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Integration of Ion Mobility MS^E after Fully Automated, Online, High-Resolution Liquid Extraction Surface Analysis Micro-Liquid Chromatography

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ABSTRACT: Direct analysis by mass spectrometry (imaging) has become increasingly deployed in pre-clinical and clinical research due to its rapid and accurate readouts. However, when it comes to biomarker discovery or histopathological diagnostics, more sensitive and in-depth profiling from localized areas is required. We developed a comprehensive, fully automated online platform for high-resolution liquid extraction surface analysis (HR-LESA) followed by micro-liquid chromatography (LC) separation and a data-independent acquisition strategy for untargeted and low abundant analyte identification directly from tissue sections. Applied to tissue sections of rat pituitary, the platform demonstrated improved spatial resolution, allowing sample areas as small as 400 μm to be studied, a major advantage over conventional LESA. The platform integrates an on-line buffer exchange and washing step for removal of salts and other endogenous contamination that originates from local tissue extraction. Our carry over-free platform showed high reproducibility, with an inter-extraction variability below 30%. Another strength of the platform is the additional selectivity provided by a post-sampling gas-phase ion mobility separation. This allowed distinguishing co-eluted isobaric compounds without requiring additional separation time. Furthermore, we identified untargeted and low-abundance analytes, including neuropeptides deriving from the pro-opiomelanocortin (POMC) precursor protein and localized a specific area of the pituitary gland (i.e., adenohypophysis) known to secrete neuropeptides and other small metabolites related to development, growth, and metabolism. This platform can thus be applied for the in-depth study of small samples of complex tissues with histologic features of ~400 μm or more, including potential neuropeptide markers involved in many diseases such as neurodegenerative diseases, obesity, bulimia, and anorexia nervosa.

INTRODUCTION

Mass spectrometry imaging (MSI) is being used more often in pre-clinical and clinical research due to its many advantages over conventional imaging techniques.¹⁻³ MSI offers the possibility to correlate distribution maps of multiple molecular species simultaneously with histological and clinical features without labelling. This methodology enables the discovery of potential diagnostic and prognostic markers of diseases in a single experiment. With these unique features, MSI opens new doors for molecular-driven pathology, in various fields of histopathological diagnostics, such as identification and grading of tumors.^{4,5} MSI has also proven to be a powerful tool in drug discovery as it gives insights not only on drug and metabolite distribution and site(s) of action, but also about the biology present at the sites of drug localization relating to treatment efficacy.⁶

Matrix-assisted laser desorption/ ionization (MALDI) is by far the most popular ionization technique used to map the tissue microenvironment. In this way, molecular classification can be accurately established with different tissue types, including tumors, to improve the accuracy of diagnosis and characterize tumor heterogeneity.⁷ MALDI-MSI allows *in situ* tissue

characterization at different molecular “omics” levels—metabolomics, lipidomics, peptidomics and proteomics⁸—and has found numerous clinical applications, including oncology,⁹ psychiatric and neurodegenerative disorders,¹⁰⁻¹² cardiovascular diseases,^{13,14} ophthalmology,¹⁵ joint and cartilage-related disorders.¹⁶ Extensive efforts focused on instrument development to improve spatial resolution, throughput, and sensitivity have placed MALDI-MSI in a competitive position for clinical studies where knowledge about the tumor microenvironment is critical and hundreds of samples require analysis.¹⁷⁻¹⁹ One of the drawbacks of MALDI-MSI is the need for the application of homogenous layers of MALDI matrix on the tissue surface and the ion suppression resulting from the extraction process. This is in part due to the lack of separation technology in an imaging experiment and can degrade the sensitivity and quality of data generated.

Recently, ambient ionization techniques have proven their suitability to extract relevant information from complex biological matrices.²⁰ The main benefit of these techniques is the possibility to analyze directly surfaces with relatively less complex and time-consuming sample preparation compared to MALDI. This considerably reduces sample handling and

speeds up the whole analytical workflow. Desorption electrospray ionization (DESI)^{21,22} is increasing its application in clinical research as it provides relatively fast, sensitive, and complementary analysis to MALDI-MSI.²³⁻²⁹ DESI allows rapid classification of human tumors based on tissue-specific lipid molecular profiling.³⁰ One of the most promising applications would be to use the information at the time of surgery on resected specimens to guide surgical resections that could improve management of patients.³¹ Liquid extraction surface analysis (LESA), based on a liquid microjunction (LMJ) surface sampling, was introduced in 2008 and has been employed for profiling biological matrices from localized tissue area in various studies, including drug distribution and metabolism,³²⁻³⁶ microbiology,³⁷ small molecule antibody-drug conjugate catabolites,³⁸ lipidomics,^{32,39} proteomics,⁴⁰⁻⁴² and native mass spectrometry for non-covalent complex studies.^{43,44} Although often criticized for the poor spatial resolution achievable (i.e., 1.2–2.0 mm with 1 μ L of solvent deposited on the top of the surface), LESA is an excellent tool for conducting profiling experiments from a selected spot, such as quick metabolite screening or parent drug localization to an organ. Complementary to quantitative whole-body autoradiography that provides distribution information on radiolabeled material, LESA followed by MS detection enables differentiation of a parent drug from its metabolites.

Chromatography-free approaches create ionization suppression effects, which enables the identification of analytes of high abundance⁴⁵ but limits protein/peptide identification because of the high degree of cell or tissue complexity. Frequently, compounds are co-desorbed; to deal with structural isomers and/or isobaric compounds (e.g., interfering isotopic clusters), chromatography-free approaches often suffer from the absence of separation before the mass analysis. Indeed, high resolution is not adequate to distinguish isomers and in some cases tandem mass spectrometry (MS/MS) does not provide enough selectivity. Therefore, additional information and improved selectivity are required to provide adequate identification of biologically relevant analytes. The addition of a post-desorption and post-ionization gas-phase ion mobility separation (IMS) after MALDI, DESI, and LESA has demonstrated to resolve isobaric species and reduce chemical noise.^{29,34,46} However, this method does not account for tissue-specific ionization suppression.⁴⁵ Because the surface sampling and the ionization processes are resolved both in space and time dimensions, LESA allows for the manipulation of the extracted material in the liquid phase prior to ionization of molecular content.⁴⁷ As a consequence, LESA can easily incorporate a liquid-based separation after the surface sampling process, which is not possible with MALDI or DESI ionization techniques. Kertesz *et al.* developed a LMJ-SS approach followed by HPLC-ESI-MS for the analysis of drugs and metabolites in whole-body thin tissue sections, which helps to distinguish isomeric phase II metabolites of propranolol.⁴⁸ Continuous-flow LMJ-SS coupled online with HPLC/MS also enables the extraction, separation, and detection of proteins and low-molecular-weight compounds (e.g., drugs of abuse) from tissue sections and dried blood spots. Usually, the surface of dried blood spots is sufficiently hydrophobic for maintaining a stable liquid junction, even with high-aqueous-content solvents.⁴⁹ This innovative LMJ-SS-HPLC-MS/MS approach was also used to investigate the distribution of specific markers within normal human pituitary gland and pituitary adenoma tissue sections, to discriminate between tumor and non-tumor tissues.⁵⁰ However, such

targeted approach requires sample clean-up prior to analysis and will not allow broad screening for potential biomarkers.

In the present work, we describe a platform developed to improve both poor spatial resolution achieved with “conventional” LESA and ionization suppression effect. The platform was modified for high-resolution (HR)-LESA for direct analysis of endogenous peptides from a 400- μ m area from pre-clinical tissue samples.⁵¹ The HR-LESA was integrated with an online washing step to remove salts and other contaminants, the key source of ion suppression. Micro-LC (μ LC) was used to separate the analytes of interest from endogenous sample matrix compounds, an additional source of ion suppression. This platform allows isomeric separation due to the implementation of μ LC further improved by ion mobility. After this μ LC separation, the compounds were analyzed by MS, which combines IMS with high-definition (HD) MS^E, a data-independent acquisition method. Finally, we automated the entire HR-LESA- μ LC-HDMS^E platform by implementing new software. This platform is presented here as a complementary addition to the field of mass spectrometry imaging since it opens doors to a more in-depth profiling of spatial extractions of biological tissues.

EXPERIMENTAL SECTION

Chemicals and reagents. ULC/MS-grade water, ULC/MS-grade acetonitrile (ACN), and 99% formic acid were purchased from Biosolve (Valkenswaard, NL). Microscopic glass slides were purchased from Thermo Scientific (Braunschweig, DE). Leucine enkephalin standard was provided by a Waters Q-ToF Qualification Standards Kit (Etten-Leur, NL) and prepared at a concentration of 5 ng/ μ l in ACN/water (50/50; v/v) and used as lock mass.

Murine tissue sectioning. Healthy Wistar Han rat pituitary gland tissue samples were provided by the Department of General Surgery of the Maastricht University Medical Center (MUMC+). The fresh-frozen wild type and transgenic APP_{KM670/671NL}/PS1_{L166P} mice were supplied by the Bio-Imaging Lab, University of Antwerp. Tissues were cryo-sectioned (Microtome cryostat Thermo Scientific, Braunschweig, DE) into 12- μ m-thick tissue sections and subsequently thaw-mounted on regular microscope glass slides. These tissue sections were stored at -80 °C prior to analysis. Right before the analysis, samples were thawed at room temperature and desiccated for 30 min. Hematoxylin and eosin (H&E) staining was performed on these tissue samples after HR-LESA- μ LC-MS analysis.

H&E staining protocol. H&E staining was performed on the same sections used for HR-LESA- μ LC-HDMS^E experiments. After MSI analysis, the residual matrix was gently removed by dipping the glass slides in ethanol for 2 minutes. Sections were then washed in successive bathes (96% EtOH, 70% EtOH and deionized water – 3 min each). The haematoxylin (Merck, Darmstadt, Germany) staining was then performed for 3 min, slides were then washed in gently running tap water for 3 min, followed by eosin staining for 30 s, washing under running tap water for 1 min and finally immersed in 100% EtOH for 2 min. Slides were finally dehydrated in Xylene (30 s), covered in Etallen and coverslipped. The optical images were acquired using a MIRAX Desk scanner (Zeiss, Gottingen, Germany). Images were acquired with a magnification of 40.

Conventional LESA extraction. The LESA extraction was performed using the automated TriVersa NanoMate Advion

robot (Advion, Ithaca, NY). A 0.5- μ L volume of the extraction solvent (ACN/water/formic acid, 70/30/0.1 v/v/v) was deposited with a conductive pipette tip onto the tissue section for 5 s repeated 1 times. This extract was directly infused into the mass spectrometer (Waters Synapt G2-Si, USA) using chip-based nano-ESI, by applying a nitrogen gas pressure of 0.3 bar and voltage of 1.40 kV.

HR-LESA extraction, online washing, and μ LC separation. High spatial resolution extraction was performed using the capillary extraction arm usually used for coupling with LC-MS fraction collection. Extraction with this setup was performed with 0.5 μ L extraction solvent (ACN/water/formic acid, 70/30/0.1 v/v/v) after 5 s in contact with the tissue section. An online and automated buffer exchange was then performed, by diluting 10 times the sample with the carrier solvent (ACN/water/formic acid, 5/95/0.1 v/v/v) – to ensure compatibility with reversed-phase chromatography – and collected in a 3- μ L loop (Figure 1). Under these conditions, the local extraction was achieved at a spatial resolution of 400 μ m, and the total workflow from extraction solvent aspiration to filling in the sample loop takes \sim 3 min.

Following this extraction, the sample was trapped onto a trap column (ACQUITY UPLC M-class Symmetry C18, 100 \AA , 5 μ m, 300 μ m \times 50 mm, Waters, City, ST) and washed with the carrier solvent for 2 min to remove salts and other possible interferences/contamination. After the 2-min online washing, the trap column was back-flushed onto the μ LC IonKey column (iKey BEH C18 Separation Device, 130 \AA , 1.7 μ m, 150 μ m \times 50 mm, Waters, Milford, MA) for chromatographic separation as follow: a 13-min gradient from 1% to 85% solvent B (ACN/formic acid, 100/0.1) was used for elution of peptides. The column was washed for 3 min at 85% solvent B prior to the column equilibration at 1% solvent B for 5 min. The trap column was equilibrated at 1% solvent B for 4 min.

Mass spectrometry. All MS experiments were conducted on a Waters Synapt G2-Si system operated in positive ionization mode (in sensitivity mode). General operating parameters were: capillary voltage = 4 kV; source temperature = 80 $^{\circ}$ C; sampling cone voltage = 40 V; and a desolvation temperature = 150 $^{\circ}$ C. The default collision energy was set at 4 eV in full MS scan mode. IMS was performed using nitrogen as a drift gas at a flow rate of 90 mL/min. The TRAP DC entrance was set to 0 V and the wave height was set to 40 V. The velocity of the IMS wave was used to separate the ions over the total 200 ms. The start velocity was set at 1200 m/s and the end velocity at 400 m/s. Data-independent HDMS^E acquisition was conducted for the analysis of the pituitary gland and further identification of extracted endogenous peptides. These measurements were performed in the TRANSFER T-wave using a collision energy ramp from 20 to 45 eV. The detector voltage was set at 2500 V, and data was acquired within a mass range of m/z 50–2000.

Software. The LESAs extraction was controlled by a beta version of the LESAs Plus software (Advion, UK), and MassLynx 1.4 (Waters, USA) was used for controlling the online washing step and the μ LC separation. Data were processed and visualized using Mass Lynx 1.4 (Waters, USA) and DriftScope v2.5 (Waters, USA). The identification of the neuropeptides and proteins was performed using Progenesis QI for proteomics v2.0.5556.29015 (Non Linear Dynamics, USA). For this identification, a species-specific FASTA file was created, and non-specific digest reagent was selected. The amount of missed cleavages was set at 3 and the post-

translational modifications (PTMs) allowed in this MS^E search were N-acetylation, M-oxidation, and C-carbamidomethylation.

RESULTS AND DISCUSSION

Here, we describe the HR-LESA- μ LC-MS platform and its performance for spatial analysis of neuropeptides. For this purpose, two animal models were used. The first experiments rely in the investigation of neuropeptides in rat pituitary gland tissues, which consists of two different regions with different biological functionalities: the adenohypophysis (anterior lobe and intermediate lobe) and the neurohypophysis (posterior lobe). Therefore, due to the morphology of the tissue (\sim 2 \times 3 mm), local extraction at high spatial resolution is crucial for molecular characterization of both regions. The second set of experiments is performed on wild type and transgenic mice expressing amyloidosis to assess the potential of the platform to study neuropeptides potentially involved in Alzheimer's disease progression. In this case, the sampling areas include regions with high expression of amyloidosis, such as the cerebral cortex, hippocampus and cerebellum, which also require precise sampling.

Analytical platform. Spatial resolution. In a conventional LESAs set-up, the extraction is achieved using a conductive pipet tip – which, depending on the solvent composition, can lead to a large sampled area up to 2 mm.⁵² This is potentially a limiting factor when analyzing small objects such as the anatomical features of the pituitary gland or any other organs that require precise sampling. In our HR-LESA- μ LC-MS platform (Figure 1), we modified the extraction system to improve the spatial resolution and reduce the size of the extracted areas. By using a silica capillary instead, we were able to significantly improve the spatial resolution to 400 μ m – as assessed under microscopic evaluation of the tissue section after the liquid extraction took place (Figure 2). With an extracted area of 400 μ m, the molecular content from the anterior and posterior lobe of the pituitary gland can be accurately extracted. We illustrate the ability to unambiguously distinguish between the adenohypophysis and neurohypophysis of the pituitary in Figure 2. Equal spot sizes were observed from the mouse brain tissue section in the H&E image after HR-LESA- μ LC-HDMS^E (Figure S-1).

Reproducibility of the extraction. To investigate the extraction reproducibility and sample carry-over of the HR-LESA setup, extraction of a leucine enkephalin standard was performed followed by flow injection analysis. A 5-ng leucine enkephalin solution (prepared in ACN/H₂O; 50/50; v/v) was spotted onto a hydrophobic plate and air-dried. The extractions of 5 consecutive spots using 0.9 μ L of 50% ACN + 0.1% HCOOH and the modified HR-LESA extraction system resulted in a coefficient of variation of 30% (based on the surface area from the extracted ion chromatogram at m/z 556.28 (protonated species [LeuEnk + H]⁺) for each extracted spots; $n=5$; Figure S-2).

Integration of online clean up between extraction and chromatographic separation. The tissue sample was placed in the sample plate holder as shown in the photograph in Figure 1. This tissue extract was then collected in a 384-well plate and diluted with carrier solvent. The diluted extract was 'injected' by the same fused silica capillary, collected in the 3- μ L loop, and, after switching the 6-port valve, trapped onto

1 the C₁₈ trap column for an online washing with water/ACN
2 (99/1; v/v) to remove all water-soluble matrix compounds.
3 The trap column was back-flushed to separate the remaining
4 sample on an Ionkey separation device using a reversed phase
5 μ LC separation.

6 *Reproducibility of the chromatographic separation and*
7 *sample carry-over.* HR-LESA combined with the online
8 washing and μ LC separation IonKey system was evaluated.
9 The chromatographic peak of leucine enkephalin was found at
10 a retention time of 16 min. This extraction was performed in
11 triplicate to test the retention time reproducibility followed by
12 two blank extractions to study carry-over. The relative
13 standard deviation of the retention time was < 0.12 % and the
14 carry-over in the first blank extraction was < 1.1 % using the
15 absolute peak area, which demonstrates a good reproducibility
16 of chromatographic separation (Figure S-3). The minimal
17 occurrence of sample carry-over was due to the online clean-
18 up of the sample loop during the analysis. In addition to the
19 leucine enkephalin standard extraction, reproducibility of the
20 chromatographic separation of neuropeptides from mouse
21 brain and corresponding carry-over has been investigated.
22 Relative standard deviation values vary from 7.1% to 25.4%
23 and the sample carry-over is <0.8% (Figures S-4, S-5, and S-
24 6). These values were obtained from three tissue extractions
25 followed by a blank extraction in use of the absolute
26 chromatographic peak area. While the implementation of μ LC
27 into our platform significantly minimizes the amount of
28 ionization suppression, the reproducibility could be
29 considerably improved by the addition of an isotope-labelled
30 internal standard to the tissue sample.

31 **Post-surface sampling chromatographic separation.**

32 Having demonstrated that the HR-LESA provided higher
33 spatial resolution, good reproducibility, and minimal sample
34 carry-over, we implemented a μ LC separation to enable
35 isomeric separation and further sample clean-up. Isomers have
36 the same mass and the same molecular formula, in contrast to
37 isobaric compounds, which have the same nominal mass but
38 differ in molecular formula. MS alone is insufficient to
39 separate and accurately identify isomeric compounds (even
40 with high mass resolution instrumentation). Furthermore, ion
41 suppression often occurs and is considered the primary cause
42 of irreproducibility in MS. Because LC is a powerful tool for
43 separation of these isomeric compounds,⁵² we integrated an
44 online washing step and μ LC separation after the surface
45 sampling and prior to electrospray ionization (Figure 1).
46 Compared to conventional LESA, the HR-LESA approach
47 with online washing and μ LC separation showed an increase
48 in sensitivity and selectivity for leucine enkephalin in the
49 adenyphopphysis region of rat pituitary online (Figure 3).
50 When we performed our HR-LESA- μ LC-HDMS^E approach
51 for the analysis of pituitary, 67 compounds could be
52 chemically identified, while out of this list (Table S-2) only 5
53 and 4 compounds could be found in the conventional LESA-
54 HDMS and LESA-MS data, respectively. For the mouse brain
55 cerebral cortex analysis, 14 compounds could be identified
56 with HR-LESA- μ LC-HDMS^E while out of this list (Table S-4)
57 only 3 and 2 compounds were observed in the conventional
58 LESA-HDMS and LESA-MS data, respectively. In view of
59 the even larger spot size of LESA-MS, the minimization of
60 ionization suppression by μ LC is crucial for detection of lower
abundant compounds. Although the implementation of μ LC in
our approach requires a longer analysis time (30 min/spot)
compared to conventional LESA-MS (1 min/spot), this can be

justified due to the enhanced detection limits of the HR-
LESA- μ LC-HDMS^E platform compared to conventional
LESA-HDMS and LESA-MS.

Interestingly, in the pituitary data, we observed the presence of
a triply charged species (Figure 3d) co-eluting with the leucine
enkephalin, which was not detected with the conventional
LESA extraction (Figure 3b). This difference can be explained
by the occurrence of ion suppression, which was minimized
by HR-LESA- μ LC-MS. The extracted ion chromatogram
(XIC) based on the protonated molecule signal of leucine
enkephalin (m/z 556.28 \pm 0.05) extracted from the
adenohypophysis region showed a retention time of 16 min,
similar to that of the monoisotopic peak of the triply charged
species (m/z 555.65, Figure 4a).

However, the corresponding mass spectrum shows a different
isotope pattern belonging to a triply charged species (Figure
4b, green asterisk). This clearly exposes the limitations of LC
separation and need for additional separation power to
increase selectivity and accurate identification. For this reason,
an additional gas-phase IMS was added to the analytical
workflow to increase the analytical content of the data.

Ion mobility separation (IMS) of isobaric species. We
employed tri-wave ion mobility to achieve additional
separation: the TRAP T-Wave region was used for trapping
and accumulating ions, IMS T-Wave region for subsequent
separation, and the TRANSFER T-Wave region to focus the
ions and also as fragmentation cell when performing data-
independent HDMS^E measurements.

IMS was performed from the anterior lobe HR-LESA
extraction (m/z 556.28 eluting at t_R = 16 min; Figure 5a). The
extracted ion mobility drift time spectrum (Figure 5b) depicts
two drift time peaks, which can be labelled using the
corresponding mass spectra as leucine enkephalin (Figure 5d)
and its co-eluting compound (Figure 5c). The mass spectra
extracted at this specific retention time displayed clean signals
with overall improved sensitivity. The observed isotope
pattern of the triply charged species has a mass of 1664 Da
after deconvolution. The advantage of the integration of IMS
after chromatographic separation is the additional separation
power that is gained to separate co-eluting isomeric species,
without compromising the overall analysis time. For the
identification of this co-eluting species and other extracted
proteins (Figure S-7), HDMS^E analysis was performed.
HDMS^E is a data-independent acquisition (DIA) mode that
includes both high and low collision energy measurements
alternated in one parallel analysis using the retention time to
match precursor and product ions. Species can be identified
in one analysis without requiring additional data-dependent
MS/MS experiments by performing HDMS^E analysis.

**Identification of neuropeptides and proteins present in the
pituitary gland.** We sought to identify neuropeptides and
proteins in the rat pituitary samples using our platform by
integrating data-independent acquisition (DIA). We chose the
DIA approach because data-dependent acquisition (DDA)
often neglects low-abundance precursor ions, which limits the
discovery of untargeted analytes and markers.⁵³

One DIA approach, MS^E, collects TOF mass spectra with and
without fragmentation by alternating the energy of the
collision cell between low and high values.⁵³ Another
approach, sequential window acquisition of all theoretical
fragment-ion spectra (SWATH), was developed on hybrid
QqTOF mass analyzers that offer resolving power of 20,000–
40,000.⁵⁴ Both approaches were demonstrated to be

particularly powerful in detecting low-abundance analytes for further metabolite and peptide identifications. We integrated high-definition MS^E (HDMS^E) acquisition—including ion mobility separation—into our platform.

HDMS^E detects both precursor ions and fragments of the precursor ions fragmented independently on their abundance.⁵⁵ Progenesis QI for proteomics software uses an algorithm that also performs a database search considering retention time, mass accuracy, and PTMs.⁵³ However, due to the DIA nature of these analyses, this algorithm is based on physicochemical properties of the peptides and proteins. These characteristics are used to calculate the correlation to models with regards to hydrophobicity and gas-phase separation and are, therefore, applicable to reversed-phase LC and IMS. In similar strategy to DDA to calculate false-positive identification rates, we applied decoy and species-specific databases. Sensitivity and selectivity are significantly increased because of the repetitive approach of this algorithm. After the identification of the most abundant protein, this data is removed from the database, and another search is executed to identify the second most abundant protein. After removing the data from the second most abundant protein, a third search is performed. This process is continued until all proteins are identified.

In the extracts from the rat pituitary gland, we identified vasopressin and POMC (Figure S-8), based on 17 and 25 peptides, respectively (Table S-1; Table S-2 lists the peptides found).⁵³ These peptides, which are deriving from the pro-opiomelanocortin (POMC) precursor protein, are important signaling molecules in regard to feeding behavior and, therefore, involved in diseases like obesity, bulimia, and anorexia nervosa.^{56,57} In the mouse brain cerebral cortex extracts, we identified 14 peptides (Table S-3; Table S-4 lists the peptides found).

After applying *de novo* peptide sequencing to the fragmentation spectrum of the co-eluting species at *m/z* 1664 at the retention time of 16 min, this compound was identified as N-acetylated alpha-melanocyte stimulating hormone based on nine fragment ions: *y*₁, *b*₁, *y*₂, *y*₇⁺², *y*₅, *y*₁₀⁺², *y*₁₂⁺², *y*₇, and *y*₈, deriving from P01194 (124–136). The MS/MS spectrum can be found in the supplementary data (Figure S-9). Based on the MS^E data, the singly charged species was identified as leucine enkephalin (Figure S-10).

CONCLUSION

This work reports the development of an automated and integrated platform combining the advantages of both spatial sampling with LESA and chromatographic separation (μLC) for the direct analysis of tissue sections with potential clinical, pre-clinical relevance (Figure 1). In addition, the platform was strengthened by the integration of ion mobility spectrometry (IMS) and data-independent acquisition (DIA) for both the separation and the identification of neuropeptides from a complex tissue extract from a 400-μm area. Improvement over the “conventional LESA” of the spatial resolution capabilities of the surface-sampling process was achieved, down to a spatial resolution of 400 μm, by modifying the sampling probe (Figure 2). The platform demonstrated strong reproducibility, minimal carry-over, increased sensitivity, technical reproducibility, and identification of isobaric compounds (Figure 3–5).

The advantages of this integrated platform hold strong potential for pre- and clinical applications. Currently, the

extraction efficacy is limited to soluble proteins with the number of protein and peptide identifications at fewer than 500. In particular, low-abundance analytes can be identified in complex and small tissue samples, as demonstrated with rat pituitary and mouse brain here. Information about protein isoforms, important in many neurodegenerative diseases, is predicted to be elucidated with the application of our platform with classical in-gel proteomics.⁴² This approach paves the way for imaging researchers to increase the total number of proteins and peptides identified and enable regional quantification-based on-tissue analysis.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Supporting information (PDF)

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Figures

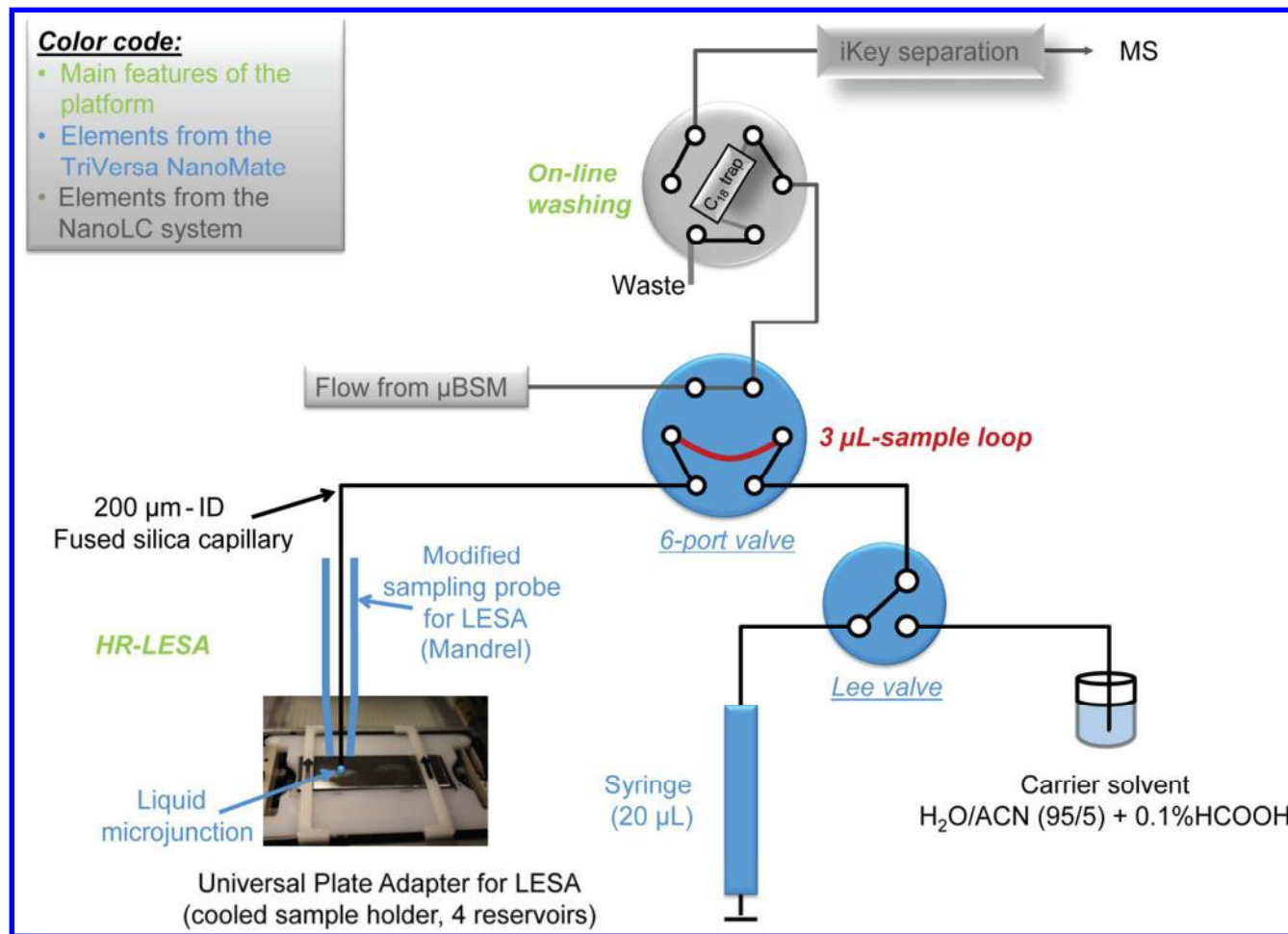


Figure 1. Schematic representation of flow connections between the elements of the μ LC system (in grey) and the automated sampler device (blue). Main features of the analytical platform are indicated in green. The route of the sample loop is shown in red. The 6-port valve is in the “sample loading” position. HR: high spatial resolution. LESA: liquid extraction surface analysis. BSM: binary solvent manager.

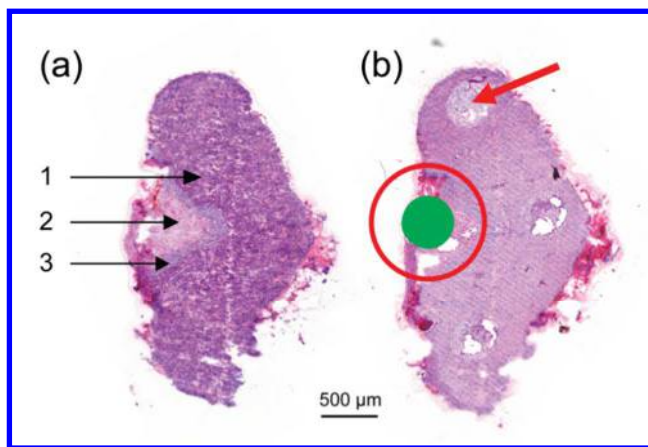


Figure 2. H&E stained images of the pituitary gland before (a) and after (b) HR-LESA extraction show an improvement in spatial resolution with HR-LESA (i.e. 400 μm area represented by a green dot in b) compared to conventional LESA (area shows the spot size of a typical LESA extraction (i.e., 1000 μm -diameter, red circle in b). In (b), the arrow indicates the trace after an extraction with the capillary. Anatomical features of the pituitary gland containing the adenohypophysis and the neurohypophysis. Anatomical features: 1. Anterior lobe; 2. Posterior lobe; 3. Intermediate lobe.

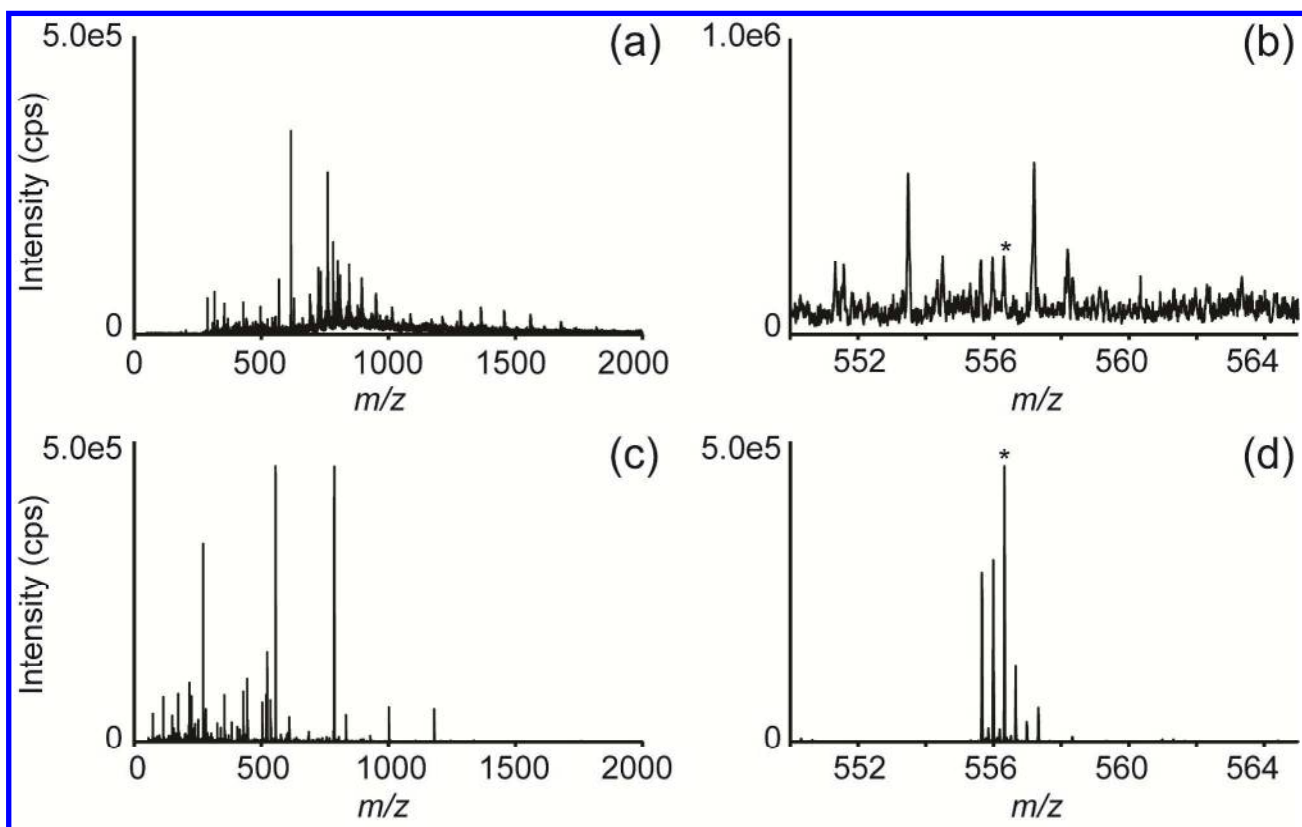


Figure 3. Sensitivity of LESA-MS versus HR-LESA- μ LC-MS: The total mass spectrum (a, c) and a zoomed-in mass spectrum (b, d) of the adenohypophysis region of rat pituitary gland using LESA-MS (a, b) compared to HR-LESA- μ LC-MS (c, d). The zoomed mass range displays a low-intensity peak corresponding to the protonated molecule of leucine enkephalin (marked with *), which is known to be present in this region. This is extracted from the ion chromatogram at retention time at 16 min. The zoomed mass range from HR-LESA- μ LC-MS shows the presence of leucine enkephalin co-eluting with a triply charged species, which was not observed with LESA-MS.

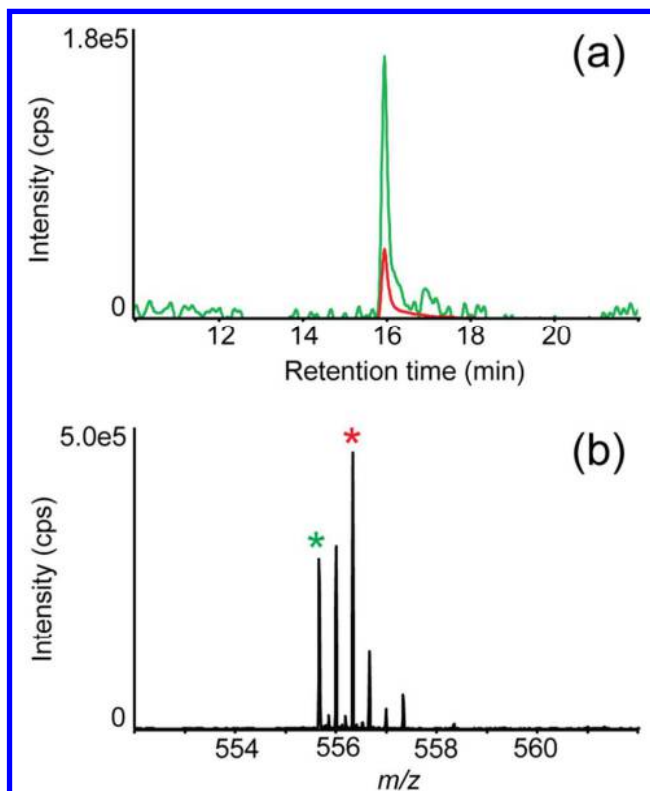


Figure 4. The extraction of two co-eluting compounds: the XIC (a) and corresponding mass spectra (b). The green XIC represents the chromatogram extracted from the monoisotopic peak of the triply charged species at m/z 555.65. The red XIC represents the chromatogram extracted from leucine enkephalin at m/z 556.28.

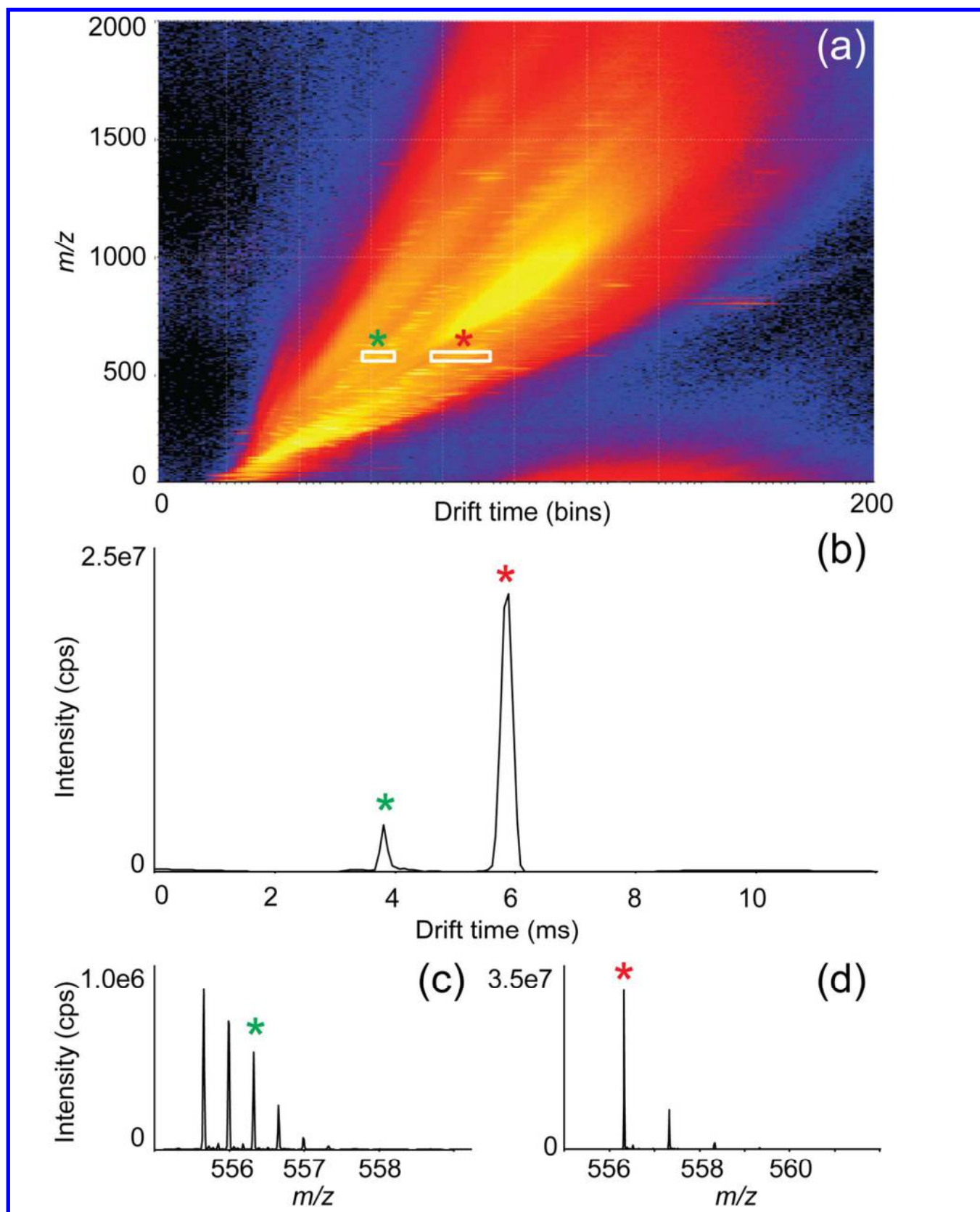
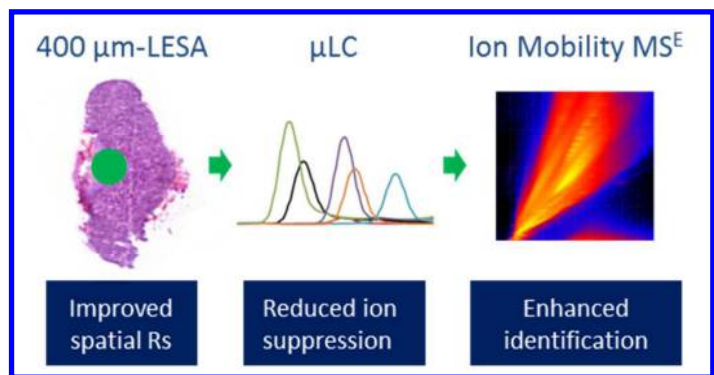


Figure 5. The ion mobility (a) of the extract from the anterior lobe of pituitary gland is depicted. The extracted ion mobility drift time spectrum (b) shows the separation of two co-eluting compounds. The extracted mass spectra at drift time 3.8 ms (c) and 5.6 ms (d) show the separation of both compounds, indicated in red (leucine enkephalin) and green (N-acetylated alpha-melanocyte stimulating hormone).

For TOC only



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