Anal. Chem. **2006,** *78,* 7145−7153

MALDI-MS Direct Tissue Analysis of Proteins: Improving Signal Sensitivity Using Organic Treatments

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Direct tissue analysis using MALDI-MS allows the generation of profiles while maintaining the integrity of the tissue, displaying cellular localizations and avoiding tedious extraction and purification steps. However, lower spectral quality can result from direct tissue analysis due to variations in section thickness, the nature of the tissue, and the limited access to peptides/proteins due to high lipid content. To improve signal sensitivity, we have developed a tissue-washing procedure using organic solvents traditionally used for lipid extraction, i.e., $CHCl_3$, hexane, toluene, acetone, and xylene. The increased detection for peptides/proteins (m/z 5000−30 000) is close to 40% with chloroform or xylene, and 25% with hexane, while also improving sample reproducibility for each solvent used in the present study. This strategy improved matrix cocrystallization with tissue peptides/proteins and more importantly with cytoplasmic proteins without delocalization. The extracted lipids were characterized by nanoESI-QqTOF/MS/MS using the precursor ion mode, lithium adducts, or both and were identified as phospholipids including phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and lysophosphatidylinositol, confirming membrane lipid extraction from the tissues.

Matrix-assisted laser desorption/ionization (MALDI)[1,2] is now an extensively used and hard to circumvent technique for biomolecular analysis of peptides/proteins, glycoproteins, sugars, lipids, and even oligonucleotides. It is a revolutionary technology for proteomic studies when combined with traditional separation techniques such as gel electrophoresis or liquid chromatography. However, such strategies lead to loss of the localization of the biomolecules inside the tissue. Using direct analysis of tissues and by extension molecular imaging by mass spectrometry, this difficulty can be overcome.[3,4] Since, its introduction by Caprioli and collaborators, MALDI direct analysis and imaging of peptides/proteins were successfully used for the analyses of different tissues and different applications.[4−8] More recently, MALDI imaging has been extended to lipid and drug analyses.[9−15] Several fundamental developments have also contributed to improvements in MALDI imaging performance in instrumentation, sample preparation, and informatics treatment. Several applications have already been established for this technology for drug biodistribution studies,[13,16] biomarker discovery, or understanding molecular mechanisms.[4,6,17,18] The application of MALDI imaging is a promising technique for pathologies such as cancers or diseases related to aging or metabolic disorders.

Increasing the representation and the reproducibility of analysis is difficult since tissues are highly complex matrixes including a wide range of biomolecules such as peptides/proteins, oligonucleotides, sugars, and lipids present in various quantities. In

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10.1021/ac060565z CCC: $33.50
Published on Web 09/15/2006

this regard, tissue sections are more complex samples to analyze than separated or purified ones. For example, it is observed that analytical quality, especially for peptides/proteins, is excellent for freshly dissected tissue samples but that signal intensity decreases drastically with the length of time of conservation. Signal decrease is due to a low signal-to-noise ratio and poor reproducibility. After one-year storage, even at −80 °C, it is difficult to obtain good peptide/protein spectra for tissues. This phenomenon of tissue aging must be pointed out when experiments in direct profiling or MALDI imaging are undertaken. In fact, it is not always possible to analyze freshly prepared samples especially when working in the clinical field, where samples are difficult to collect and are stored in centralized tissue repositories. It is therefore of even greater importance to find tissue treatments that can ensure a good signal for peptides/proteins from such samples by MALDI. In a previous publication, the addition of an ethanol washing step was introduced to reduce salts and hemoglobin contaminants on the tissue surface.[19] To make further improvements, compatible with protein profiling, we tested washing tissues with organic solvents traditionally used for lipids extraction, e.g., chloroform,[20,21] hexane,[22] or xylene. This strategy resulted in marked improvements of matrix access to peptides and proteins in the tissue sections and more particularly to the ones located in the cytoplasm without any delocalization of the proteins.

EXPERIMENTAL SECTION

Materials. α-Cyano-4-hydroxycinnamic acid (HCCA), sinapinic acid (SA), angiotensin II, Des-Arg-bradykinin, substance P, ACTH 18−39, ACTH 7−38, and bovine insulin were obtained from Sigma-Aldrich and used without any further purification. Trifluoroacetic acid (TFA) was purchased from Applied Biosystems. Acetonitrile p.a. and methanol p.a. from J. T. Baker.

Tissue Preparation. Adult male Wistar rats weighing 250−350 g (animal welfare accreditation by the French ministry of the agriculture N*o*. 04860) maintained under standard care were used. Animals were sacrificed by decapitation and immediately dissected to remove the brain. Frozen sections of 15 μm were performed on a cryostat and immediately transferred onto the MALDI stainless steel plate. The matrix solution was applied to several sections after 10 min of drying at room temperature. The rest were kept for organic solvent treatments prior to matrix deposition and MALDI analysis.

Tissue Treatment. Tissue sections were quickly rinsed using different organic solvent. Typically, the rinsing was performed with a glass syringe, using twice 200 μL of solvent/cm^2 of tissue. During the operation, the MALDI plate was inclined to drain excess solvent and remove impurities or compounds extracted by the treatment. After complete drying, matrix was applied onto the tissue and dried at room temperature before MALDI analysis.

Mass Spectrometry. (a) MALDI-MS Direct Analysis. MALDI-TOF mass spectra were performed on a Voyager-DE STR mass spectrometer (Applied Biosystems, Framingham, MA) with delayed extraction (DE) and a 337-nm pulsed nitrogen laser. Either HCCA or SA was used at concentrations of 10 and 20 mg/mL, respectively, in acetonitrile (ACN)/0.1% TFA−H_2O (2:1, v/v). Both matrixes were applied onto the tissue using a micropipet (typically 20 μL for a whole rat brain slice) and then dried at room temperature. For compounds with m/z<10 000, HCCA matrix was used and external calibration was performed using a mixed solution of peptides (bradykinin 1.6 μM, substance P 1.6 μM, ACTH 18−391.6 μM, ACTH 7−383.2 μM, bovine insulin 4.8 μM, and bovine ubiquitin 4.8 μM in H_2O). For higher masses, SA was preferred as matrix and hemoglobin chain α (major) and hemoglobin chain β (major) peaks were used as internal calibrants.[5] Slices were visualized in the mass spectrometer using a color CCD camera (Sony). Each recorded mass spectrum is resulting from the average of 200 laser shots on the area of interest. Acquisition parameters were set as follow: HCCA matrix (mass range 1000−10000): acceleration voltage 25 kV, first grid voltage 94%, guide-wire voltage 0.05%, and extraction delay time 200 ns. SA matrix (mass range 2000−30000): acceleration voltage 25 kV, first grid voltage 94%, guide-wire voltage 0.2%, and extraction delay time 300 ns.

(b) MALDI Imaging Experiments. For MALDI-IMS, imaging was performed on an Ultraflex LIFT-TOFTOF (Bruker Daltonics, Bremen, DE) using the ionic matrix HCCA/ANI for peptides (below 5000 Da) in positive reflector mode[15] and sinapinic acid (20 mg in ACN/TFA 0.1% in H_2O (1:1, v/v)) for compounds over 5000 Da in positive linear mode. For a better crystallization on the tissue, the ionic matrix was prepared by increasing the volume of base (1.5 equiv) added in HCCA (10 mg) dissolved in a volume of 1 mL of ACN/0.1% TFA−H_2O (2:1, v/v).

Acquisition was realized using a 337-nm, pulsed nitrogen laser, with a repetition rate of 50 Hz. For images reconstruction the FlexImaging v. 1.0.6.0 software (Bruker Daltonics) was used. For positive mode, 12 000 points covering the whole slice with 100 laser shots per position were scanned. From each position, the software measures an average mass spectrum with its coordinates on the slice. Images were reconstructed using the same parameters for treated and nontreated sections.

(c) Nano ESI-QqTOF/MS and MS/MS Experiments. Analyses were performed on a QSTAR pulsar quadrupole time-of-flight mass spectrometer (MDS Sciex) equipped with a nano-electrospray ion source (Proxeon Biosystems). Spectra were acquired in both the positive and negative modes after dissolving compounds in $CH_3OH/CHCl_3$ (9:1, v/v). Collision energy was set at 45 eV for collisional activation. Precursor ion scan experiments were performed in both the positive and negative mode (5 scans at 800 ms), with 0.5 amu increments, at unit resolution for the Q1 quadrupole. Acquisitions were done with Analyst QS software and treated with the same software or "m/z" (Genomic Solutions Inc.). For lithium adduct ions, samples were spiked using a 2 mM LiCl solution.

Immunohistochemistry. Immunohistochemistry experiments were performed according to classical protocols. Briefly, tissues were placed in 0.1 M PBS buffer (pH 7.4) at 4 °C for 30 min and then incubated at room temperature with 500 μL of a second buffer (0.1 M PBS/1% BSA/1% normal goat serum/0.05% Triton X 100) for 30 min. The same buffer was used to dilute the oxytocin peptide antibody (1/200), and incubation was performed for 80 min at 30 °C. After 3 times washing in PBS, sections were

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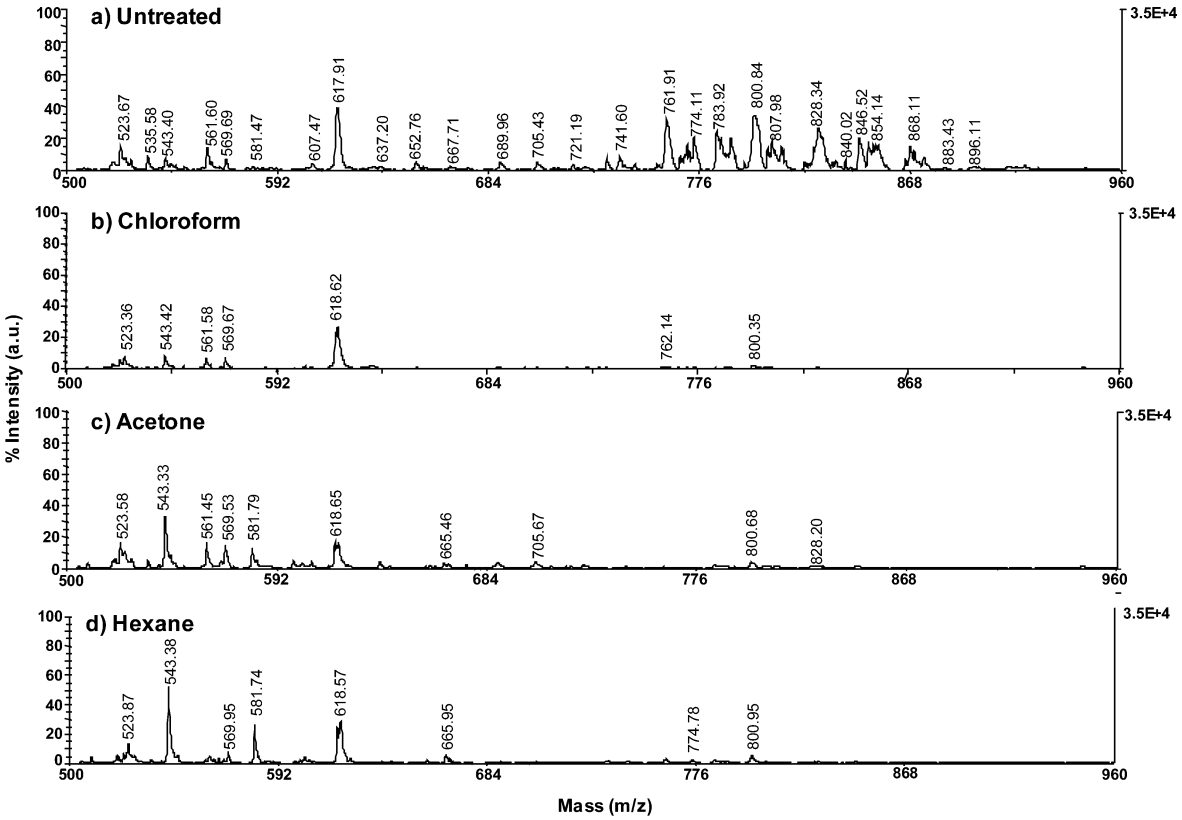

Figure 1. Low mass range region of the MALDI-TOF mass spectra resulting from the direct analysis in linear positive mode with HCCA as matrix of rat brain tissue sections from a tissue block conserved 6 months at −80 °C.

incubated with peroxidase-conjugated secondary antibody (anti-rabbit IgG 1/100 developed in goat; Jackson Immunoresearch Inc. Europe Ltd.) during 80 min at 30 °C. After another 3 washing steps, the sections were incubated in 3,3′-diaminobenzidine tetrahydrochloride (DAB) with 0,.05% H_2O_2 for detection. The DAB reacts with the peroxidase group of the secondary antibody, leading to a brown precipitate. Tissues were then compared using microscopy after additional washing in phosphate buffer and ultrapure water.

The procedure and the characterization of the antioxytocin used were according to the one from Salzet et al.[23]

RESULTS AND DISCUSSION

The following organic solvents, chloroform, hexane, acetone, toluene, and xylene, were tested on rat brain tissue sections. Tissues were stored (−80 °C storage) for periods ranging from freshly dissected to one-year storage, from which signals for peptides/proteins were very weak when compared to freshly dissected slices. The main difficulty with these experiments was linked to variations between animals and in the chemical composition of the different areas of the brain. Thus, care was taken to analyze the same area of each section on adjacent sections. To obtain a statistical analysis, experiments were repeated at least 5 times for each parameter. The first set of experiments was performed using HCCA as the matrix. In general, for long storage after treatment, the analyses revealed a signal increase in peptides/proteins as compared to untreated samples for all solvents. A more detailed inspection of the mass spectra clearly shows a major signal decrease in the mass range 500−1000 for all solvents (Figure 1). This effect is particularly visible in chloroform-treated tissue. Signal loss in this region could be attributed to reduction of lipids by chloroform extraction, which is in good agreement with the chemistry of lipid extraction[24−26] and lipid direct analysis.[27,28] It must be noted that partial lipid removal simplifies the MALDI mass spectra in the low-mass range, thus allowing the identification of low-mass peptides that were masked by the high abundance of lipid peaks. As shown in Figure 1, the peak profiles are slightly different depending on the solvent use, which suggests specific extraction of certain classes of lipids depending on the treatment.

To study the increase of signal intensity in the mass ranges corresponding to proteins, experiments were repeated with SA as a matrix. This experimental set confirmed the increase in peak intensities for the mass range 5000−30 000 with chloroform and xylene showing a particularly clear effect (Figure 2) in comparison to untreated tissues. Table 1 summarizes the averaged results for all experiments. From the mass spectra (Figure 2), increased intensity is clearly observed after solvent treatment of the tissue. Table 1 also shows an increase in the number of detected compounds. On average, 26 more compounds were detected after

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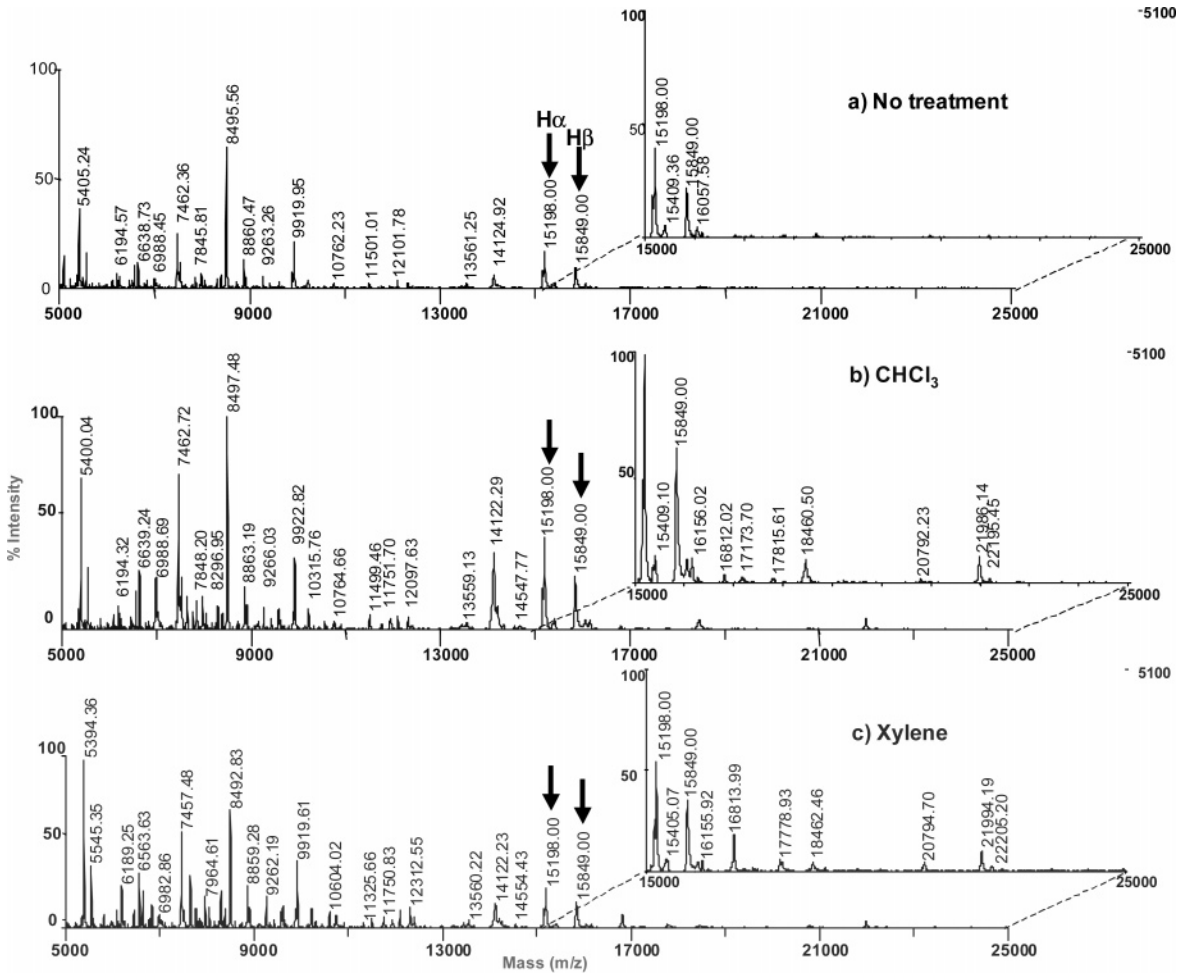

Figure 2. *m*/*z* >5000 region of the MALDI-TOF mass spectra resulting from the direct analysis in linear positive ion mode with SA as matrix of rat brain tissue sections from a tissue block conserved 6 months at −80 °C. (a) Untreated sample, after tissue treatment with (b) chloroform and (c) xylene (the arrows indicate the peaks corresponding to hemoglobin α chain (major) or Hα and hemoglobin *β* chain (major) or H*β* used as internal calibrants).

Table 1. Average Number of Detected Compounds, Standard Deviation, and Calculated Increase Detection for Peptides/Proteins of *m*/*z* >5000 Determined from the Mass Spectra Recorded on Untreated Rat Brain Sections versus Organic Solvent Treated Ones

treatment	*n*[a]	no. of detected compds	standard deviation (%)	increase in detection (%)
chloroform	10	81	22	34
hexane	5	75	28	25
toluene	5	68	22	13
xylene	5	86	13	44
acetone	5	64	29	7
untreated	10	60	34	0

[a] Number of experiments.

treatment with xylene and 21 more with chloroform, for an increase in detection of peptides/proteins of 44 and 34%, respectively. A decrease in standard deviations and an improvement in reproducibility were registered after treatment of the tissue for all solvents tested. The same trend was observed when treating freshly dissected tissues. However, the effect was less significant for these tissues and was more dependent on the rat brain region probed; i.e., it presumably depended on the lipid versus peptide/protein composition of the region. Hence, increase in protein detection is directly related to lipid decrease, presumably due to lipid extraction, giving easier access to intracellular proteins by breaching the cell membrane. To optimize protein analyses, detected peak numbers and intensities were studied as a function of the solvent volume used for the rinsing step. Volumes of 50−500 μL were tested. Treatment times were not considered as a variable due to the high volatility of some solvents. Under our experimental conditions, we did not observe major differences with increased volumes. A solvent volume of 200 μL/cm^2 appeared to be sufficient for optimal results. On the other hand, the washing procedure has to be repeated twice to get optimal reproducibility. Rinsing of the sample was preferred to dipping in bath of solvent to avoid loss of tissue, through detachment from the support. Since ethanol tissue treatment was previously described by Schwartz et al.[19] to remove salts, we therefore compared ethanol treatment to chloroform and xylene treatments (Figure 3). Our data show that signal intensity, signal-to-noise ratio, and number of peaks were always higher using either xylene or chloroform.

Considering each of the solvent properties, we conclude that improved detection and reproducibility is related to the decreased lipid levels in the tissue sections. However, since low-abundance

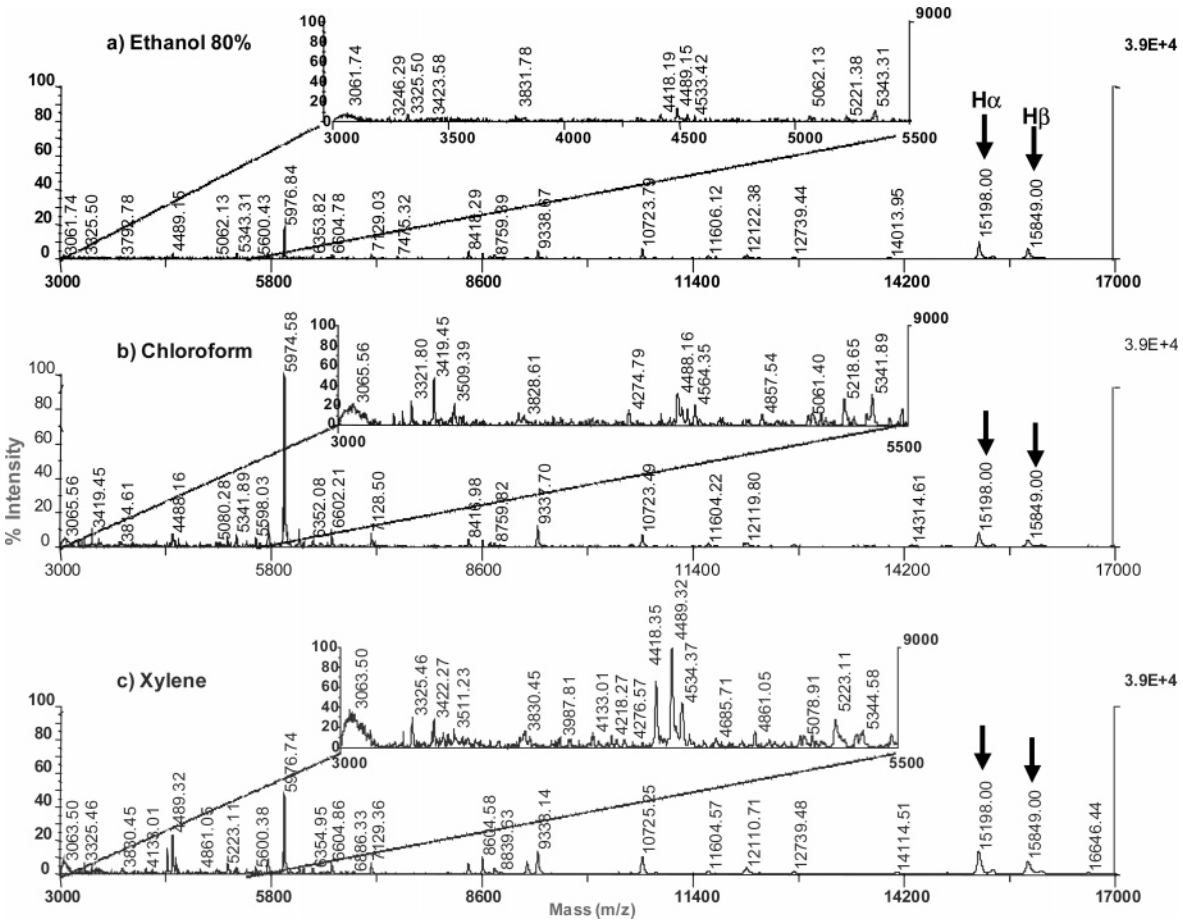

Figure 3. *m*/*z* >3000 region of the MALDI-TOF mass spectra resulting from the direct analysis in linear positive ion mode with SA as matrix of rat brain tissue sections from a tissue block conserved 6 months at −80 °C, after tissue treatment with (a) 80% ethanol, (b) chloroform, and (c) xylene (the arrows indicate the peaks corresponding to hemoglobin α chain (major) or Hα and hemoglobin β chain (major) or Hβ used as internal calibrants).

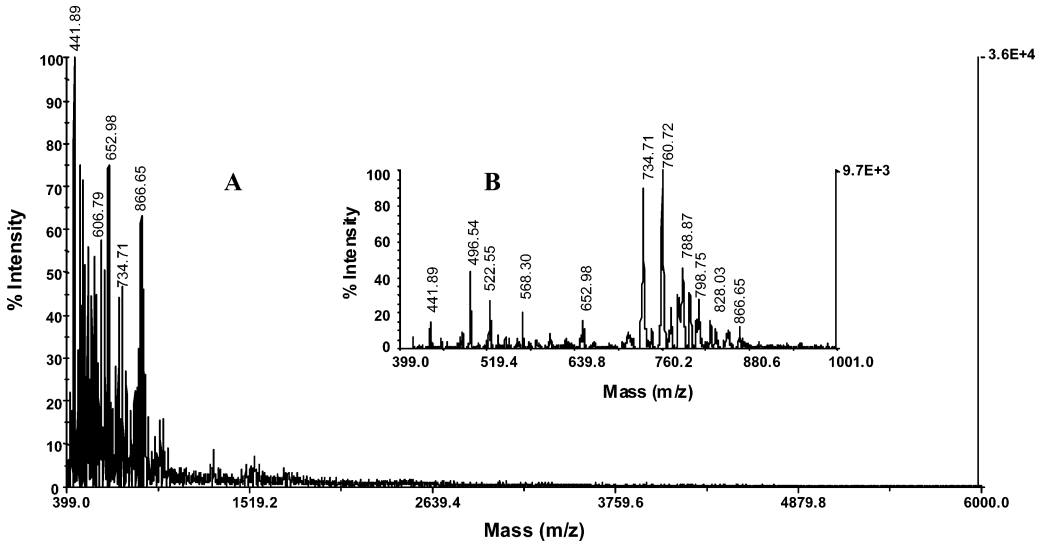

Figure 4. MALDI-TOF mass spectra using HCCA as matrix in the linear positive mode of the chloroform rinsing solution collected after tissue treatment. (A) 400−6000 and (B) 400−1000 *m*/*z*.

peaks corresponding to lipids remain observable on the mass spectra (Figure 1), washing did not allow the total removal of lipids. We conclude that increased detection is most likely due to the opening of the lipidic bilayer and that the solvent acts more as a "peeling" agent. This effect would permit a better cocrystallization of peptides/proteins contained in the cytoplasm within the MALDI matrix crystals.

To evaluate the validity of this hypothesis, washing solutions were analyzed after tissue treatment. Five rinsing solutions of each solvent were collected after the tissue treatment. The solutions

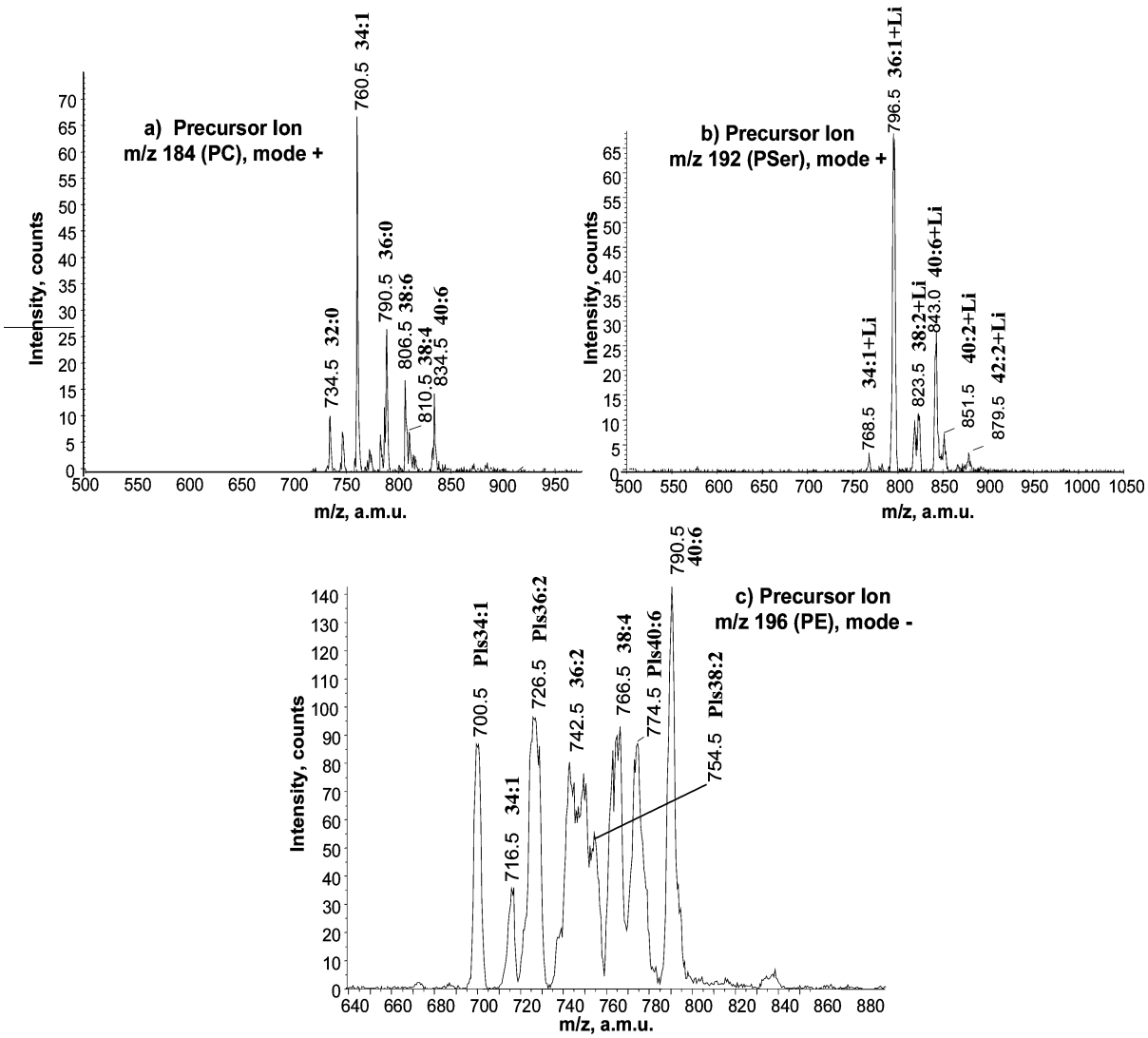

Figure 5. CID precursor ion spectra using nanoESI-QqTOF by analyzing the extracts collected after tissue treatment with chloroform for (a) *m*/*z* 184 (phosphatidylcholine) in positive mode, (b) *m*/*z* 192 (phophatidylserine) with lithium adducts in positive mode, and (c) *m*/*z* 196 (phosphatidylethanolamine or plasmonylethanolamine) in negative mode.

were evaporated under a N_2 stream, dissolved in chloroform/methanol, and then analyzed by MALDI-TOF and nanoESI-QqTOF. MALDI-TOF analyses were performed with both HCCA and SA matrixes. In none of the cases, did we observe peptide/protein peaks that would usually be seen in the tissue section as shown in Figure 4A for chloroform with HCCA as matrix. On the other hand, the presence of lipids was confirmed for all solvents. Also shown in Figure 4B for chloroform with HCCA, lipids peaks are observed in the 400−1000 mass range and signals around *m*/*z* 800 are characteristic of phospholipids. The large number of lipids detected in one extract suggests the presence of a different class of lipids. A more detailed characterization of these lipids present in the chloroform extract was undertaken on a nanoESI-QqTOF by studying their CID spectra. As previously described,[29−31] differentiation in lipid forms can be achieved on the basis of characteristic product ions. Phosphatidylinositol (PI) and lisophosphatidylinositol (LPI) present a specific fragment ion at *m*/*z* 241, plasmenylethanolamine (PlsEtn) and phosphatidylethanolamine (PtdEtn) at *m*/*z* 196, and phosphatidylcholine (PC) at *m*/*z* 184. By performing precursor ion scan mode analysis, each form of lipids can be assigned. In the case of the phosphatidylserine family (PSer), only neutral loss allows the identification of these compounds. As neutral loss mode is not available on a QTOF instrument, lithium salts were used to form lithium adducts and obtain a characteristic fragment at *m*/*z* 192 as described by Ekroos et al.[32] Figure 5 presents the CID ion precursor spectra obtained for precursor ions corresponding to PC, PlsEtn, PtdEtn, and PSer families. For each type of lipid, several compounds are identified. PlsEtn can be distinguished from PtdEtn on the base of the molecular mass and the absence of detection of the second acyl group in MS/MS mass spectra (Figure 6a) as described in the work of Brouwers et al.[33] The position of the fatty acyl group on the glycerol backbone chain was elucidated according to the works

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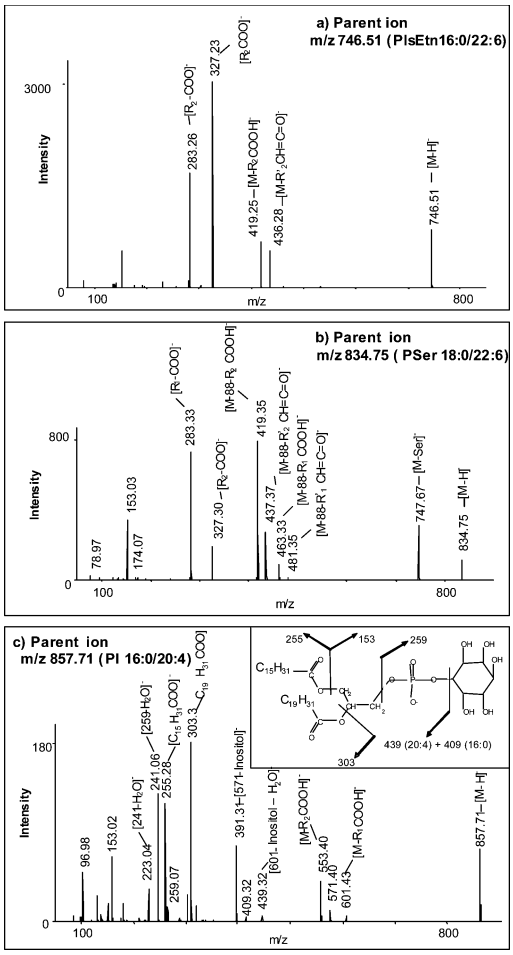

Figure 6. CID product ion spectra obtained on a nanoESI-QqTOF by analyzing the extracts collected after tissue treatment with chloroform.

Table 2. Assignment of Several Phospholipids Using Positive or Negative Precursor Ion Scanning and Product Ion Mode Experiments on NanoESI-QqTOF by Analysis of Chloroform Rinsing Extracts of Rat Brain Tissue Sections

m/z	polarity	identified lipid[a]	precursor ion
569.5	−	16:1-LPI	*m/z* 241 characteristic product ion of $[M-H]^-$ PI and LPI families
571.5	−	16:0-LPI	
599.5	−	18:0-LPI	
619.5	−	20:4-LPI	
655.5	−	22:0-PI	
857.5	−	16:0/20:4-PI	
885.5	−	18:0/20:4-PI	
909.5	−	40:6-PI	
700.7	−	34:1-PlsEtn	*m/z* 196 characteristic product ion of $[M-H]^-$ PlsEtn and PtdEtn families
716.5	−	34:1-PtdEtn	
726.5	−	36:2-PlsEtn	
742.5	−	36:2-PtdEtn	
746.5	−	16:0/22:6-PlsEtn	
750.5	−	38:4-PlsEtn	
754.5	−	38:2-PlsEtn	
766.5	−	18:0/20:4-PtdEtn	
774.5	−	40:6-PlsEtn	
790.5	−	18:0/22:6-PtdEtn	
734.5	+	16:0/16:0-PC	*m/z* 184 characteristic product ion of $[M+H]^+$PC
760.5	+	16:0/18:1-PC	
790.5	+	36:0-PC	
806.5	+	38:6-PC	
810.5	+	38:4-PC	
814.5	+	38:2-PC	
834.5	+	18:0/22:6-PC	
768.5	+	34:1-PSer	*m/z* 192 characteristic product ion of $[M+Li]^+$ PSer[f]
796.5	+	36:1-PSer	
823.5	+	38:2-PSer	
843.0	+	18:0/22:6-PSer	
851.5	+	40:2-PSer	
879.5	+	42:2-PSer	

[a] Lipid abbreviations given in text.

of Hsu and Turk[24−26] by performing CID parent ion scan as presented in example for PSer 18:0/22:6 (Figure 6b) and PI 16:0/20:4 (Figure 6c). Most abundant identified lipids are summarized by class in Table 2. Several sulfatides were also detected but in lower abundance (data not shown). For the other solvents including xylene, the same lipids were identified. As expected, the abundant detection of phospholipids confirms that treatments specifically remove lipids from tissue surface, especially membrane lipids. Moreover, PtdEtn and Pser are known to be present in high concentration in the inner monolayer of plasma membranes, while PC is dominant in the outer monolayer.[29] But, more specific lipids such as PlsEtn known to be very abundant in neuronal cells[31] were also detected.

Finally, analyses of the wash solutions from treated tissue show that no peptides/proteins are extracted from the tissue. On the contrary, many phospholipids are present, from both the outer and inner cellular membranes, confirming the hypothesis that lipid bilayer opening by the solvent facilitates matrix access to intracellular peptides/proteins for cocrystallization. Tissue treatment with solvents such as chloroform is advantageous when compared to other strategies[19] based on the reduction of salts using ethanol or other types of alcohols. Indeed, using this approach, in our experience, results in extraction of hydrophilic peptides/proteins and explains the reduction of hemoglobin signal,[19] whereas such a phenomenon is not observed with xylene/chloroform treatments as demonstrated by the washing solutions' analysis. Peptide or protein removal by solvent treatments must really be considered to prevent the potential loss of information during direct analysis of tissue.

Considering these results, increased detection limits of peptide/proteins can be obtained for tissue profiling in MALDI-MS without extraction of peptides/proteins. However, this does not mean that peptides/proteins do not migrate from their original location under the solvent treatment effect or that the tissue keeps its integrity. Knowing that solvents such as chloroform or acetone induced precipitation of proteins,[34] migration of proteins at the tissue surface by solvent dragging effects was examined. Indeed, direct tissue analysis of specific area and MALDI imaging of tissue sections fully require localization and tissue structure preservation. To confirm the latter, we used an approach based on tissue observations using optical microscopy, direct analysis of tissues, and immunohistochemistry experiments. Microscopic examination of the tissue, after treatment and solvent evaporation, shows that the tissue section has turned white. However, no change in the tissue structure was observed, demonstrating that microscope observations remain valid after tissue treatment (data not shown). To check delocalization, we studied peptide/protein signals in

(34) Jiang, L.; He, L.; Fountoulakis, M. *J. Chromatogr., A* **2004**, *1023*, 317−320.

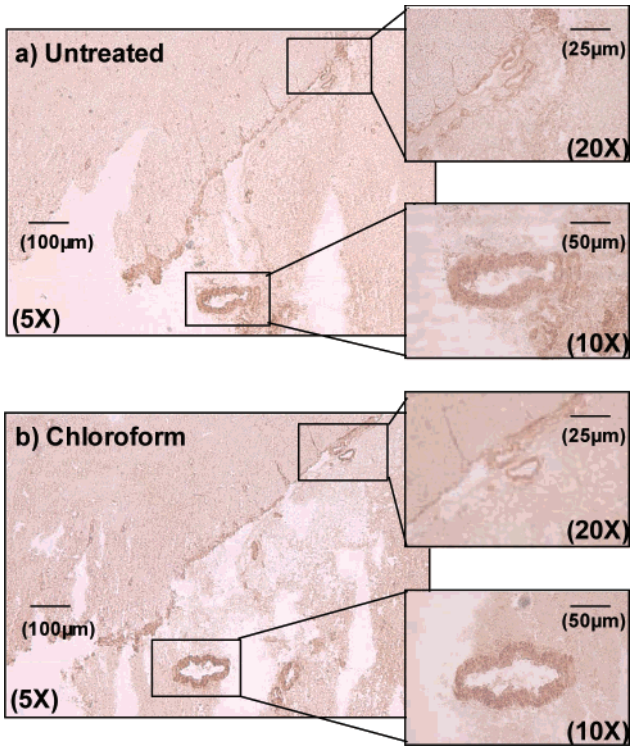

Figure 7. Immunohistochemistry staining for antibody directed against oxytocin of two adjacent rat brain sections (a) untreated and (b) rinsed with chloroform.

specific locations of treated and nontreated tissue of rat brain, known to contain specific peptides/proteins. Spectra of treated samples do not show major differences compared to nontreated ones showing no delocalization of compounds. However, immunohistochemical validation is a more powerful strategy for comparative analysis. For immunohistochemistry experiments, two adjacent rat brain sections were used, one section being treated with solvent and the other remaining untreated. The direct analysis of a section has confirmed the presence of the oxytocin peptide (M_w = 1010.2 amu) and protein precursor (M_w = 12 822.95 amu) in the tissue sections. Rabbit antibody against oxytocin was used on both sections. As shown in Figure 7 for treatment with chloroform, antibody labeling of the two sections is similar, demonstrating the same localization of the peptides and its precursor in the treated (Figure 7b) and nontreated (Figure 7a) sections. Similar results were obtained using xylene. Thus, treatment of the tissue sections does not generate any delocalization of peptides/proteins and can be used for MALDI imaging.

To study this matrix accessibility phenomenon using organic solvents, MALDI-MS imaging experiments were performed on tissues before and after treatment of adjacent rat brain sections. In this way, the potential of MALDI imaging following organic solvent improvement without delocalization can be examined. Particular attention was given to older tissue sections in these experiments (at least 6 months conservation). As seen on the images obtained after scanning (Figure 8c), it is very difficult to recognize rat brain structures in these conserved tissues sections without treatment. Following treatment with chloroform, the sections turn white and brain structures appear more clearly. For these experiments, either HCCA/ANI or SA were used as matrix depending on the mass range of interest. HCCA/ANI was used to explore the low-mass range corresponding to peptides (m/z <10 000) whereas SA was used for proteins with m/z >10 000. For identified lipids, as expected, delocalization is observed with a decrease in the ion signal intensity. For peptides such as m/z 1394 (Figure 8a) analyzed with HCCA/ANI, the effect of the tissue treatment is clear. Few ion signals with no correlated localization are observed from untreated tissues, while significant increase of signal is seen on the reconstructed images of chloroform-treated tissues, with localization much more in relation with the structures of the region. Figure 8b presents the images reconstructed from the data collected after acquisition from two adjacent rat brains sections: the first one untreated and the second one washed with chloroform and with sinapinic acid as matrix. Considering the higher mass protein, here m/z 14 306, a remarkable increase in signal intensity is observed between untreated and chloroform-treated samples. MALDI images of long conserved samples untreated and treated demonstrate clearly the effect of solvent treatment on the signal increase and localization correlation improvement of peptides/proteins with no observed delocalization.

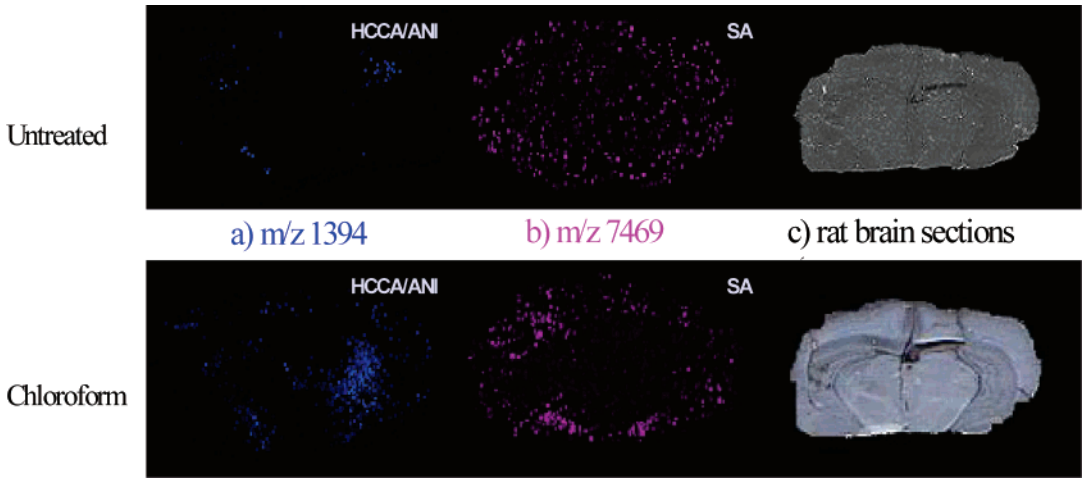

Figure 8. MALDI-LIFT-TOFTOF molecular images reconstructed from the data obtained by scanning two adjacent rat brain sections untreated (upper panel) and chloroform treated (lower panel) (a) for *m/z* 1394 with HCCA/ANI as matrix and (b) for *m/z* 14306 with SA as matrix compared to (c) pictures of the sections before matrix deposition for MALDI analysis.

CONCLUSION

Treatments with organic solvents such as chloroform, acetone, hexane, toluene, or xylene are an effective and rapid method for signal enhancement in MALDI direct tissue profiling. Solvent treatment effects provide impressive results for archived tissues which prior to these treatments did not allow proper detection. These studies demonstrate that solvent treatments remove, at least partially, lipids from the tissue surface. Among all the tested solvents, chloroform and xylene have proven to lead to the higher increase in signal intensity and number of detected peptides/proteins. Compared to previous studies with ethanol, chloroform/xylene solvent, rinsing is more specific for lipid removal and does not generate any delocalization or extraction of most soluble peptides/proteins as tested by immunohistochemistry experiments. However, this treatment does not reduce salt adducts as alcohol treatments. Application of this protocol is suitable for direct analysis profiling on tissues as well as for MALDI imaging applications. Moreover, the results suggest that it is possible to detect, after organic rinsing treatments, compounds that were masked by lipids in the tissue, like peptides/proteins present in the cytoplasm. As lipids from the outer and the inner membranes are identified from the washing extracts, we can assume that treatment opens lipid bilayers, increasing peptide/protein access for the MALDI matrix. Mostly, solvent and in particular chloroform or acetone will open the membrane layers inducing precipitation of the proteins at the surface of the tissue, resulting in cocrystallization between the matrix and the proteins. This aspect is confirmed by the white color of the tissue, suggesting precipitation. For old tissues, a layer is present at the surface of the tissue section, probably due to the degradation of lipids and possibly oxidation due to the presence of air. Treatment with chloroform or xylene probably removes this layer and partially nondegraded lipids. Such treatments are a fast and efficient method for working on tissue stored for a long time in tissue banks and open the opportunity to work with old samples stored in hospital libraries from patients with pathologies.

ACKNOWLEDGMENT

Supported by grants from Centre National de la Recherche Scientifique (CNRS), Ministère de L'Education Nationale, de L'Enseignement Supérieur et de la Recherche (ACI Jeunes Chercheurs ACI JC4074 to I.F.), le Conseil Régional Nord-Pas de Calais to M.W., and the Canadian Institutes of Health Research (CIHR to R.D.). Also supported by a collaboration agreement between Bruker Daltonics GmbH and the Laboratoire de Neuroimmunologie des Annélides. The authors acknowledge Adeline Page, the Proteomic Plateforme of Lille, IFR 118, Patrick Ducoroy, the Proteomic Plateforme of Dijon, IFR 100 and thank Dr. A. S. Woods for her critical reading.

Received for review March 28, 2006. Accepted June 16, 2006.

AC060565Z