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Solid Ionic Matrixes for Direct Tissue Analysis and MALDI Imaging

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Direct analysis of tissue by MALDI-MS allows the acquisition of its biomolecular profile while maintaining the integrity of the tissue, giving cellular localization, and avoiding tedious extraction and purification steps. However, direct tissue analysis generally leads to some extent to a lowered spectral quality due to variation in thickness, freezing tissue date, and nature of the tissue. We present here new technical developments for the direct tissue analysis of peptides with ionic liquid made of matrix mixtures (α-cyano-4-hydroxycinnamic acid (CHCA)/2-amino-4-methyl-5-nitropyridine and α-cyano-4-hydroxycinnamic acid/*N*,*N*-dimethylaniline (CHCA/DANI)). The properties of these direct tissue analysis matrixes, especially CHCA/aniline when compared to CHCA, 2,5-dihydroxybenzoic acid, and sinapinic acid, are as follows: (1) better spectral quality in terms of resolution, sensitivity, intensity, noise, number of compounds detected, and contaminant tolerance, (2) better crystallization on tissues, i.e., coverage capacity, homogeneity of crystallization, homogeneity of crystal sizes, and time of crystallization, (3) better analysis duration in term of vacuum stability, (4) better resistance to laser irradiation especially for high-frequency lasers, (5) better ionic yield in negative mode, and (6) enough fragmentation yield to use the PSD mode on sections to get structural information. Applied to MALDI imaging on a MALDI LIFT-TOF with a 50-Hz laser frequency, these ionic matrixes have allowed the realization of a new type of image in both polarities and reflector mode using the same tissue section. These results give a new outlook on peptide tissue profiling by MS, characterization of compounds from tissue slices, and MALDI-MS high-quality imaging.

Since its introduction in the mid 1980s,[1−3] matrix-assisted laser desorption/ionization (MALDI) mass spectrometry has become a powerful tool in the field of biological research and is used for the detection, identification, and characterization of peptides and proteins from complex mixtures. More recently, MALDI imaging was initiated by the Caprioli group[4−6] and is now developed by several laboratories.[7−11] These developments were applied to several biological questions demonstrating the potential of MALDI in the medical field. In fact, direct analysis by MALDI is now used for pharmaceutical or proteomics questions to detect drugs[12−14] for biomarkers discovery, or diagnosing pathology such as colon cancer,[15] prostate adenocarcinomas,[16] neuroendocrine carcinomas,[16] and brain tumors.[17]

However, direct analysis of crude samples by mass spectrometry requires maximum resolution, sensitivity, and dynamic mass range since tissues are very complex systems presenting a wide range of molecules interacting together, some of which are expressed in very low concentration in comparison to some others or presenting very close m/z ratio. To obtain optimal performances, such a technique still requires new developments either in instrumentation (e.g., new detectors,[18] higher repetition rate lasers, ion microscope images,[10] and SMALDI[7] technique for high-resolution imaging) or in sample preparation. Thus, one alternative is to develop new MALDI matrixes since a matrix is fundamental

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§ Plateforme Protéomique.

(1) Karas, M.; Hillenkamp, F. *Anal. Chem.* **1988**, *60*, 2299−2301.
(2) Karas, M.; Hillenkamp, F.; Beavis, R. C.; Chait, B. T. *Anal. Chem.* **1991**, *63*, 1193A−1203A.
(3) Tanaka, K.; Waki, H.; Ido, Y.; Akita, S.; Yashida, Y.; Yohida, T. *Rapid Commun. Mass Spectrom.* **1988**, *2*, 151−153.
(4) Caprioli, R. M.; Farmer, T. B.; Gile, J. *Anal. Chem.* **1997**, *69*, 4751−4760.
(5) Stoeckli, M.; Farmer, T. B.; Caprioli, R. M. *Nat. Med.* **2001**, *7*, 493−496.
(6) Chaurand, P.; Schwartz,. S. A.; Caprioli, R. M. *Anal. Chem.* **2004**, 87A−93A.
(7) Spengler, B.; Hubert, M. *J. Am. Soc. Mass Spectrom.* **2002**, *13*, 735−748;
(8) Li, L.; Garden, R. W.; Sweedler, J. V. *Trends Biotechnol.* **2000**, *18*, 151−160.
(9) Rubakhin, S. S.; Jurchen, J. C.; Monroe, E. B.; Sweedler, J. V. *Drug Discovery Today* **2005**, *10* (12).
(10) Luxembourg, S. L.; Mile, T. H.; McDonnell, L. A.; Heeren, R. M. A. *Anal. Chem.* **2004**, *76*, 5339−5344.
(11) Fournier, I.; Day, R.; Salzet, M. *Neuroendocrinol. Lett.* **2003**, *24*, 9−14.
(12) Reyzer, M. L.; Hsieh, Y.; Ng, K.; Korfmacher, W. A.; Caprioli, R. M. *J. Mass Spectrom.* **2003**, *38*, 1081−1092.
(13) Rohner, T. C.; Staab, D.; Stoeckli, M. *Mech. Aging Dev.* **2005**, *126*, 177−185.
(14) Bunch, J.; Clench, M. R.; Richards, D. S. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 3051−3060.
(15) Chaurand, P.; DaGue, B. B.; Pearsall, R. S.; Threadgill, D. W.; Caprioli, R. M. *Proteomics.* **2001**, *1*, 1320−1326.
(16) Masumori, N.; Thomas, T. Z.; Chaurand, P.; Case, T.; Paul, M.; Kasper, S.; Caprioli, R. M.; Tsukamoto, T.; Shapell, S. B.; Matusik R. J. *Cancer Res.* **2001**, *61*, 2239−2249.
(17) Schwartz, S. A.; Weil, R. J.; Johnson, M. D.; Toms, S. A.; Caprioli, R. M. *Clin. Cancer Res.* **2004**, *10*, 981−987.
(18) Chaurand, P.; Hayn, G.; Matter, U.; Caprioli, R. M., Poster presented at the 52nd ASMS Conference, Nashville, TN, 2004.

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for the desorption/ionization process, thus contributing to spectral quality, i.e., peak resolution, sensitivity, intensity, and noise. Actually, α-cyano-4-hydroxycinnamic acid[19] (CHCA), 2,5-dihydroxybenzoic acid[20] (2,5-DHB), and sinapinic acid[21] (SA) are the three matrixes commonly used for the analysis of peptides/proteins. For direct analysis of tissues, matrix choice is even more restrictive since homogeneity of crystallization becomes a crucial parameter in such conditions. In this respect, CHCA and SA are good candidates for direct analysis whereas 2,5-DHB crystallization is too heterogeneous for such applications except if a special spotting systems is used. Nevertheless, direct tissue analysis generally leads to some extent to a lowered spectral quality due probably to the tissue, e.g., thickness, freezing date, or type of tissue. Thus, development of new matrixes for tissue analysis is therefore a necessity for addressing such problems.

The properties of a new matrix for MALDI imaging as compared to CHCA, SA, or 2,5-DHB must be (1) better spectral quality in term of resolution, sensitivity, intensity, noise, number of compounds detected, and tolerance for contaminants, (2) better crystallization on tissues, i.e., covering capacity, homogeneity of crystallization, homogeneity of crystal sizes, and time of crystallization, (3) better analysis duration in term of vacuum stability, and (4) better resistance to laser irradiation especially for high-frequency lasers.

As previously described by Armstrong et al.,[22] coupling ionic liquids to MALDI matrixes could generate better matrixes. In fact, ionic liquids are increasingly used particularly in organic synthesis[23] and present lots of different interesting properties such as solvent recycling, low vapor pressure, or electrical conductivity.[24] Ionic liquids can be obtained from an acid/base reaction leading to liquids presenting the general formula [A^-, B^+]. Classical MALDI matrixes being acidic, acid/base reactions were already tested and could be performed using different bases to synthesize salt complexes with different MALDI ionic matrixes (2,5-DHB, CHCA, sinapinic acid, and 3-HPA[22,25]).

In the present study, we report that, in many cases, the solid ionic matrixes' performance is equal or higher than their classical analogues. Particularly, a significant signal and sensitivity improvement were observed for aniline and dimethylaniline derivatives. Considering the properties of some liquid ionic matrixes as previously described by Mank and co-workers,[26] and in particular shot-to-shot reproducibility, softer desorption properties, increase of signal/noise ratio, broader applicability, and high stability under vacuum conditions, we were interested in using solid ionic matrixes for peptide analysis directly from tissue samples. Experiments on rat brain sections demonstrate the use of such solid ionic matrixes for direct analysis of tissues especially looking in terms of shot-to-shot reproducibility, higher stability under vacuum, crystallization homogeneity, signal intensity in both positive and negative modes, sensitivity, and performances in PSD mode. Used to perform molecular imaging, solid ionic matrixes have also shown interesting properties compared to classical matrixes with very low ablation rate and especially for high laser repetition rates (>20 Hz) allowing several scans of the same tissue section without any loss in signal intensity.

EXPERIMENTAL SECTION

Materials. CHCA, aniline (ANI), *N*,*N*-dimethylaniline (DANI), *N*,*N*-diethylaniline (DIENI), triethylamine (Et_3NH), 2-amino-4-methyl-5-nitropyridine (2A4M5NP), 3-aminoquinoline (3AQ), pyridine (PY), 1,8-diazabicyclo[5.4.0]undec-7ene (DBU), piperidine (PIP), angiotensin 2, 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 and acetonitrile p.a. and methanol p.a. from J. T. Baker.

Sample Solutions. (a) Calibration Mixture. External calibration was performed using a solution of standard neuropeptides 1.6 μM bradykinin, 1.6 μM substance P, 1.6 μM ACTH 18−39, 3.2 μM ACTH 7−38, 4.8 μM bovine insulin, and 4.8 μM bovine ubiquitin in 0.1% TFA/H_2O. External calibration was performed on sample deposited on the sample holder. Thus, with the variations of tissue slices thickness (ranging from 15 to 20 μm), differences of calibration were noticed when comparing mass spectra from different tissue slices.

(b) Sensivity Tests. Substance P at 2.5 μM in 0.1% TFA/H_2O was diluted 9 times in water in order to get concentrations ranging from 1 pmol/μL to 125 amol/μL.

(c) Intensity Tests. For positive mode analysis, substance P was used at 0.8 μM. ACTH 18−39 was used at 3.2 μM for negative mode.

Preparation of Ionic Matrixes. All solid ionic matrixes (CHCA/DANI, CHCA/2A4M5NP, CHCA/3AQ) and liquid ionic matrixes (CHCA/DIENI, CHCA/DBU, CHCA/Et_3NH, CHCA/PIP) can be produced using classical protocols used for ionic liquids synthesis.[22,23] The 50-mg samples of CHCA, SA, or other MALDI matrix were dissolved in 20 mL of methanol. An equimolar amount of base was added. The solution was mixed for 1 h, and the solvent evaporated in a vacuum evaporator for 45 min (T = 50 °C, P = 40 mbar). The resulting compound was placed in a desiccator for 30 min to eliminate residual solvent and stored at −20 °C. Just before use, the ionic matrixes were prepared by dissolving 10 mg of compound in 1 mL of acetonitrile/water (2:1, v/v, 0.1% TFA).

Both solid ionic matrixes used in this study, CHCA/ANI and CHCA/PY, can also be prepared just before use, following a faster protocol: 1 equiv of base (4.8 μL for CHCA/ANI and 4.29 μL for CHCA/PY) was added to a solution of 10 mg/mL CHCA (1 mL) in acetonitrile/water (2:1, v/v, 0.01% TFA). The mixture was then vortexed and sonicated for 10 min before application to the tissue.

For classical CHCA and SA, 10 and 20 mg of the matrix were dissolved respectively in 1 mL of acetonitrile/water (2:1, v/v, 0.1% TFA/H_2O).

Tissue Preparation. Adult male Wistar rats weighing 250−350 g (animal use accreditation by the French ministry of the agriculture N*o*. 04860) were used in the study and maintained under standard care. Animals were sacrificed by decapitation and

(19) Beavis, R. C.; Chaudhary, T.; Chait, B. T. *Org. Mass Spectrom.* **1991**, *27*, 156−158.
(20) Stropat, K.; Karas, M.; Hillenkamp, F. *Int. J. Mass. Spectrom. Ion Processes* **1991**, *111*, 89−102.
(21) Beavis, R. C.; Chait, B. T. *Rapid Commun. Mass Spectrom.* **1989**, *12*.
(22) Armstrong, D. W.; Zhang, L. K.; He, L.; Gross, M. L. *Anal. Chem.* **2001**, *73*, 3679−3686.
(23) Welton, T. *Chem. Rev.* **1999**, *22*, 2071−2083.
(24) Barhdadi, R.; Courtinard, C.; Nedelec, J. Y.; Troupel, M. *Chem. Commun.* **2003**, *12*, 1434−1435.
(25) Carda-Broch, S.; Berthold, A.; Armstrong, D. W. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 553−560.
(26) Mank, M.; Stahl, B.; Boehm, G. *Anal. Chem.* **2004**, *10*, 2939−2950.

Table 1. Signal Evolution in Positive and Negative Modes Using CHCA/2A4M5NP or CHCA/DANI versus CHCA

matrix	mode	analyte	n^a	signal[b] intensity range	average[b] intensity	rsd[c] (%)
CHCA/2A4M5NP	+	SP	10	14000−29000	21000.0	20.0
CHCA	+	SP	10	1830−14500	5938.5	63.0
CHCA/2A4M5NP	+	apoMb	5	1851−14500	2011.0	7.0
CHCA	+	apoMb	5	670−1528	1084.0	31.6
CHCA/2A4M5NP	−	ACTH 18−39	10	2208−8564	4380.5	50.0
CHCA	−	ACTH 18−39	10	1116−7226	1982.5	65.5
CHCA/DANI	−	ACTH 18−39	5	5529−18000	10218.0	48.0

[a] *n*, number of experiments (sample/matrix preparation and analyses) using new preparations each time. [b] Signal intensity values are given for $[M + H]^+$ or $[M - H]^-$ ions in counts number (a.u.). [c] Rsd, relative standard deviation.

immediately dissected to remove the brain. Frozen sections of 15 or 20 μm were obtained in a cryostat and transferred onto the MALDI stainless steel plate.

Sample Preparation for MALDI/MS Analysis. For direct analysis, 20 μL of matrix solution was applied onto the frozen section using a micropipet. The sample was then allowed to dry at room temperature.

For classical analysis, 1 μL of sample solution and 1 μL of matrix solution were mixed on the MALDI plate according to the procedure of the dried-droplet preparation.

MALDI-MS Analysis. MALDI-TOF mass spectra were acquired on a Voyager-DE STR mass spectrometer (Applied Biosystems, Framingham, MA) using delayed extraction and a pulsed nitrogen laser at 337 nm.

(a) Classical Analysis in Linear Mode. Acquisition parameters were set as follows: acceleration voltage, 20 kV; first grid voltage, 94%; guide wire voltage, 0.05%; extraction delay time, 200 ns.

(b) Direct Analysis in Linear Mode. Acquisition parameters were as follows: acceleration voltage, 25 kV, first grid voltage, 94%; guide wire voltage, 0.05%; extraction delay time, 200 ns.

(c) Direct Analysis in Reflector Mode. The following parameters were used: acceleration voltage, 25 kV; first grid voltage, 75%; guide wire voltage, 0.05%; extraction delay time, 200 ns. Each recorded mass spectrum results from the average of 400 laser shots on the area of interest, considering four or five different localizations depending on the size of the cut. Slices were visualized in the mass spectrometer with a color CCD camera (SONY).

(d) PSD Mode. The following parameters were used: acceleration voltage, 25 kV; first grid voltage, 72%; extraction delay time, 200 ns. The ion precursors were selected using the timed ion gate (12 mm) of the instrument. Acquisition of the product ions was usually done at 1.0, 0.98, 0.85, 0.75, 0.60, 0.41, 0.27, 0.19, 0.12, 0.067, and 0.05 mirror ratios, and the resulting individual spectra (each an average of 200 shots) were stitched to produce a composite product ion spectrum. In the case of in situ direct analysis, only the three first windows (1.0, 0.98, 0.85) were used.

MALDI Imaging. For MALDI-IMS, imaging was performed on the Ultraflex LIFT-TOF (Bruker Daltonics, Bremen, DE) in reflector positive and negative modes, using a 337-nm pulsed nitrogen laser and with a repetition rate of 50 Hz. For image reconstruction, the FlexImaging v. 1.0.6.0 software (Bruker Daltonics) was used. For positive mode, 10 000 points covering the whole slice with 100 laser shots per position were scanned, and for negative mode, 8000 points with 100 shots were performed. From each position, the software records an average mass spectrum with its coordinates on the slice. Images were reconstructed using the same parameters for CHCA and for ionic matrix.

RESULTS AND DISCUSSION

Several ionic matrixes were synthesized and first tested on standard compounds. Three of these matrixes (solid) were further studied regarding their properties and are presented below. In particular, two new solid ionic matrixes, CHCA/2A4M5NP and CHCA/DANI, were compared with the previously described CHCA/ANI.[22]

Studies with Standards. CHCA/DANI was obtained from a reaction between CHCA and the base *N*,*N*-dimethylaniline. On the other hand, CHCA/2A4M5NP was synthesized by acid/base reaction between two classical matrixes, CHCA and a basic matrix, 2A4M5NP.[27] Both matrixes were evaluated for the production of MS ion signal in positive and negative modes by recording 400 laser shots moving slowly around over the whole spot of a mixture of substance P (500 fmol/μL) or ACTH 18−39 (1 pmol/μL).

(a) Evaluation of Spectral Quality in Positive Mode. The first step was to compare the energy threshold for ion production of substance P and ACTH 18−39, respectively, with CHCA/DANI, CHCA/2A4M5NP, and CHCA/ANI matrixes. In all cases, variations were always less than 3% between CHCA and these solid ionic matrixes. Furthermore, in all our intensity studies, the laser energy was set 20% higher than the energy threshold for peptide ions production in order to obtain high enough signal. In the positive mode, a significant signal increase was observed using CHCA/2A4M5NP ionic matrix (Table 1). The signal was 3.5-fold higher with the ionic matrix in the case of substance P than with CHCA. For apomyoglobin, the signal was also higher by a factor of 1.8 with the solid ionic matrix, demonstrating the usefulness of this matrix for both peptide and protein analysis. However, we must note that the apomyoglobin signal is wide, resulting in low resolution. This can probably be attributed to matrix adducts as observed for 2A4M5NP in its nonionic form.[27] For CHCA/ANI and CHCA/DANI on substance P, results were in the same order as for CHCA/2A4M5NP (data not shown) and for CHCA/ANI comparable to what was previously obtained by Armstrong et al.[22] for bradykinin.

(b) Evaluation of Spectral Quality in Negative Mode. Negative mode could be advantageous for direct analysis of tissue, especially for detection of compounds such as phosphorylated

(27) Fitzgerald, M. C.; Parr, G. R.; Smith, L. M. *Anal. Chem.* **1993**, *65*, 3204−3211.

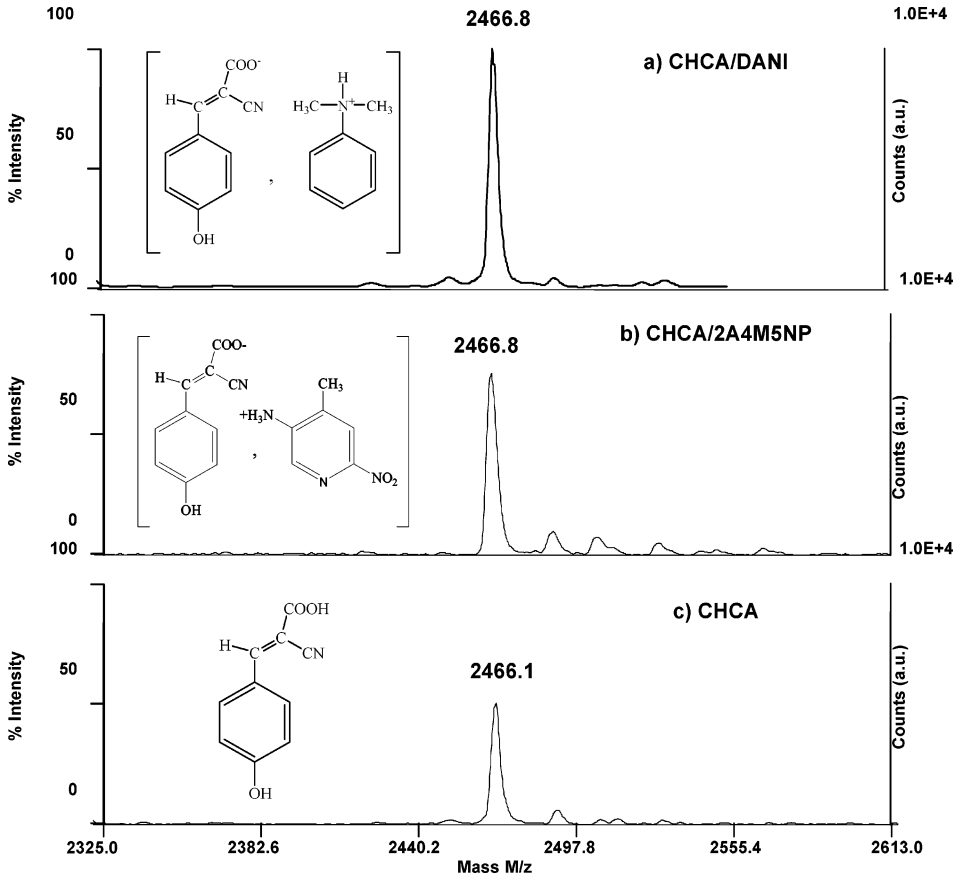


Figure 1. Typical MALDI mass spectrum obtained for ACTH 18−39 (1 pmol) in the linear negative ion mode using CHCA/DANI (a), CHCA/2A4M5NP (b), and CHCA (c) as matrix. Ionic matrix formula is enclosed to each mass spectrum.

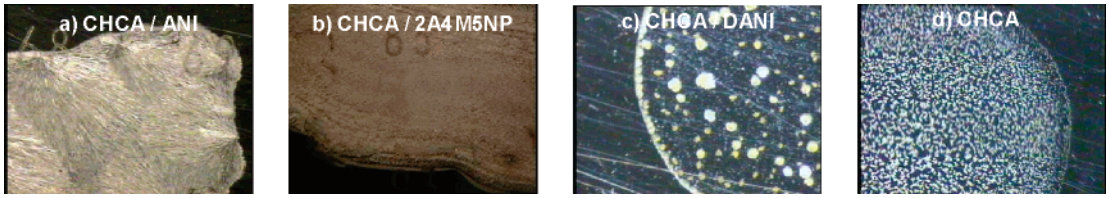
peptides,[28] lipids,[29,30] or phospholipids[31] that may show extensive adduct signals in positive mode. It can also be used to obtain complementary structural information using PSD.[32] Reduction of salt signals lead to a resolution increase and easier interpretation of data. Generally, negative ion mode is not extensively used in MALDI because ion production yields with conventional matrixes give low counts of negative ions. Here, we have tested the ionic matrixes in order to find out whether they may give better ion yields in this mode than conventional matrixes. Ionic matrixes have been previously tested for low molecular weight compounds[33] (amino acid), but no studies on peptides/proteins have yet been done. In negative mode, the best signals were recorded for the three solid ionic matrixes CHCA/ANI, CHCA/DANI, and CHCA/2A4M5NP (Table 1). A 2-fold increase in signal was observed as presented for ACTH 18−39 with solid ionic matrixes (Figure 1a and b) in comparison with CHCA (Figure 1c). In the case of CHCA/2A4M5NP, this increase could be attributed to the 2A4M5NP group of the ionic matrix. The basic properties of this group could help in the ionization step (deprotonation of the analyte) by enhancing proton transfer from the analyte to the matrix. In this respect, for classical matrix deprotonation of the analyte would be more difficult since the matrix only shows acidic properties. Ionic matrixes are salts that display the characteristics of both acidic and basic groups, as was observed in previous organic studies.[23] Looking to these results, CHCA/DANI and especially CHCA/ANI have proved to be the best matrixes in term of signal intensity enhancement in both the positive and negative modes.

(c) Sensitivity Tests. To detect biomolecules in tissue, matrixes must be very sensitive due to the low amount of material contained in a tissue section of 15 μm (for each species of peptide) and the low accessibility of molecules embedded in cells or tissue. This can be particularly critical when tissue is analyzed for discovering potential biomarkers.

Sensitivity of CHCA/2A4M5NP and CHCA/DANI was tested using substance P and ACTH 18−39 peptides at different concentrations in both negative and positive modes. Best results were obtained using CHCA/DANI. The limit of detection was found to be 250 amol in the positive mode and 100 fmol in the negative mode. These values have to be compared to CHCA for which the minimum amount detectable has been found in our experimental conditions to be respectively 1 and 250 fmol. Although, CHCA/2A4M5NP with 150 fmol has shown better sensitivity than CHCA in the negative mode, it appeared to be 100 times less sensitive than CHCA in positive mode. CHCA/ANI was found to be more sensitive than CHCA on substance P in the positive mode as previously observed by Armstrong et

(28) Asara, M.; Allison, J. *J Am. Soc. Mass Spectrom.* **1999**, *10*, 35−44.
(29) Schiller, J.; Süb, R.; Arnhold, J.; Fuchs, B.; Lebig, J.; Muller, M.; Petkovic, M.; Spatlehotz, H.; Zschörnig O.; Arnold, K. *Prog. Lipid Res.* **2004**, *43*, 449−488.
(30) Al Saad, K. A.; Zabrouskov, V.; Siems, W. F.; Knowles, N. R.; Hannan, R. M.; Hille, H. H., Jr. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 87−96.
(31) Estrada, R.; Yappert, M. C.; *J. Mass Spectrom.* **2004**, *39*, 412−422.
(32) Clipston, N. L.; Jai-Nhuknan, J.; Cassady, C. J. *Int. J. Mass Spectrom.* **2003**, *222*, 363−381.
(33) Moghaddam, M. Z.; Heinzle, E.; Tholey, A. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 141−148.

Table 2. Evaluation of Several Ionic Matrixes, in Terms of Crystallization on the Tissue Slice and Intensity of Signal (Both Linear and Reflector Modes) Considering Positive Voltages[a]



matrix	form	crystallization on tissue[b]	signals [b] linear	signals [b] reflector
CHCA/ANI[c]	solid	++++	+++++	+++++
CHCA/DANI[d]	solid	++	+++	+++
CHCA/DIEANI[c]	liquid	+	−	−
CHCA/2A4M5NP[d]	solid	+++++	++	−
CHCA/DBU[d]	liquid	+	−	−
CHCA/PY[c]	solid	+	−	−
CHCA/ET_3NH[c]	liquid	+	−	−
CHCA/PIP[d]	liquid	+	−	−
CHCA/2AQ[c]	solid	+	−	−
CHCA	solid	+++	+++	++

[a] Photographs of the matrix crystals of CHCA/ANI (a), CHCA/2A4M5NP (b), and CHCA/DANI (c) in comparison with conventional CHCA (d). [b] ++++, best results; +, worst results; and −, no signal recorded. [c] Adapted from Armstrong et al.[22] [d] New ionic matrixes.

al..[22] for bradykinin. In the negative mode, sensitivity was the same as for CHCA/DANI (100 fmol).

Direct Analysis of Tissue in Positive Mode Using Ionic Matrixes. Based on previous studies using standards, several ionic matrixes were chosen for direct analysis, either for their properties in term of ion signal intensity, sensitivity, or their ability to crystallize. According to these criteria "aniline derivatives" are of great interest for their high sensitivity. On the other hand, pyridine derivatives gave high increase of ion signal in a wide mass range. Finally, some other matrixes such as liquid matrixes with Et_3N or diethylaniline or some solid ionic matrixes[33,34] result in more homogeneous crystallization than classical preparation. Several types of ionic matrixes were tested in order to find those with the best crystallization, sensitivity, and signal intensity potentials when applied to tissues.

(a) Study of Crystallization on Tissue. To compare crystallization pattern, matrixes were simply applied on the whole tissue surface using a micropipet without using a sprayer or any other techniques for improving crystallization. Two matrixes, CHCA/ANI (Table 2a) and CHCA/2A4M5NP (Table 2b), have given a very thin crystal layer covering the entire tissue. Very small and homogeneously distributed crystals were observed in the case of CHCA/2A4M5NP. For classical matrixes, spotting generally gives irregular crystals covering only 50% of the tissue.[35] It must also be noted that ionic matrixes have a high vacuum stability,[25] making them very suitable for direct tissue analysis or MALDI imaging since experiments are longer than for classical MALDI. For CHCA/DANI (Table 2c), crystallization leads to the formation of big red/orange crystals covering most of the area of the tissue. Coverage can be improved by increasing the concentration of the matrix (10 mg in 500 μL of acetonitrile/0.1% TFA in water 2:1, v/v,). However, the size of crystals decreases the homogeneity of the coverage in comparison with classical CHCA (Table 2d). For other ionic matrixes, crystallization is generally at the rim of the slice (CHCA/PY) or crystals grow in packets covering only some parts of the cut (CHCA/DIEANI, CHCA/ET_3NH). This lack of homogeneity in coverage can complicate the analysis and makes these matrixes not very suitable for direct analysis.

Thus, CHCA/2A4M5NP and CHCA/ANI have shown to be the best suited ionic matrixes for direct analysis of tissue in term of crystallization. These matrixes were then studied in term of signal intensity. Data are summarized Table 2.

(b) Direct Analysis in Linear and Reflector Mode (Positive Mode). For comparison of intensity, one spot of an ionic matrix and one spot of CHCA were applied very close together on the same slice. This experiment was repeated over 5 times on several slices in order to check out the reproducibility.

In linear mode, the best signals were obtained using the ionic matrix CHCA/ANI at the same laser energy above the threshold for ion production. For this matrix on rat brain sections, peptides present a better signal intensity using the solid ionic matrix than CHCA (Table 3). This increase is especially obvious for peptides at m/z 2507.8 and 4289.1. Comparing the number of peptides detected with both matrixes, all peptides observed with CHCA are retrieved with CHCA/ANI with at least the same intensity. However, a few peptides are only detected with the solid ionic matrix, e.g., m/z 3076.4 and 3459.7 (Table 3). This demonstrates the advantage of solid ionic matrixes for proteomic analysis. For CHCA/DANI, good signal intensity was observed too, and for peptides at m/z ~1800, CHCA/DANI was better than CHCA. For higher masses, signal was equivalent to the signal intensity observed with CHCA. In the same way, CHCA/2A4M5NP and CHCA/PY give less response than CHCA (data not shown).

In the case of liquid ionic matrixes CHCA/DIENI and CHCA/ET_3NH, no good signal was recorded from direct analysis. This

(34) Li, Y. L.; Gross, M. L. *J. Am. Soc. Mass Spectrom.* **2004**, *15*, 1833−1837.
(35) Schwartz, S. A.; Reyzer, M. L.; Caprioli, R. M. *J. Mass Spectrom.* **2003**, *38*, 699−708.

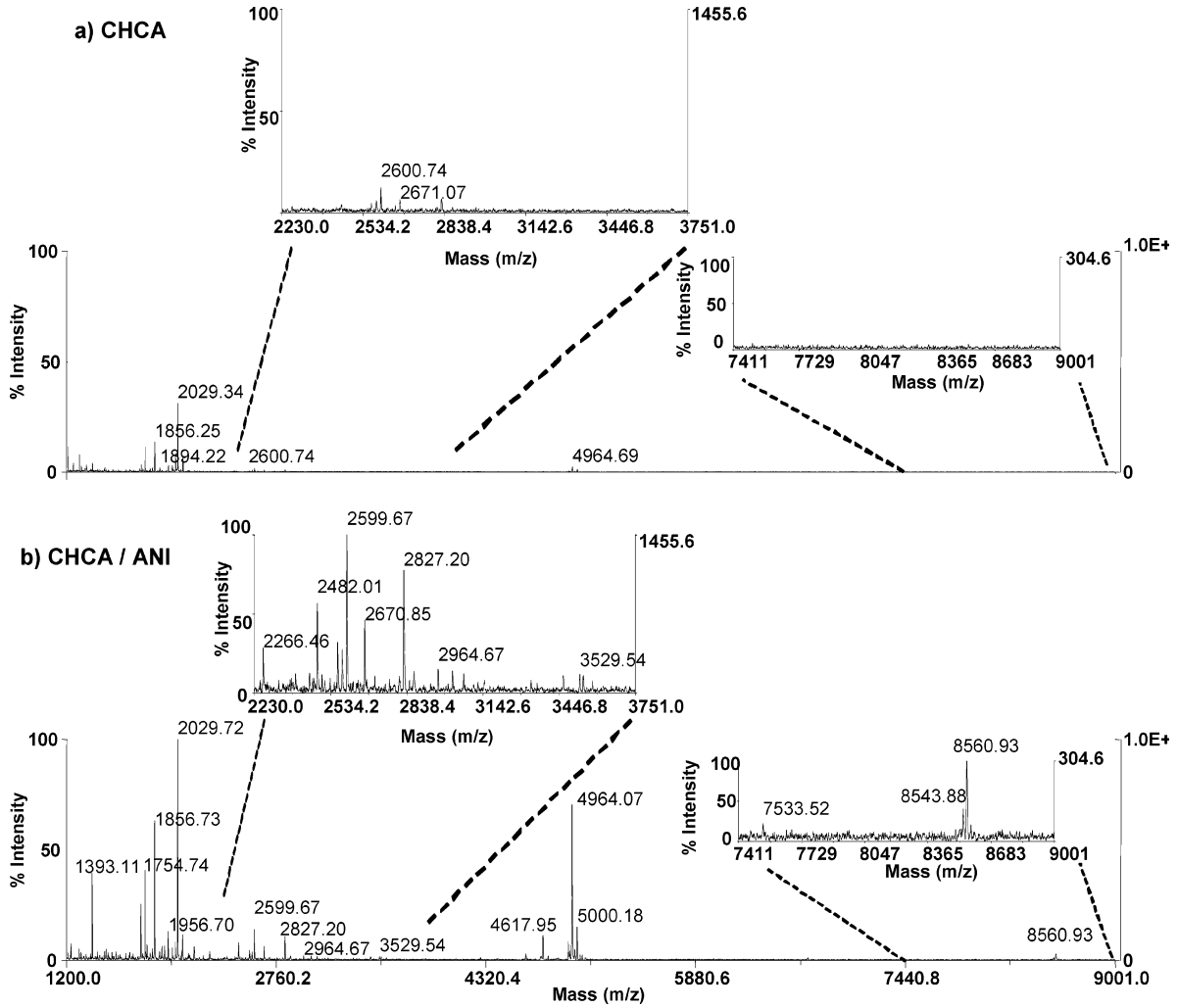


Figure 2. MALDI mass spectra recorded during direct analysis of a rat brain section in reflector positive mode using (a) CHCA and (b) CHCA/ANI for mass range 1200−9000.

was attributed to the important laser fluence required for desorption/ionization of molecules using these matrixes. This phenomenon is observed in addition to the increase of fluence generally required for all experiments when working with tissue slices. However, this result could also be explained by assuming a poor incorporation of the molecules embedded in the tissue into the matrix crystals or some critical parameters obstructing the desorption process. The different acquisition parameters of the instrument were modified (grid voltage, laser fluence, or delay time), but no interesting spectra were recorded.

In the reflector mode, the increase of signal using CHCA/ANI (Figure 2b) and CHCA/DANI compared to CHCA (Figure 2a) was confirmed. Increase of intensity for the two ionic matrixes is easily observed in the mass spectra. In all cases, CHCA/ANI has always shown higher signal intensity than CHCA. (Figure 2) Moreover, ions in the mass range m/z 2230−3750 and especially at m/z 8600 are observed whereas nearly no ions are detected above m/z 5000 for CHCA. A similar trend in signal increase was observed using CHCA/DANI but to a less extent than for the aniline derivative (data not shown).

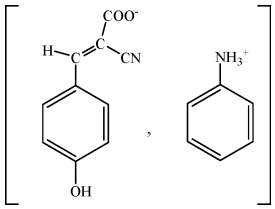
Thus, ionic matrixes as CHCA/ANI result in better accurancy and sensitivity on tissue in the reflector mode for an extensive mass range (m/z 1000−10 000) in comparison with CHCA.

We also compared mass spectra recorded with SA to those obtained with CHCA/ANI ionic matrix for direct analysis. SA was tested due to its common use as matrix for direct tissue analysis.[35] As expected, better signals were detected using the ionic matrix for mass range below m/z 5000.

Other ionic matrixes formed between sinapinic acid and aniline, *N*,*N*-dimethylaniline, or *N*,*N*-diethylaniline base were also synthesized and tested. However, the results obtained were generally equivalent to the classic SA matrix (data not shown).

(c) In Situ Direct PSD. The signal increase for CHCA/DANI and CHCA/ANI was used in conjuction with PSD to study peptide fragmentations directly from tissue sections. In spite of the known fact that ionic matrixes produce less fragmentation[34] than CHCA, the signal increase should be sufficient to overcome this deficiency. However, PSD directly from tissues is always problematic, leading to very few fragment ions even for small peptides. CHCA is known to be the matrix of choice for fragmentation by PSD. Even when using CHCA, scarce fragmentations have been observed when directly probing tissue. When PSD was performed with the ionic matrixes, a higher fragmentation yield was observed in tissue with CHCA/ANI (Figure 3). Some mirror ratio windows (below 0.75) have given nearly no fragment ions. This in situ PSD was repeated several times on different slices from the same brain,

Table 3. Typical Variation of Signal Intensity between CHCA/ANI and CHCA for Direct Analysis on Rat Brain Slice in the Mass Range *m/z* 1000−10 000 for 16 Peptides



m/z obsd	*m/z* calcd	probable assignment[a]	sequence	intensity[b] matrix CHCA	intensity[b] matrix ANI	increase factor ANI/CHCA
1087.4	1087.4	Arg vassopressin	CYFQNCPRG	**283**	**325**	1.1
1513.9	1514.7	melanotropin γ	YVMGHFRWDRF	**328**	**654**	2.0
1641.0	1639.7	somatostatin	AGCKNFFWKTFTSC	**284**	**644**	2.3
1690.2	1689.9	neurotensin	QLYENKPRRPYIL	**139**	**674**	4.8
1789.1		unknown		**1610**	**4190**	2.6
1844.0	1845.8	C-terminal flanking peptide	ALNSVAYERSAMQNYE	**143**	**425**	3.0
1859.1		unknown			**5896**	3.0
2271.3	2273.1	prolactin releasing peptide PrPR2	TPDINPAWYTGRGIRPVGRF	**411**	**848**	2.1
2351.1	2351.16	CLIP (1-21)	PVKVPNVAENESAEAFPLEF	**100**	**283**	2.8
2506.2	2507.8	unknown		**182**	**3417**	18.8
3076.4		unknown			**220**	
3459.7	3461.6	C-flanking peptide of NPY	SSPETLISDLLMRESTENAP-RTRLEDPSMW		**168**	
4289.1		unknown		**131**	**1816**	13.9
4970.1		unknown		**7187**	**57000**	7.9
6221.4		unknown		**103**	**1125**	10.9
6176.6		unknown			**130**	

[a] Assignments based on molecular weight measurements.[4,11] [b] Analysis were performed at the same laser intensity (considering energy threshold for ion production)

from different brains, and for different peptide ion precursors. Reproducible fragmentation was observed for each parent ion using the same acquisition parameters. Different delay time, accelerating voltage, or laser intensity were studied in order to optimize the fragmentation. However, no total PSD was obtained. PSD fragmentation can be obtained from precursor ion masses of up to *m/z* 2000 (e.g., *m/z* 1785). For CHCA/DANI, little fragmentation was observed for molecules in the low-mass range (<*m/z* 1000). For higher masses, no significant fragmentation was observed probably due to the weak intensity of the parent ion (data not shown).

To understand the poor fragmentation rate observed in tissue, we checked out the hypothesis that it could be due to the presence of salts. Salt adducts are known to be very stable in the gas phase and consequently lead to poor fragmentation rates. If some of the compounds observed are cationized ions, they will not result in any fragmentation. In this respect, if negative mode analyses are performed, this will remove any ambiguity as to the nature of the ions since in classical conditions mainly $[M - H]^-$ ions are observed. However, considering, for example, *m/z* 1785 peptide, presenting very few fragmentations in PSD in positive mode, we have performed the same analysis in negative mode, where the peptide peak is observed at *m/z* 1783 and corresponds to the $[M - H]^-$ ion. The mass shift is of only two mass units as compared to the positive mode, demonstrating that the ions are in their protonated $[M + H]^+$ form in the positive mode. Thus, difficulties of fragmentation in positive mode on tissue sections cannot be attributed to adduct ions and must probably come from more complex factors. Low fragmentation rates could suggest that ions formed from tissue slices would have less internal energy.

(d) Direct Analysis in Negative Mode. The different solid ionic matrixes were tested in the negative mode for direct tissue analysis. Three ionic matrixes, CHCA/DANI, CHCA2/A4M5NP, and CHCA/ANI, have given particularly good results in negative mode. A significant increased improvement was observed with these matrixes in comparison to CHCA, confirming the results previously obtained with standards. CHCA/DANI and especially CHCA/ANI were the matrixes giving the highest increase in intensity and the best sensitivity. This phenomenon is illustrated in Figure 4 by comparing CHCA/ANI (Figure 4a) to CHCA (Figure 4b).

We also compared direct analysis in negative and positive modes on one spot by switching only the voltage polarity as presented for CHCA/DANI in Figure 5. Due to the lower sensitivity in negative mode, less intense signal was observed in this mode. Nevertheless, some compounds, e.g., *m/z* 959 or 995, can be detected with a higher intensity. Moreover, adduct suppression in the negative mode gives easier readability of the mass spectra. For the mass range below *m/z* ~1200, resolution is greatly improved (e.g., *m/z* 787 or 811, see insets) probably due to lipids' numerous adducts in positive mode.

MALDI Imaging of Rat Brain Tissue Sections. Generally, for direct analysis of peptides/proteins in tissue sections, only lasers with conventional repetition rate frequencies ranging from 2 to 20 Hz are used. The number of positions to scan to perform MALDI imaging leads to very long acquisition time, e.g., ~92 h

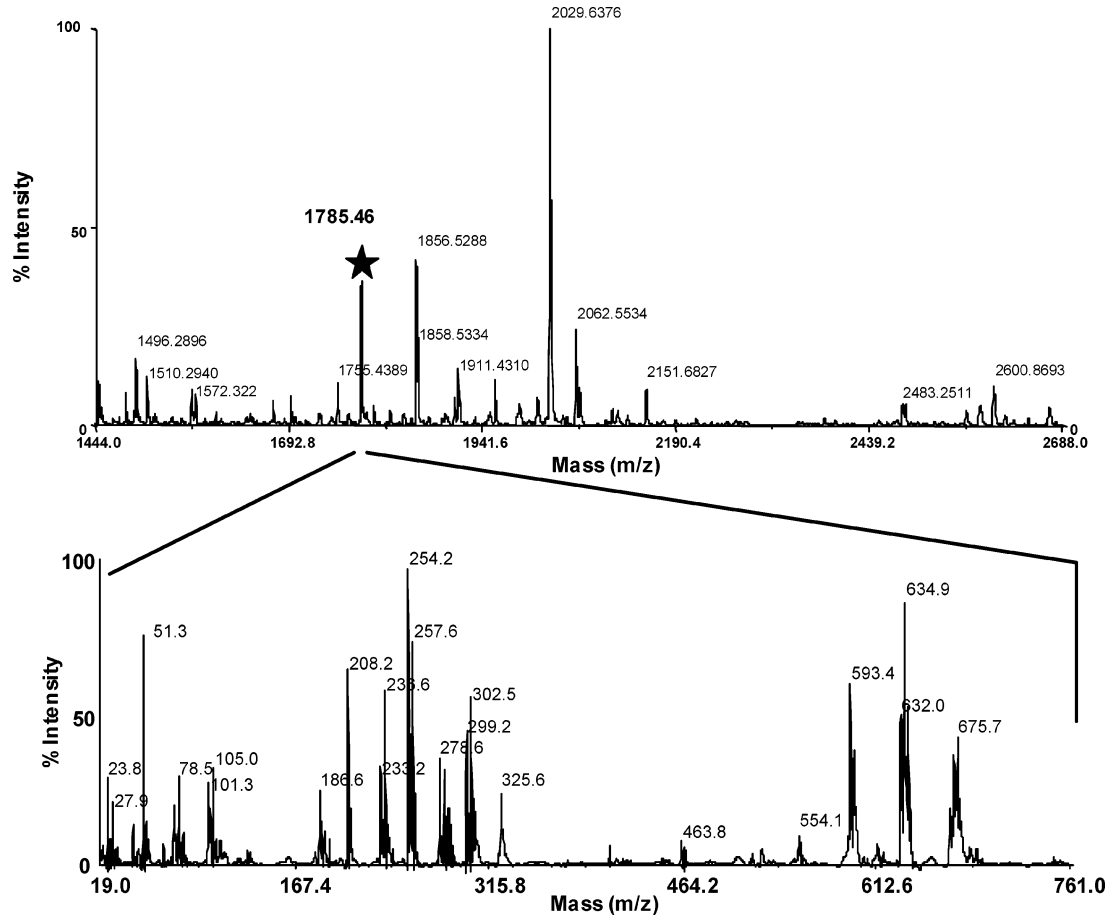


Figure 3. MALDI positive reflectron mass spectrum of a rat brain section using CHCA/ANI and the resulting PSD spectrum for the parent ion at *m*/*z* 1785 (collision gas, xenon).

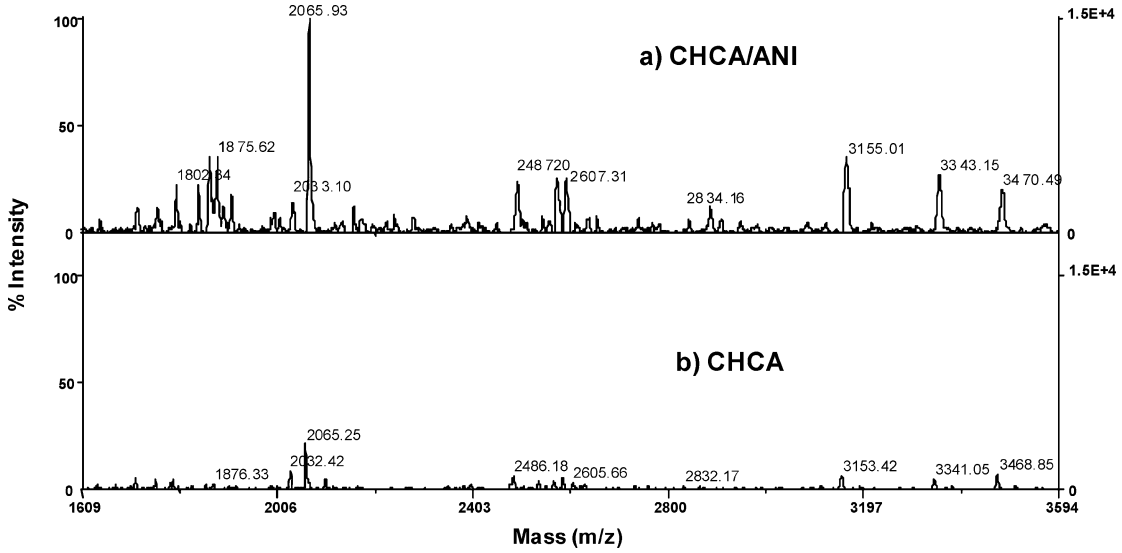


Figure 4. Direct analysis on a rat brain section in linear negative mode for (a) CHCA/ANI and (b) CHCA.

to scan 10 000 spots averaging 100 shots per position and only ~6 h for the same experiments with a 50-Hz repetition rate laser. The improvements in laser technology give access to 200-Hz repetition rate lasers. However, at such rates, conventional MALDI matrixes such as SA or CHCA are much less suitable for analysis resulting in abundant material removal during the ejection process. Consequently, after several hours of acquisition, intensity is much lower and some signals are not observed any more, leading in loss of resolution of the resulting images. In contrast, ionic matrixes are more resistant under the same conditions. Direct analysis of rat brain tissue sections using CHCA/ANI shows that there is very little material ejection and the morphology of the matrix crystals has not changed after the scan (no damages observed). The size of the matrix crystals could give a first explanation of these results. CHCA/ANI (Table 2a) presents quite large crystals compared to CHCA (Table 2d). In more fundamental

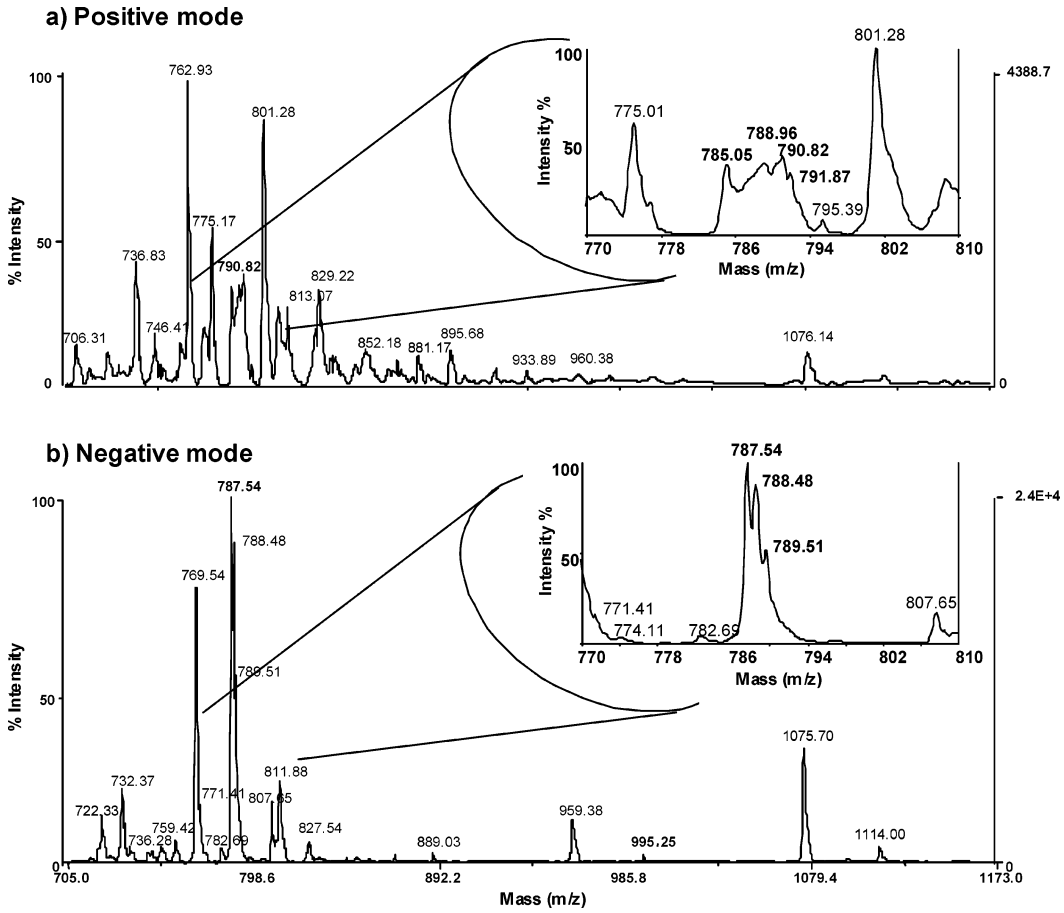


Figure 5. Typical linear MALDI mass spectra for the direct analysis of a rat brain section (a) in positive and (b) in negative modes at the same localization using CHCA/DANI. The insets present a zoom in the mass range 770−810.

studies, Sadeghi and Vertes[36] have demonstrated that smaller crystals are completely volatilized under laser shot irradiation whereas larger crystals undergo a kind of layer by layer ("peeling") evaporation. Thus, considering matrix covering capacity and resistance to laser irradiation, high signal intensity, and sensitivity capabilities, CHCA/ANI was chosen among all the ionic matrixes for MALDI imaging. Experiments were performed using a MALDI-LIFT-TOF analyzer with a 50-Hz repetition rate laser. Images were obtained by scanning the whole rat brain section in 10 000 positions, averaging 100 shots per position in reflector mode. Direct analysis and imaging parameters were optimized using CHCA, and the same method was applied for an ionic matrix. A first scan was acquired in positive mode, and the section was rescanned on the same positions in negative mode. Finally, a last scan was performed in the positive mode again. Using CHCA/ANI, no major decrease in signal intensity was noticed between the first and the third scans (Figure 6). For CHCA, decrease of intensity was clearly observed as illustrated in Figure 6 for m/z 1224. After three scans, the section was still totally covered with ionic matrix, which was not the case with classical CHCA. This inevitably leads to loss in image resolutions, since some peaks are not observed any more in the corresponding mass spectra (e.g., m/z 2062 and 1224).

Several images reconstructed from both positive and negative data for some m/z ratios are shown in Figure 7a and b. The images demonstrate a lesser delocalization for CHCA/ANI than CHCA considering a classical deposition using a micropipet. This phenomenon is well illustrated by the molecular image reconstructed for peptide at m/z 2015 (Figure 7a). This was attributed to the very fast and homogeneous crystallization of this matrix on the tissue. The comparison of the images using CHCA and the ionic matrix shows a better resolution for CHCA/ANI with a higher signal intensity and detection. Rat brain regions can be easily recognized by comparing MALDI ionic matrix imaging to rat brain maps (Figure 7c). For instance, in positive mode, peptides at m/z 2015 are found to be in forceps minor of corpus callosum (fmi) and anterior commissure (aca), but peptide at m/z 4741 is in the rest of brain. For m/z 2028 in negative mode and m/z 2030 in positive mode, similar localization was observed despite the poorer detection in negative mode for other regions than corpus callosum.

Better sensitivity using CHCA/ANI was confirmed for several ions as for m/z 4741 (positive mode) or 2092 (negative mode). Consequently, precise localization of these peptides using MALDI imaging is impossible using CHCA when it can be done using CHCA/ANI ionic matrix.

CONCLUSIONS AND APPLICATIONS

Taken together, we have developed and tested ionic matrixes for tissue analysis by MALDI. Ionic matrixes have particularly interesting properties, when compared to classical matrixes for

(36) Sadeghi, M.; Vertes, A. *Appl. Surf. Sci.* **1998**, *127−129*, 226−234.

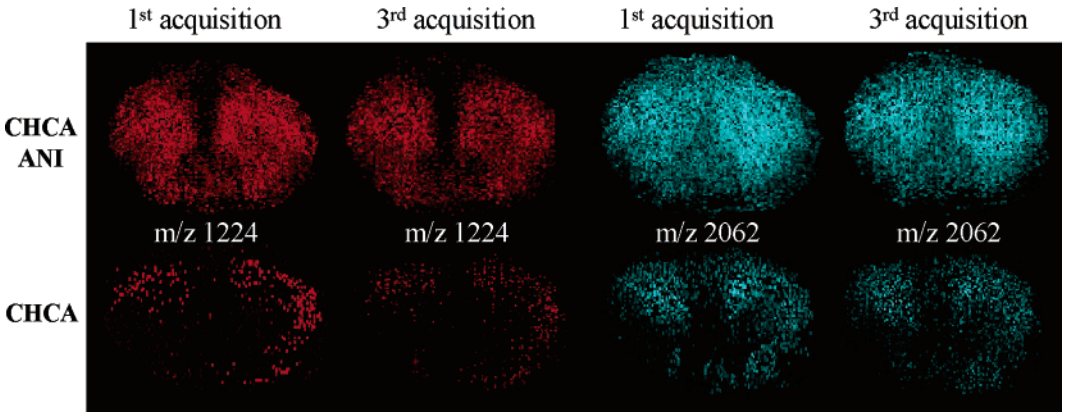


Figure 6. MALDI-IMS using MALDI LIFT-TOF in reflector mode (50-Hz laser repetition rate, 10 000 positions scanned), with ionic matrix CHCA/ANI and with CHCA in positive mode for the first and the third acquisitions on the same rat brain slice. Images have been reconstructed using FlexImaging software and represent the repartition of a *m*/*z* in the tissue slice. Same imaging parameters were used for all acquisitions.

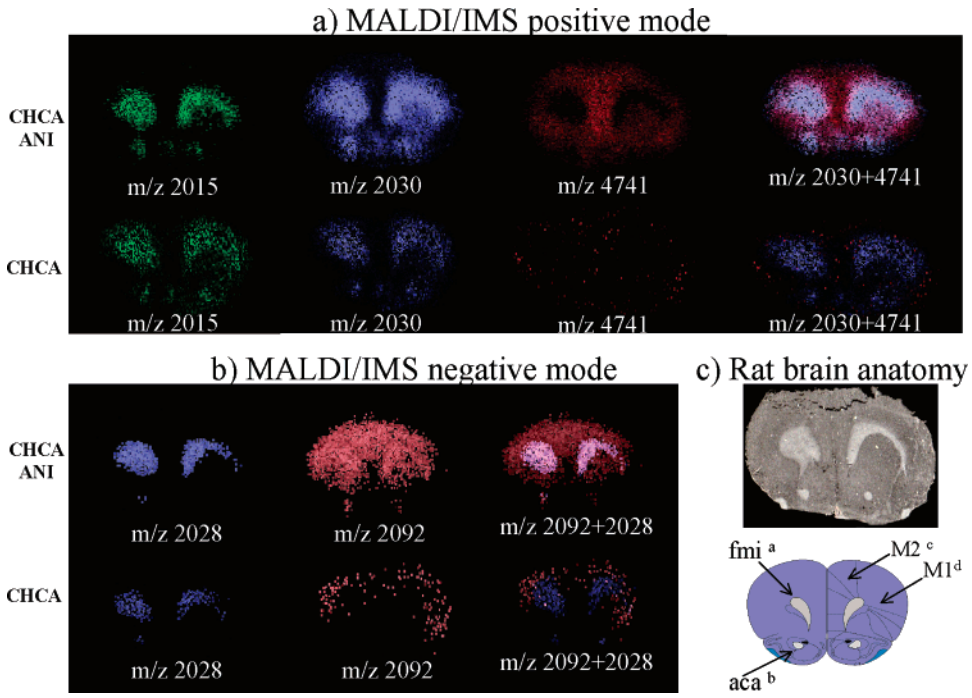


Figure 7. MALDI-IMS using MALDI LIFT-TOF in reflector mode at 50-Hz repetition rate with ionic matrixes CHCA/ANI and CHCA in positive (a) and negative modes (b). MALDI imaging can be compared with rat brain anatomy (c). For CHCA/ANI and CHCA, acquisitions in both polarities were performed on the same rat brain cut. Images have been reconstructed with the same parameters for ionic matrix and CHCA using FlexImaging software and represent the repartition of a *m*/*z* in the tissue slice. Images with two colors correspond to the superposition of two *m*/*z* images ([a]forceps minor of corpus callosum, [b]anterior commissure, and [c,d]mortor cortex).

peptide direct tissue analysis: (1) a better signal intensity and sensitivity, (2) homogeneous crystallization patterns, (3) the possibility of in situ partial fragmentations, (4) a better vacuum stability, (5) a high resistance to laser ablation especially using a high repetition rate laser, and (6) the possibility to analyze compounds in positive and negative modes using the same slice for MALDI-MS high-quality imaging.

CHCA/2A4M5NP, CHCA/DANI, and especially CHCA/ANI appeared to be of great interest in solution or on tissue, with a highly significant enhancement signal. In negative mode analysis, an easier interpretation of mass spectra by suppression of adduct ions can be helpful in the future for analysis of a specific class of compounds such as phosphorylated peptides or lipids.

Applied to the study of peptide profiling in tissue, aniline derivative ionic matrixes gave better results than CHCA. CHCA/ANI has shown great potential both in positive and negative modes as well as for peptide sequence tag by PSD with unambiguous assignment after comparison of peptides coming from different tissues using in situ PSD. Moreover, to access more information, molecular maps can be reconstructed by scanning the tissue sample. MALDI imaging performed on rat brain slices comparing ionic matrix CHCA/ANI to CHCA has shown higher signal intensity, sensitivity, better image quality and peptide localization, reproducibility, and remarkable resistance to laser ablation with the ionic matrix. Thus, with CHCA/ANI, it was possible to scan several times the whole rat brain section both in positive and in negative modes without observing consecutive damage of matrix crystals. We performed, for the first time, imaging in both modes on the same slice, using a high-frequency laser and good accuracy in reflector mode.

Thus, ionic matrix as CHCA/ANI with its remarkable properties, is an important development in direct tissue analysis and MALDI imaging techniques.

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