 tissue sections. Furthermore, MAIV-MS/MS analysis has been achieved in both standards and
19 tissue protein extraction analysis. The fragmentation efficiency of multiply charged species has
20 been greatly improved in comparison to that of the singly charged ions. We also demonstrated
21 that MAIV-MS could be used for the analysis of labile PTMs on peptides because of the soft
22 ionization nature of MAIV.

2. Materials and methods

2.1. Materials

Methanol (MeOH), ethanol (EtOH), acetonitrile (ACN), acetic acid (AA) and formic acid (FA) were purchased from Fisher Scientific (Pittsburgh, PA). 3-nitrobenzonitrile (3-NBN), α -cyano-4-hydroxycinnamic acid (CHCA), insulin (bovine), cytochrome C (bovine heart), lysozyme (chicken egg white) and myoglobin (equine heart) were purchased from Sigma Aldrich Inc. (St. Louis, MO). Peptide standard bradykinin was purchased from American Peptide Company (Sunnyvale, CA). Kinase domain of insulin receptor was purchased from AnaSpec (Fremont, CA) and glycosylated erythropoietin (EPO) 117-131 was purchased from Protea Biosciences Group, Inc. (Morgantown, WV). All standards and reagents were used without additional purification.

2.2. Sample preparation

2.2.1. Peptide and protein standard preparations. Peptide and protein stock solutions with concentration of 10 mg mL^{-1} were prepared by dissolving standards in water (0.1% FA). Standards with concentrations of 1 mg mL^{-1} , $100 \text{ } \mu\text{g mL}^{-1}$, $10 \text{ } \mu\text{g mL}^{-1}$ and $1 \text{ } \mu\text{g mL}^{-1}$ were prepared by serial dilutions in water (0.1% FA). Samples were stored at $-20 \text{ }^{\circ}\text{C}$ until analysis.

2.2.2. Tissue analysis. Animal experiments were conducted following institutional guidelines (UW-Madison IACUC). Female Sprague-Dawley rats were anesthetized, perfused with chilled phosphate buffered saline, decapitated and removed brains. The brain tissues were either stored in Eppendorf tubes for protein extraction or embedded in gelatin solution (100 mg

1 mL⁻¹ in MilliQ water) for tissue sectioning. Tissue samples were snap frozen and stored in -80 °C until analysis.

A 3x volumes of chilled acidified MeOH solution (MeOH:H₂O:AA (v/v/v) 90 : 9: 1) was added to the brain tissue sample for neuropeptide extraction. The brain tissue was manually homogenized using a glass homogenizer and then centrifuged at 16,100 × g for 10 minutes. The supernatant was obtained and dried down in a speed vacuum concentrator and was reconstituted in water (0.1% FA) prior to desalting. A 10 µL C₁₈ ZipTip (EMD Millipore, Darmstadt, Germany) was used for salt removal. The ZipTip was first conditioned with ACN and equilibrated with water (0.1% FA). After loading the tissue extract, the ZipTip was washed with water (0.1% FA) for 3 times and the sample was eluted with 10 µL of ACN:H₂O:FA (v/v/v) 49.95:49.95:0.1. The eluted peptide solution was then used for MS analysis.

Cryosectioned rat brain tissue was used for *in situ* biomolecule profiling. Gelatin embedded tissue was sectioned into 12 µm slices in a cryostat (Microm HM525, Thermo Scientific, Bremen, Germany), thaw mounted onto a glass slide (75x25x1mm) and stored in a desiccator at -80 °C until analysis.

2.3. MALDI-MS, MAIV-MS and MS/MS set up

CHCA matrix solution was prepared by dissolving 10 mg of CHCA in 1 mL solution of ACN:EtOH:H₂O:FA (v/v/v/v) 84:13:2.997:0.003. 3-NBN matrix solution was prepared as described by Inutan and Trimpin [23, 24]. Briefly, 10 mg of 3-NBN was dissolved in 50 µL acetonitrile, then mixed with 150 µL solution of ACN:H₂O:FA (v/v/v) 49.95:49.95:0.1. The matrix solution was made freshly before every experiment and kept at above 25 °C to avoid recrystallization. Other solvent combinations, including MeOH/water/FA and EtOH/water/FA,

1 were also tested for optimization. For MALDI-MS analysis, 1 μ L CHCA matrix was mixed with
2 1 μ L analyte (standard or tissue extract) in an Eppendorf tube. The mixture was deposited on a
3 stainless steel sample plate. For MAIV-MS analysis, 1.5 μ L 3-NBN matrix was deposited on top
4 of 0.5 μ L analyte directly on a stainless steel sample plate. Analyte and 3-NBN matrix could not
5 be pre-mixed in an Eppendorf tube before depositing on the target plate, since 3-NBN
6 crystallizes immediately upon mixing. One spot was prepared for each time of data acquisition.
7 For *in situ* analysis, 1.5 μ L matrix was spotted directly onto tissue sections.

8 The MALDI-LTQ-Orbitrap XL platform (Thermo Scientific, Bremen, Germany) was
9 used for all data acquisitions. For all experiments, survey crystal positioning system (CPS) was
10 used as the plate motion mode and 1 microscan was acquired per step. Laser energy of 10.5 μ J
11 was used for all MALDI-MS analysis, and laser energy of 0.1 μ J (the minimum laser setting on
12 the instrument in order to trigger data acquisition) was used for MAIV-MS. For full scan, FTMS
13 mode was used with a resolving power of 100,000 at m/z of 400. Automatic gain control (AGC)
14 was set at a target value of $1e^6$ and maximum laser shots of 80, indicating that a total ion count of
15 $1e^6$ or 80 laser shots have to be reached for each microscan. Both HCD and CID were used to
16 acquire MS/MS data. AGC target for MS/MS was set to be $1e^5$ and isolation window of 3 m/z
17 was chosen for all parent ions. Normalized collisional energy was optimized for each parent ion.
18 Database searching and *de novo* sequencing were performed by PEAKS 7 (Bioinformatics
19 Solution Inc., ON, Canada) and verified manually.

20 3. Results and discussion

21 MAIV is a novel soft ionization technique that generates multiply charged ions from
22 solid state samples without laser ablation or high voltage. Although a quantitative mechanism for

MAIV has not yet been formulated, properties of the matrices used in these methods have offered clues. A recent review by McEwen and Larsen suggests two related matrix-dependent mechanisms. Matrices which produce greater ion yields at high temperatures, such as 2-nitrophenylglycinol and 2,5-dihydroxyacetophenone, may melt into liquid droplets within the heated inlet and become ionized in the pressure gradient, thus leading to an ESI-like charged droplet event. More volatile matrices which produce greater ion yields at lower temperatures, such as 3-NBN, may form highly charged clusters from a crystal-shearing or triboluminescence process during sublimation [30, 31]. Due to the low energy ionization processes, it is a promising tool to analyze labile biomolecules without extensive in-source or post-source fragmentation. In this study, we have adapted MAIV to the HRAM MALDI-LTQ-Orbitrap XL platform for both extracted and *in situ* tissue analysis of peptides and proteins. Moreover, we have demonstrated improved HCD and CID fragmentation efficiency of MAIV produced multiply charged peptide ions.

3.1. Optimization of MAIV-MS conditions using peptide and protein standards

MAIV-MS Optimization. 3-NBN concentration, solvent composition and laser energy were optimized. Different 3-NBN concentrations (10, 50 and 100 mg mL⁻¹) were tested. Only 50 mg mL⁻¹ 3-NBN allowed production of multiply charged ions under MAIV-MS condition. 100 mg mL⁻¹ 3-NBN cannot be fully dissolved, whereas 10 mg mL⁻¹ 3-NBN sublimates too rapidly in the intermediate vacuum source resulting in the absence of detectable signal. Different solvents, including ACN, MeOH, EtOH and water, were tested to dissolve 3-NBN. Only ACN was able to dissolve the 3-NBN powder. A small amount of water (less than 40% of total volume) could be added into the ACN dissolved 3-NBN solution without inducing recrystallization. Different laser energies (0.1, 5, 10, 15, 20, 25 and 30 μJ) were tested using 100 μg mL⁻¹ insulin

standard (0.1 μJ , which can be ignored, was the smallest laser energy value allowed to trigger data acquisition). Multiply charged ions were observed at every laser energy tested. Although the exact mechanism of MAIV is not fully understood yet, this observation suggests that the ionization process of MAIV does not involve laser, which allows a softer ionization compared to MALDI. Based on these results, 50 mg mL^{-1} 3-NBN dissolved in ACN: H_2O :FA (v/v/v) 62.45:37.45:0.1 and 0.1 μJ laser energy were used for all subsequent MAIV-MS data acquisition.

3.2. Characterization of peptide and protein standards

250 $\mu\text{g mL}^{-1}$ protein standards: insulin (Figure 1a), cytochrome C (Figure 1b), lysozyme (Figure 1c) and myoglobin (Figure 1d) were used to evaluate the HRAM MAIV-MS system for the generation of multiply charge ions. Most of the multiply charged ions appeared between m/z 700-2000. While most of the singly charged ions were observed below m/z of 700 and very small amount of ions were observed above m/z of 2000. For insulin, +3, +4 and +5 charged ions were detected with high mass accuracy (0.58 ppm in average) and high resolution (100,000 at m/z 400). Charge states +7 to +15 of cytochrome C and +8 to +12 of lysozyme were all detected and well resolved. Myoglobin (16952.30 Da) was the largest protein standard we tested using this platform and as many as 18 positive charges attached to the intact protein were observed. Isotopic peaks of +5 charged insulin, +14 charged cytochrome C, +13 charged lysozyme and +15 charged myoglobin were well resolved (Figure 1, zoomed in spectra) with the superior resolving power provided by the orbitrap.

To evaluate the sensitivity of HRAM MAIV-MS, serial dilutions of peptide and protein standards were tested. The lower limit of detection (LLOD) was determined by the lowest concentration where analyte ions could be detected. The LLODs of bradykinin, insulin,

1 cytochrome C, lysozyme and myoglobin were determined as 250 ng mL⁻¹ (236 nM), 250 ng mL⁻¹
2 (43.6 nM), 2.50 µg mL⁻¹ (202 nM), 2.50 µg mL⁻¹ (175 nM) and 250 µg mL⁻¹ (14.7 µM),
3 respectively. The sensitivity for large proteins is limited on this platform as the detection of large
4 proteins with Orbitrap is difficult. Modifications of instruments are required for extended mass
5 range detection on Orbitrap [32].

6 MAIV-MS/MS by CID and HCD was also achieved using the LTQ-Orbitrap XL platform.
7 To demonstrate the improved fragmentation efficiency on the multiply charged ions, bradykinin
8 was chosen for MS/MS comparisons between +1 and +2 charged ions (Figure 2). HCD
9 experiments were performed with MALDI produced +1 charged ion (Figure 2a), and MAIV
10 produced +1 (Figure 2b) and +2 charged ions (Figure 2c) respectively. The fragmentation
11 patterns of the +1 charged ions generated by MALDI and MAIV were similar: a few b and y ions
12 were observed along with some a-type ions. Compared to +1 charged ions, +2 charged ions
13 generated by MAIV showed significantly improved HCD fragmentation efficiency and almost
14 all of the b and y ions were observed with the exception of b₇ ion.

15 As mentioned in introduction, we hypothesize that MAIV-MS can reduce in-source
16 fragmentation of peptides and proteins with PTMs as MAIV does not require high energy (laser
17 or high voltage) to ionize molecules. To test this hypothesis, phosphorylated kinase domain of
18 insulin receptor and glycosylated EPO fragments were analyzed by MALDI-MS and MAIV-MS
19 respectively (Figure 3). Under MALDI condition, in source fragmentation occurred to both
20 phosphopeptide (Figure 3a) and glycopeptide (Figure 3b). Singly charged phosphopeptide ion
21 was observed along with neutral loss fragment ions such as [M-p+H]⁺, [M-p-H₂O+H]⁺ and [M-
22 2p-H₂O+H]⁺ in MS¹ scan. For the glycosylated peptide, both intact glycosylated EPO ion and Y₀
23 ion (EPO losing the GalNAc) were observed in MS¹ scan. In contrast, under MAIV condition,

doubly charged intact ions of both phosphopeptide and glycopeptide were observed as the base peak and very limited number of fragments were detected in MS¹ scan. Neutral loss fragment ions such as [M-p+H]⁺ of the phosphopeptide and EPO Y₀ ions were detected but with much lower intensities compared to the base peaks (about 12% of the base peak). MAIV-MS/MS of the doubly charged glycosylated EPO ion was performed by both CID and HCD (Supplemental Figure 1). The detected fragment ions were mostly b and y ions without the modification along with some ions having GalNAc preserved on the serine residue. The slow heating fragmentation techniques such as CID and HCD pose limitations for more comprehensive structural analysis by MS/MS because most of the labile PTM groups are often detached from the peptide backbone during the fragmentation process [33]. Coupling MAIV-MS to electron-transfer dissociation or electron-capture dissociation, which are advantageous to preserve labile PTM groups during dissociation that allows pinpointing the modification site, will be explored for PTM analysis in future studies [34, 35].

3.3. MAIV-MS Analysis of Animal Brain Tissue

In addition to peptide and protein standards, analysis of complex samples in solution and *in situ* were also demonstrated using MAIV-MS. Neuropeptide and protein extracts from rat pituitary gland and rat brain were analyzed by MAIV-MS (Figure 4a-b). Highly charged ions were detected and protein species were identified based on accurate mass matching. It was observed that majority of the multiply charged ions were detected in the tissue extracts of both rat pituitary gland and entire brain (Table 1). Among the 861 mass spectral peaks (with signal to noise ratios greater than 3 and charge state assigned by Xcalibur) detected from rat pituitary gland extracts, only 11% of the peaks were singly charged. Majority of peaks (69%) had charge states between +2 to +4, and 20% of the peaks had charge states greater than four. Molecules as

large as 14 kDa were detected with a charge state of 15. For the whole brain tissue extract, peaks with higher charge states were observed; a large portion of peaks (68%) had charge states greater than +4, 29% of the peaks were singly charged, and only 3% of the peaks were between +2 to +4.

MAIV-HCD-MS/MS analysis was performed with different charge states of an endogenous neuropeptide SYSMEHFRWGKPV (N-term: diacetyl, C-term: amide) from the tissue extracts of a rat pituitary gland (Supplemental Figure 2). The HCD MS/MS analyses of +2 and +3 charged ions showed highly abundant b and y ions, whereas the MS/MS of the +1 charged precursor ions produce barely detectable signals. As expected, the HCD fragmentation efficiency of the multiply charged ions was significantly improved compared to that of the singly charged ions.

Rat brain cryosections were used to demonstrate *in situ* analysis by MAIV-MS (Figure 4c). A drop (1.5 μ L) of 3-NBN matrix was applied to the occipital cortex of a 12 μ m rat brain section for profiling analysis. Singly charged neuropeptide (α -MSH), multiply charged protein (ubiquitin 6+) and several singly charged lipids (cardiolipin/CL) were detected from the tissue sections. In summary, MAIV-MS is suitable for the analyses of large molecules extracted from tissue as well as on tissue slices.

3.4. Challenges

As 3-NBN is a volatile matrix, it usually sublimates within 5 minutes in an intermediate vacuum MALDI source. MS imaging, which usually requires much longer acquisition time, cannot be achieved by MAIV-MS at this stage. Moreover, detection of large proteins is still a problem with Orbitrap. The sensitivity of large protein (such as myoglobin) detection using MAIV-MS is mediocre, thus only the most abundant proteins in tissue could be detected.

1 Slowing down the sublimation rate and enhancing the sensitivity/dynamic range for protein
2 analysis remain to be addressed in order to make MAIV-MS a more useful and versatile
3 analytical tool.

4 **4. Conclusion**

5 MAIV is a novel ionization technique that generates multiply charged ions during a
6 triboluminescence process under vacuum without the use of high voltage or laser ablation. As an
7 attractive alternative to MALDI for off-line and *in situ* analysis of biological samples, MAIV
8 coupling with HRAM instrument platforms such as LTQ-Orbitrap, expands the mass detection
9 range of matrix-assisted ionization methods and improves the fragmentation efficiency because
10 of the production of multiply charged ions. In this study, we demonstrated the capability of
11 HRAM MAIV-MS in detecting biomolecules as large as 18.7 kDa with 18 charge states in an
12 orbitrap mass analyzer. MAIV-MS analysis of peptides and proteins were demonstrated with
13 both extracts from rat brain and *in situ* tissue profiling. Compared to singly charged ions
14 produced by conventional MALDI, multiply charged ions produced by MAIV enabled an
15 improved fragmentation efficiency for both peptide standards and endogenous peptides from
16 complex tissue samples. Moreover, MAIV-MS can also be used as an alternative tool for labile
17 PTM analysis due to the fact that neither laser ablation nor high voltage is involved in the
18 ionization process, which decreases the chance of in source fragmentation. Collectively, HRAM
19 MAIV-MS offers a versatile tool for *in situ* protein analysis, sequencing via improved
20 fragmentation and more complete PTM analysis with enhanced performance.

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Table Caption

Table 1. Summary of MAIV-MS tissue analysis results of tissue extracts from a rat pituitary gland and a whole rat brain.

Sample	Total Peaks*	Singly Charged Ion%	2-4 Charged Ion %	>4 Charged Ion %	Max. M.W. (Da)	Max. Charges
Pituitary Gland	861	11.27	68.64	20.09	14175	15
Whole Brain	223	29.15	3.14	67.71	15602	10

* Only peaks with signal to noise ratio greater than 3 and charge state assigned by Xcalibur were counted.

Figure Captions

Figure 1. MAIV-MS analyses of protein standards: insulin, M.W. 5734.51 (a), cytochrome c, M.W. 12360.97 Da (b), lysozyme, M.W. 14295.81 Da (c) and myoglobin, M.W. 16952.30 Da (d).

Figure 2. Comparison of MALDI-HCD-MS/MS and MAIV-HCD-MS/MS using $10\text{ }\mu\text{g mL}^{-1}$ bradykinin. (a) HCD MS/MS spectrum of $[\text{M}+\text{H}]^+$ bradykinin ion (m/z 1060.57) produced by MALDI, normalized collisional energy was 40. (b) HCD MS/MS spectrum of $[\text{M}+\text{H}]^+$ bradykinin ion (m/z 1060.57) produced by MAIV, normalized collisional energy was 43. (c) HCD MS/MS spectrum of $[\text{M}+2\text{H}]^{2+}$ bradykinin ion (m/z 530.79) produced by MAIV, normalized collisional energy was 43.

Figure 3. MALDI-MS (a, b) and MAIV-MS (c, d) analysis of a phosphopeptide standard and a glycopeptide standard. Kinase domain of insulin receptor (a, c) and glycosylated erythropoietin (EPO) fragment 117-131 (b, d) were analyzed under MALDI-MS (a, b) and MAIV-MS (c, d) conditions respectively.

Figure 4. HRAM MAIV-MS analysis of different tissue samples: (a) neuropeptide and protein extracts from a rat pituitary gland; (b) neuropeptide and protein extracts from a whole rat brain; (c) *in situ* neuropeptide and protein profiling on the occipital lobe of rat brain cortex. Putative identifications of biomolecules were assigned by accurate mass matching.

1 **Figures**

Chen *et al.*, Figure 1

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GRAPHICAL ABSTRACT