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**SOLID IONIC MATRICES FOR DIRECT TISSUE ANALYSIS AND MALDI
IMAGING.**

R. Lemaire^{1,2}, J.C. Tabet², P. Ducoroy³, J.B. Hendra³, M.Salzet¹, I. Fournier^{1*}

*Corresponding Author

¹Laboratoire de Neuroimmunologie des Annélides, UMR-CNRS 8017, Bât SN3, 1^{er} étage,
Université des Sciences et Technologies de Lille, 59655 Villeneuve d'Ascq Cedex, France

²Synthèse, Structure et Fonction de Molécules Bioactives, UMR-CNRS 7613, Boite 45,
Université Pierre et Marie Curie, 4 place Jussieu, 75252 Paris Cedex 05, France

³ Plateforme Protéomique, IFR 100, 8 Boulevard du Maréchal de Lattre de Tassigny, 21000
Dijon, France.

ABSTRACT

Direct analysis of tissues by MALDI-MS allows the acquisition of profiles 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 new developments for direct tissue analysis concerning analysis of peptides/proteins with ionic liquid synthesized with matrices mixtures (α -cyano-4-hydroxycinnamic acid/2-amino-4-methyl-5-nitropyridine (CHCA/2A4M5NP) and α -cyano-4-hydroxycinnamic acid/N,N-dimethylaniline (CHCA/DANI)). The properties of these direct tissue analysis matrices, especially CHCA/ANI when compared to α -cyano-4-hydroxycinnamic acid (CHCA), 2,5-dihydroxybenzoic acid (2,5-DHB) and sinapinic acid (SA) are : 1) better spectral quality in term of resolution, sensitivity, intensity, noise, number of compounds detected, contaminants tolerance, 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 4) better resistance to laser irradiation especially for high frequency lasers 5) better ionic yield in the negative mode 6) enough fragmentation yield to use the PSD mode on cuts to get structural information. Applied to MALDI Imaging on a MALDI LIFT-TOF with a 50Hz laser frequency, these ionic matrices have allowed the realization for a new type of image in both polarities and reflector mode using the same cut. These results open a new field in peptides profiling by MS on tissues, characterization of compounds on slices and MALDI Imaging.

INTRODUCTION

Since its introduction in the mid 80's^{1,2,3}, Matrix Assisted Laser Desorption/Ionization (MALDI) mass spectrometry has become a powerful tool in the field of biological researches and is used for the detection, identification and characterization of peptides and proteins from complex mixtures. More recently, MALDI techniques were developed by Caprioli^{4,5,6} and different groups^{7,8,9,10,11} for direct analysis of tissue and molecular imaging. Their studies demonstrate the potentiality of MALDI in the medical field. In fact, MALDI direct analysis is now used for pharmaceutical or proteomics questions to detect drugs^{12,13,14} or for new biomarkers discovery, pathology diagnosis such as colon cancer¹⁵, prostate adenocarcinomas¹⁶, neuroendocrine carcinomas¹⁶, and brain tumors¹⁷.

However, direct analysis of tissues using mass spectrometry requires to have maximum resolution, sensitivity and dynamic mass range since tissues are made of a very complex mixture of molecules, some of which are expressed at a very low level in comparison with some others and can present very close m/z ratio. Although, to obtain optimal performances, such a technology still requires new developments either on the instrumental part (e.g. cryodetector to increase detection¹⁸, higher repetition rate laser, Ion microscope images¹⁰ and SMALDI⁷ technique for high resolution imaging) or on MALDI techniques. Thus, one alternative is to develop new MALDI matrices since matrix is fundamental for desorption/ionization process in MALDI. Thus, spectral quality *i.e.* peak resolution, sensitivity, intensity and noise, is dependent on the choice of the matrix. Actually, for peptide/protein analysis, α -cyano-4-hydroxycinnamic acid¹⁹ (CHCA), 2,5-dihydroxybenzoic acid²⁰ (2,5-DHB) and sinapinic acid²¹ (SA) are matrices commonly used. 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 deposition 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 nature of the tissue etc... Thus, development of new matrices for tissues analysis is therefore a necessity for circumscribing such problems.

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

As previously described by Armstrong²², coupling ionic liquids to MALDI matrices, could generate much matrices. In fact, ionic liquids are increasingly used particularly in organic synthesis²³ and present a lot of different interesting properties such as solvent recycling, low vapor pressure or electrical conductivity²⁴. Ionic liquids can be obtained from an acid /base reaction leading to liquids presenting the general formula $[A^-, B^+]$. Classical MALDI matrices being acidic, acid/base reactions were already tested and can be performed using different bases to synthesize salt complexes with different MALDI ionic matrices (2,5-DHB, CHCA, sinapinic acid and 3 HPA^{22,25}).

In the present study, we report that in many cases, that solid ionic matrices performance is equal or higher to their classical analogues. Particularly a significant signal and sensitivity improvement were observed for aniline and dimethylaniline derivatives. In this context, taking into account, the shot to shot reproducibility was well described by Mank and co-workers²⁶, the high stability under vacuum conditions, the crystallization, the signal intensity, the sensitivity, and the possibility to perform post-source decay (PSD) experiment, studies in

both the positive and negative modes. We were interested in testing solid ionic matrices for peptidomic studies on rat brain tissue. Finally, we performed MALDI Imaging on a MALDI-LIFT-TOF with 50 Hz laser frequency and ionic matrices, and developed a new type of image in reflector mode in both polarities using the same tissue section which demonstrate the resistance of those matrices to laser irradiation.

EXPERIMENTAL SECTION

Materials. α -cyano-4-hydroxycinnamic acid (CHCA), aniline (ANI), N,N-dimethylaniline (DANI), N,N-diethylaniline (DIENI), triethylamine (Et₃NH), 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.

Calibration mixture. External calibration were performed using a solution of standard neuropeptides 1.6 μ M of Bradykinin, 1.6 μ M of Substance P, 1.6 μ M of ACTH 18-39, 3.2 μ M of ACTH 7-38, 4.8 μ M Bovine Insulin and 4.8 μ M Bovine Ubiquitin in 0.1% TFA/H₂O. External calibration was performed on sample deposited on the sample holder. Thus, with the variations of tissue slices thickness (ranging from 15-20 μ m), differences of calibration were noticed when comparing mass spectra from different tissue slices.

Sensitivity tests. Substance P at 2.5 μ M in 0.1% TFA/H₂O was diluted 9 times in water in order to get concentrations ranging from 1pmol/ μ L to 125amol/ μ L.

Intensity tests. For positive mode analysis, Substance P was used at 0,8 μ M. ACTH 18-39 was used at 3,2 μ M for negative mode.

Preparation of ionic Matrices (IM). All solid ionic matrices (CHCA/DANI, CHCA/2A4M5NP, CHCA/3AQ), and liquid ionic matrices (CHCA/DIENI, CHCA/DBU, CHCA/Et₃NH, CHCA/PIP) can be produced using classical protocols used for ionic liquids synthesis^{22,23}. 50 mg of CHCA, SA, or other MALDI matrix are dissolved in 20 mL of methanol. An equimolar amount of base was added. The solution was mixed for one hour, and the solvent evaporated in a vacuum evaporator for 45 minutes (T= 50°C, P= 40 mbar). The resulting compound was placed in a dessicator for 30 minutes to eliminate residual solvent and stored at -20°C. Just before use the ionic matrices were prepared by dissolving 10 mg of compound in 1 mL acetonitrile/water (2:1, v/v, 0,1% TFA).

Both solid ionic matrices used in this study, α -cyano-4-hydroxycinnamic acid/aniline (CHCA/ANI) and α -cyano-4-hydroxycinnamic acid/pyridine (CHCA/PY), can also be prepared just before use, following a faster protocol : 1 equivalent of base (4.8 μ L for CHCA/ANI and 4.29 μ L for CHCA/PY) was added to a solution of 10 mg/mL of CHCA (1 mL) in acetonitrile/water (2:1, v/v, 0.1% TFA). The mixture was then vortexed and sonicated for 10 minutes before application to the tissue.

For classical CHCA and SA, 10 mg and 20 mg of the were dissolved respectively in 1 mL of acetonitrile/water (2:1, v/v, 0.1% TFA/H₂O).

Tissue preparation. Adult male Wistar rats weighing 250-350 g (animal use accreditation by the French ministry of the agriculture N° 04860) were used in the study and maintained under

standard care. Animals were sacrificed by decapitation and immediately dissected to remove the brain. Frozen sections of 15 μm 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, a volume of matrix solution was applied onto the frozen section using a micropipette. 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, USA) using delayed extraction (DE) and a pulsed nitrogen laser at 337 nm.

Classical analysis in linear Mode. acquisition parameters were set to : acceleration voltage: 20 kV ; 1st grid voltage: 94% ; guide-wire voltage: 0.05% ; extraction delay time: 200 ns.

Direct analysis in linear mode. acquisition parameters were: acceleration voltage: 25 kV, 1st grid voltage: 94%, guide-wire voltage: 0.05%, extraction delay time: 200 ns.

Direct analysis in reflector mode. acceleration voltage: 25 kV, 1st 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).

PSD mode. acceleration voltage: 25kV, 1st grid voltage: 72%, extraction delay time: 200 ns. The ion precursors were selected using the timed ion gate (12mm) of the instrument.

Acquisition of the product ions was generally accomplished 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 and 0.85) were used.

MALDI-Imaging

For MALDI-IMS, images were performed on the Ultraflex LIFT-TOF (Bruker Daltonics, Bremen, DE) in reflector positive and negative mode, operating with a pulsed nitrogen laser emitting at 337 nm and with a repetition rate of 50 Hz. For images reconstruction the FlexImaging v. 1.0.6.0 software (Bruker Daltonics, Bremen, DE) was used. For positive mode, 10 000 points covering the whole slice with 200 laser shots per position were scanned and for negative mode 7000 points with 200 shots were performed. From each position the software record an average mass spectrum with its coordinates on the slice.

RESULTS AND DISCUSSION

In a first set of experiments two solid ionic matrices, CHCA/2A4M5NP and CHCA/DANI, were studied in details in comparison with the previously described CHCA/ANI²². They were firstly checked on standard compounds, before use for direct tissue analysis.

Studies with standards.

CHCA/DANI was obtained from the reaction between CHCA and the base N,N-dimethylaniline. On the contrary, CHCA/2A4M5NP was synthesized by acid/base reaction between two classical matrices, CHCA and a basic matrix, 2A4M5NP²⁷. Both matrices were evaluated for the production of MS ion signal in positive and in negative mode by recording

400 laser shots moving slowly around over the whole spot of a mixture of Substance P (500fmol/ μ L) or ACTH 18-39 (1pmol/ μ L) with the ionic matrix.

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 matrix (Table 1). In all cases, variation of fluence was always less than 3% between CHCA and these solid ionic matrices. Furthermore, in all our studies of intensity the laser energy was set 20% higher than the energy threshold for peptide ions giving enough signal intensity with classical matrices to record a large increase of signal with ionic matrix if necessary. In the positive mode, significant signal increase was observed using CHCA/2A4M5NP ionic matrix. The signal was 3.5 fold higher with the ionic matrix in the case of Substance P. For Apomyoglobin protein, the signal was also higher by a factor of 1.8 (Table 1) with the solid ionic matrix than with CHCA, demonstrating the possibility to use this matrix for both peptides and proteins. 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 non ionic form²⁷. In the case of CHCA/DANI in the positive mode, the signal observed was less or equal to CHCA/ANI (data not shown).

Evaluation of spectral quality in Negative mode

Negative mode can be very interesting for direct analysis of tissue, especially for detecting compounds such as phosphorylated peptides²⁸, lipids^{29,30} or phospholipids³¹ which may show extensive adduct signals in positive mode. It can also be used to obtain complementary structural information using PSD experiments³². 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 matrices gives low counts of negative ions. Here, we have tested the ionic matrices in order to know whether they may give better ion yields in this mode than conventional matrices. Ionic matrices have been previously tested for low molecular weight compounds³³ (amino acid), but no studies on peptides/proteins have been yet done. In negative mode, the best signals were recorded using both solid ionic matrices CHCA/ANI and CHCA/DANI (Figure 1a) and in the same way a good response was obtained for ACTH 18-39 with CHCA/2A4M5NP (Figure 1b). A factor of 2 in signal increase was observed with solid ionic matrices in comparison to CHCA (Figure 1c). This 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 matrices deprotonation of the analyte would be more difficult since the matrix only shows acidic properties. Ionic matrices are salts that show characteristics both of acidic group and basic groups, as was observed in previous organic studies²³. Looking to these results, CHCA/DANI and especially CHCA/ANI have proved to be the best matrices in term of signal intensity enhancement in both the positive and negative modes.

Sensitivity tests.

In order to detect compounds in tissues, matrices 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 analyzing tissues for the research of 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 mode. Best results were obtained using CHCA/DANI. The limit of detection was found to be 250 amole

in the positive mode and 100 fmole 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 fmol and 250 fmole. Although, CHCA/2A4M5NP with 150 fmol has shown better sensitivity than CHCA in the negative mode, it appeared to be 100X less sensitive than CHCA in positive mode.

Direct analysis of tissues in positive mode using ionic matrices.

Based on previous studies with standards, several ionic matrices 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 present high increase of ion signal in a wide mass range. Finally, some other matrices such as liquid matrices with Et₃N or diethylaniline or some solid ionic matrices^{33,34} give more homogeneous crystallization in classical preparation. Several types of ionic matrices were tested in order to find those with the best crystallization, sensitivity and signal intensity potentials when applied on tissue.

Study of crystallization on tissues.

To compare crystallization pattern, matrices were simply applied on the whole tissue surface using a micropipette without using sprayer or any other techniques improving crystallization. Two matrices, CHCA/ANI (Table 2a) and CHCA/2A4M5NP (Table 2b), have shown to give a very thin crystal layer covering the entire tissue. Very small and homogeneously distributed crystals are observed in the case of CHCA/2A4M5NP. For classical matrices, spotting generally gives irregular crystals covering only 50% of the tissue³⁵. It must also be noted that ionic matrices have a high vacuum stability²⁵ 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 area of the tissue. Coverage can be improved by increasing the concentration of the matrix (10mg in 500 μ L acetonitrile/0.1% TFA in water 2:1, v/v). However, the size of crystals decreases the homogeneity of the coverage in comparison to classical CHCA (Figure 2d). For other ionic matrices, 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/ET3NH,...). This lack of homogeneity in coverage can complicate the analysis and makes these matrices not very suitable for direct analysis.

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

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 cut. This experiment was repeated more than 5 times on several slices in order to check out the reproducibility (Table 3).

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 m/z 4289.1. Comparing the number of peptides detected with both matrices, all peptides observed with CHCA are retrieved with CHCA/ANI with at least the same intensity. However, few set of peptides are only detected with the solid ionic matrix as for m/z 3076.4 and m/z 3459.7 (Table 3). This demonstrates the specificity of solid ionic matrices for proteomic analysis. For CHCA/DANI, good signal intensity was observed too, and for peptides at m/z around 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 matrices CHCA/DIENI and CHCA/ET3NH, no good signal was recorded for direct analysis. This was attributed to the important laser fluence required for desorption/ionization of molecules using these matrices. This phenomenon comes in addition to the increase of fluence generally observed for all experiments when working with tissue slices. However, this result could also be explained 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 2a) and CHCA/DANI compared to CHCA (Figure 2b) was confirmed. Increase of intensity for the two ionic matrices 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 with m/z at 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 matrices as CHCA/ANI open the access to better accuracy and sensitivity on tissue in the reflector mode for an extensive mass range (m/z 1000-10000) in comparison with CHCA.

In situ direct PSD.

The signal increase for CHCA/DANI and CHCA/ANI was used to study peptide fragmentations in PSD directly on tissue sections. Despite the known characteristic of ionic matrices to produce a little less fragmentation³⁴ than CHCA, the signal increase should be sufficient to overcome this deficiency. However, PSD directly on tissues is always problematic leading to very few fragment ions even for quite 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. Thus, PSD was performed with the ionic matrices. For CHCA/ANI a higher fragmentation yield was observed in tissue (Figure 3). Although, some mirror ratio window (below 0,75) has given nearly no fragment ions. This *in situ* PSD was repeated several times on different slices from the same brain, from different brains and for different peptide ion precursors. Repeatable fragments were 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. These partial PSD can be performed for masses 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).

Trying to understand the poor fragmentation rate observed in tissue, we checked out the hypothesis of salts presence. 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 lead to any fragmentation. In this respect, if negative mode analysis are performed this will raise any ambiguity on the nature of the ions since only $[M-H]^-$ ions are observed. However, considering for example m/z 1785 peptide, presenting very few fragmentations in PSD in positive mode, we have performed negative analysis. In

negative mode, the peptide peak is observed at m/z 1783 and correspond to the $[M-H]^-$ ion. The mass shift is of only two mass units towards the low masses compared to the positive demonstrating that the ion 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.

Direct analysis in negative mode.

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

We also compared direct analysis in negative and positive modes for CHCA/DANI on one spot switching only the voltage polarity (Figure 5). Due to the lower sensitivity in negative mode, less signal was observed in this mode. Nevertheless, some compounds e.g. m/z 959 or m/z 995 can be detected with a higher intensity. Moreover, adducts suppression in the negative mode give 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.

Direct analysis using SA and Ionic matrices.

We also compared mass spectra recorded with Sinapinic acid to those obtained with CHCA/ANI ionic matrix for direct analysis (Figure 6). Sinapinic acid was tested because it

has been shown to be the most commonly employed matrix for direct tissue analysis³⁵. As expected, better signals were detected using the ionic matrix for mass range below m/z 5000 (Figures 6a and 6b) but better signal quality is nevertheless obtained with Sinapinic Acid for higher masses (Figures 6c and 6d). Particularly above m/z 10 000, ions are much more intense in the case of Sinapinic acid (e.g. 11072.72, 12348.14, 15127.19).

Ionic matrices 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 Sinapinic acid matrix (data not shown).

These results point out the necessity to perform a previous enzymatic digestion on tissue for protein analysis upper than m/z 5000 using CHCA/ANI. In the future, investigations to find a specific protein (upper than 5000) solid ionic matrix must be considered to take advantage of ionic matrices in this mass range.

MALDI Imaging of rat brain tissue sections.

Generally, for direct analysis of peptides/proteins in tissue sections, only laser with conventional repetition rate frequencies ranging from 2 to 20 Hz are used. The number of positions to scan to performed MALDI imaging lead to very long acquisition time, e.g. about 92 hours to scan 10 000 spots averaging 100 shots per position and only about 6 hours for the same experiments with a 50 Hz repetition rate laser. The improvements in laser technology give us the possibility to work with 200 Hz repetition rate laser. However at such rates conventional MALDI matrices such as SA or CHCA are much less suitable for analysis resulting in abundant material removal during the ejection process. The observation of the matrix crystals on rat brain sections after scanning the tissue with 50 Hz repetition rate laser and 200 shots by position show that matrix crystals are greatly damaged. In some tissue spots only few crystals remain. In contrast, ionic matrices are more resistant under the same

conditions (Figure 7). Direct analysis of rat brain tissue sections using CHCA/ANI show that material ejection is very little and the morphology of the matrix has not changed after the scanning (not damages observable). 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 200 shots per position. A first scan was acquired in positive mode and the section was rescanned in negative mode. No signal decrease was noticed for the second scanning and even after two scans the section is still totally covered by the matrix. Images reconstructed from both positive and negative data for some m/z ratio are shown (Figure 7). Moreover, very few delocalization was observed using CHCA/ANI. Delocalization was less than we use to observe using CHCA (Figure 7c) and deposited with a classical micropipette. Analysis performed outside the slice, but at its rim, has shown a very weak signal intensity for few peptides. This was attributed to the very fast and homogenous crystallization of the matrix on the tissue. We observed very different distribution and localization of various compounds in the images. It is also clear from the pictures that negative mode imaging is possible using ionic matrices.

Moreover, the high stability of ionic matrices under vacuum conditions and especially their resistance to laser ablation suggests a promising future for these matrices for MALDI imaging.

CONCLUSIONS AND APPLICATIONS

Taken together, we have developed and tested ionic matrices for tissue analysis by MALDI . Ionic matrices are particularly interesting properties, when compared to classical matrices for peptides/proteins direct tissue analysis: 1) a better signal intensity and sensitivity, 2) an homogenous crystallization patterns, 3) the possibility of *in situ* partial fragmentations 4) a better vaccuum stability, 5) a high resistance to laser ablation especially using high

repetition rate laser 6) the possibility to analyse compounds in positive and negative mode using the same slice.

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 on tissue, aniline derivative ionic matrices gave better results than CHCA. CHCA/ANI has shown its potential both in positive and negative modes as well as for peptide sequence tag by PSD with unambiguous attribution 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 have shown much signal intensity, 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 negative mode without observing consequent damages of matrix crystals. We realize for the first time, imaging in both modes on the same slice, using high frequency laser and comfortable accuracy on reflector mode.

Thus, ionic matrix as CHCA/ANI with its remarkable properties, will have an important development in a near future for its application in direct analysis of tissues and in MALDI imaging techniques.

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Table 1. Signal evolution in positive and negative mode using CHCA/2A4M5NP or CHCA/DANI vs 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

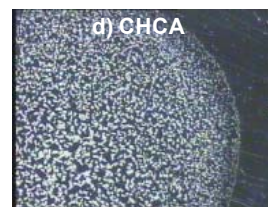
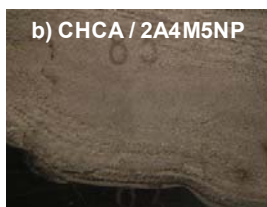
^a n number of experiments (sample/matrix preparation and analyses) using new matrices preparation each time.

^b signal intensity values are given for [M+H]⁺ or [M-H]⁻ ions in counts number (a.u.).

^c rsd is the relative standard deviation.

Table 2. Evaluation of several ionic matrices, in term of crystallization on the slice and intensity of signal in linear and reflector mode considering positive voltages. A photography of the matrix crystals of CHCA/ANI (a) CHCA/2A4M5NP (b) and CHCA/DANI (c) in comparison with conventional CHCA (d) spotted with 20μL of matrix using a micropipette and dried at room temperature are represented.

matrix	form	cristallization on tissue	signals (linear)	signals (reflector)
CHCA/ANI ¹	solid	++++ ^a	+++++	+++++
CHCA/DAN ²	solid	++	+++	+++
CHCA/DIEANI ¹	liquid	+	-	-
CHCA/2A4M5NP ²	solid	+++++	++	-
CHCA/DBU ²	liquid	+	-	-
CHCA/PY ¹	solid	+	+	-
CHCA/ET3NH ¹	liquid	+	-	-
CHCA/PIP ²	liquid	+	-	-
CHCA/2AQ ¹	solid	+	-	-
CHCA	solid	+++	+++	++

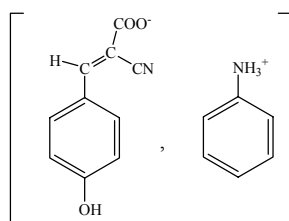


¹ adapted from Armstrong²²

² new ionic matrices

^a “++++” best results, “+”worst results and “-“ no signal recorded.

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 15 peptides.



observed m/z	calculated m/z	probable assignment ^a	sequence	intensity ^b		increase factor
				matrix CHCA	matrix ANI	
1087,4	1087,4	Arg vassopressin	CYFQNCPRG	283	325	1,1
1513.9	1514.7	melanotropin gamma	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		1983	5896	3,0
2271,3	2273.1	prolactin releasing peptide PrPR2	TPDINPAWYTGGRGIRPVGRF	411	848	2,1
2351.1	2351.16	CLIP (1-21)	PVKVYPNVAENESAEAFPLEF	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	SSPETLISDLLMRESTENAPRTRLEDPSMW		168	
4289.1		unknown		131	1816	13,9
4970.1		unknown		7187	57000	7,9
5221.4		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)

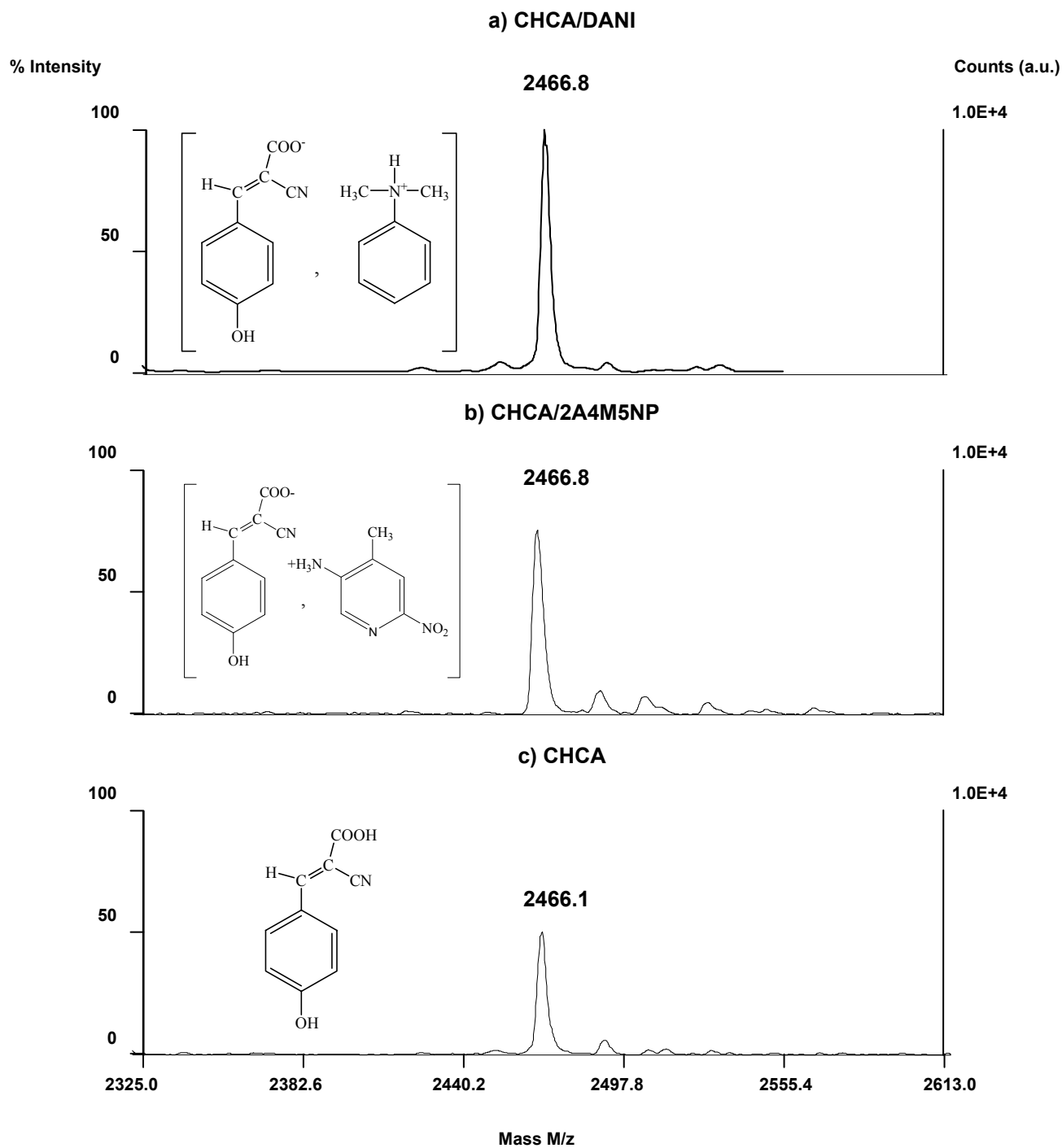


Figure 1. Typical MALDI mass spectrum obtained for ACTH 18-39 (1pmol) in the linear negative ion mode using CHCA/DANI (a), CHCA/2A4M5NP (b) and CHCA (c) as matrix. A formula are enclosed to each mass spectrum.

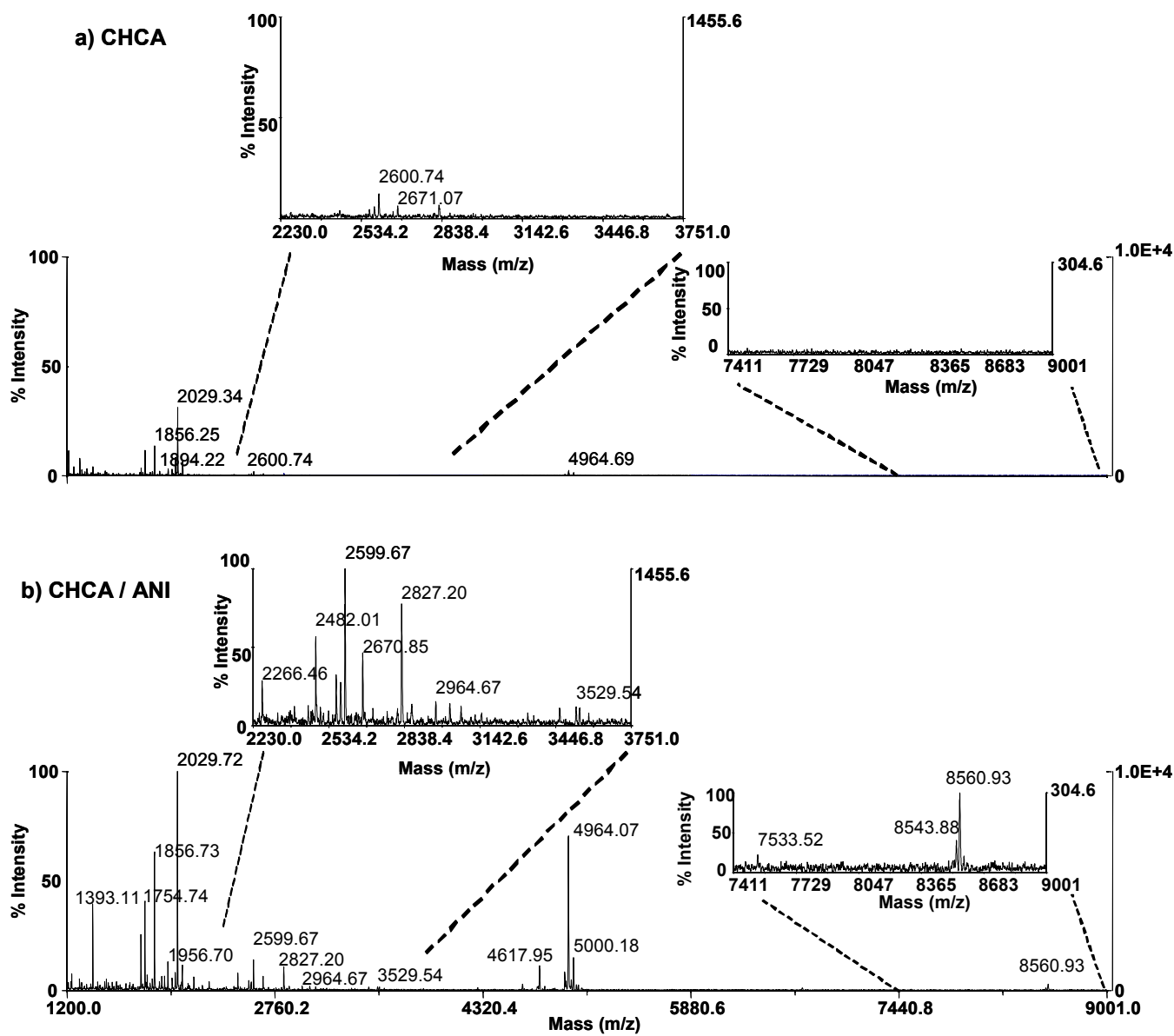


Figure 2. MALDI mass spectra recorded during direct analysis of a rat brain section in reflector positive mode using a) CHCA and b) CHCA/ANI for mass range 1200-9000 (same laser intensity in both cases corrected by energy of desorption).

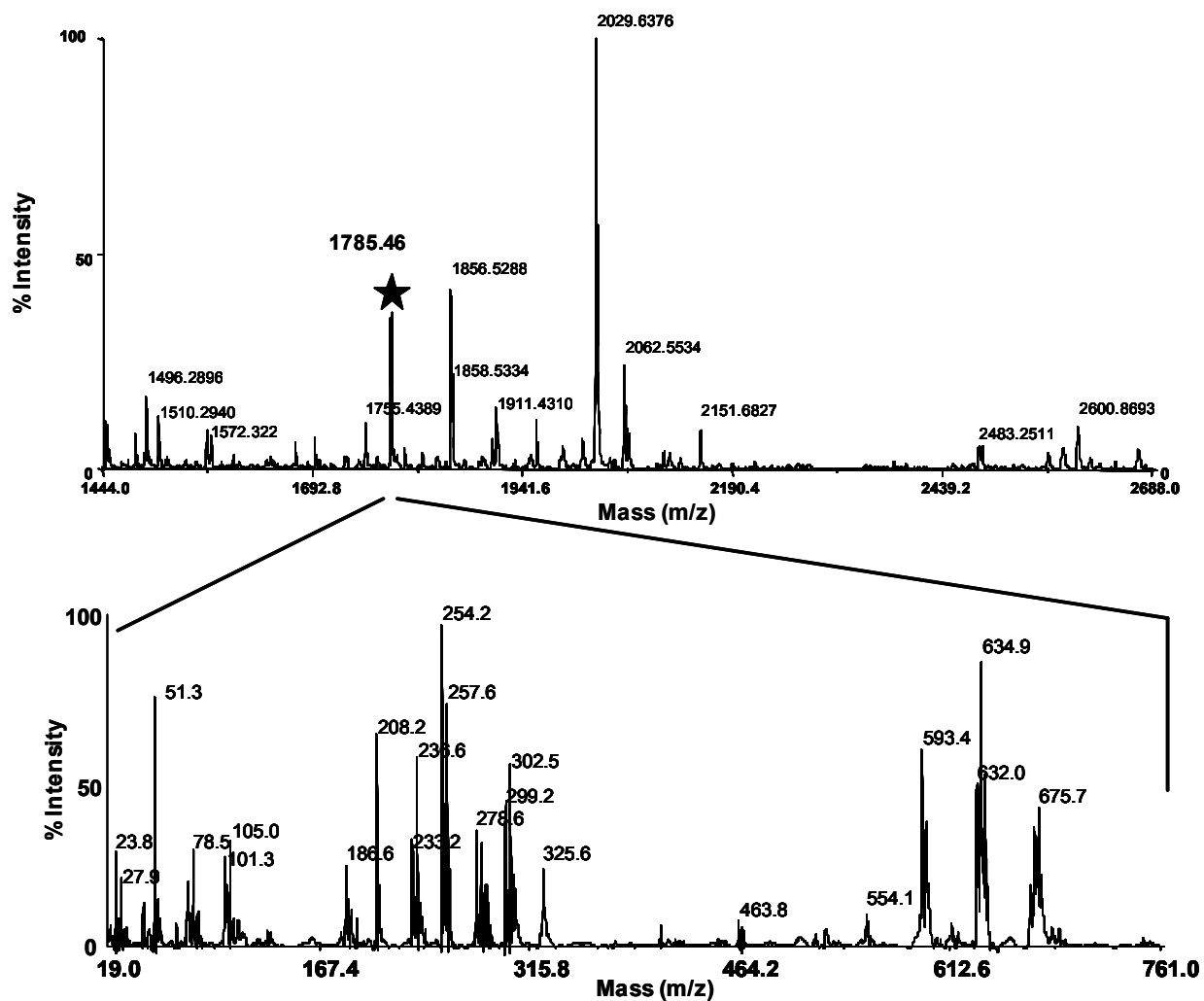


Figure 3. MALDI positive reflectron mass spectrum of a rat brain section using CHCA/ANI and the resulting PSD spectrum for the parent ion at m/z 1785 (collision gas : Xenon).

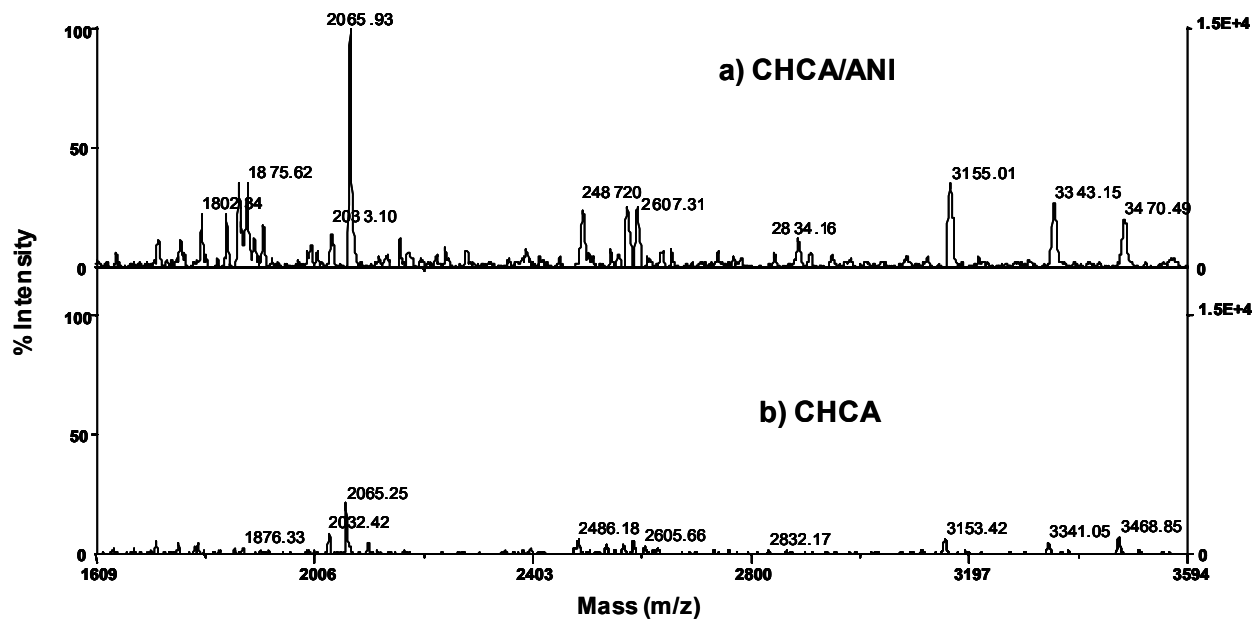


Figure 4. Direct analysis on a rat brain section in linear negative mode for a) CHCA/ANI and b) CHCA.

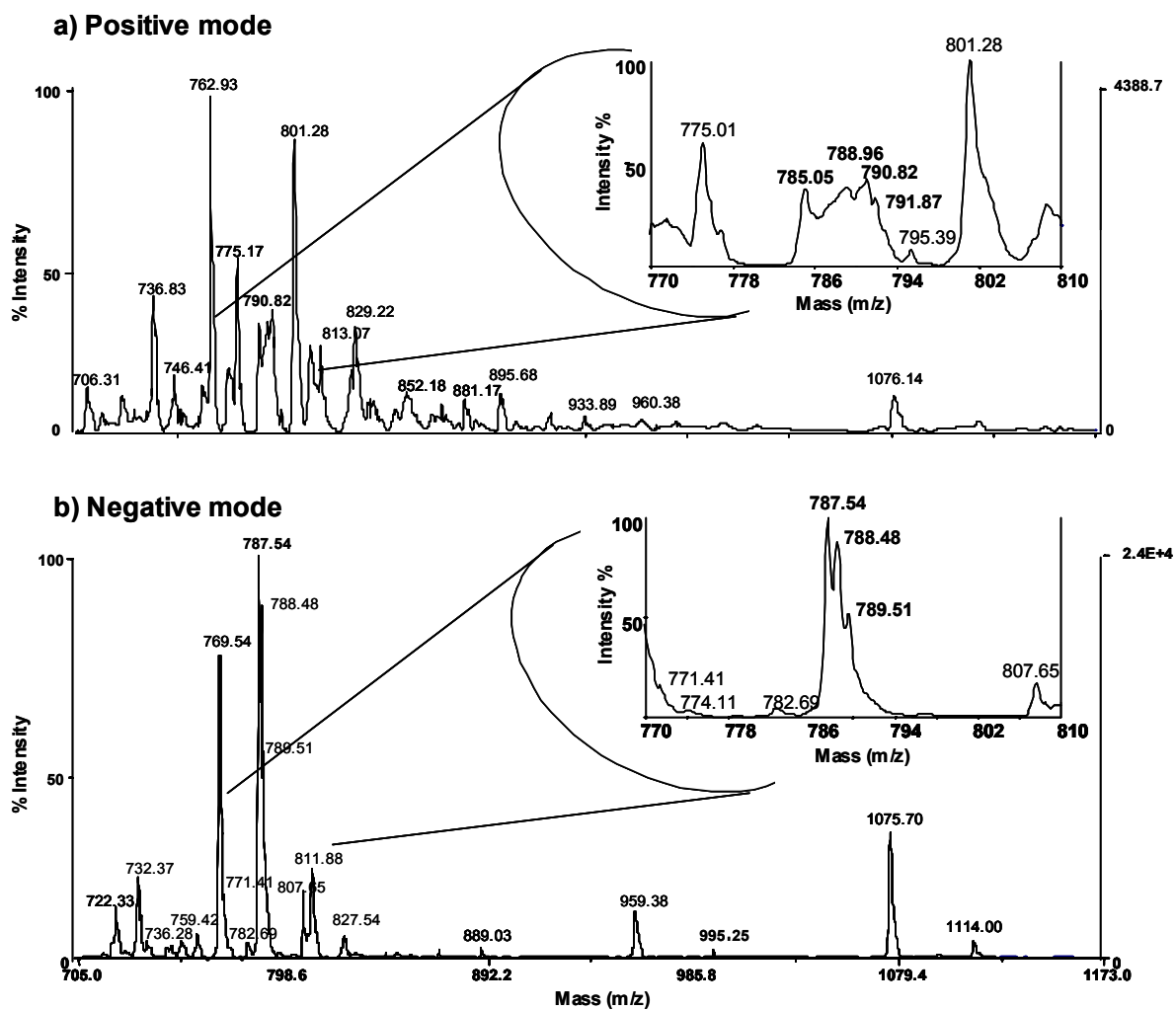


Figure 5. Typical linear MALDI mass spectra for the direct analysis of a rat brain section (a) in positive and (b) in negative mode at the same localisation using CHCA/DANI. The insets represent a zoom on the mass range 770-810.

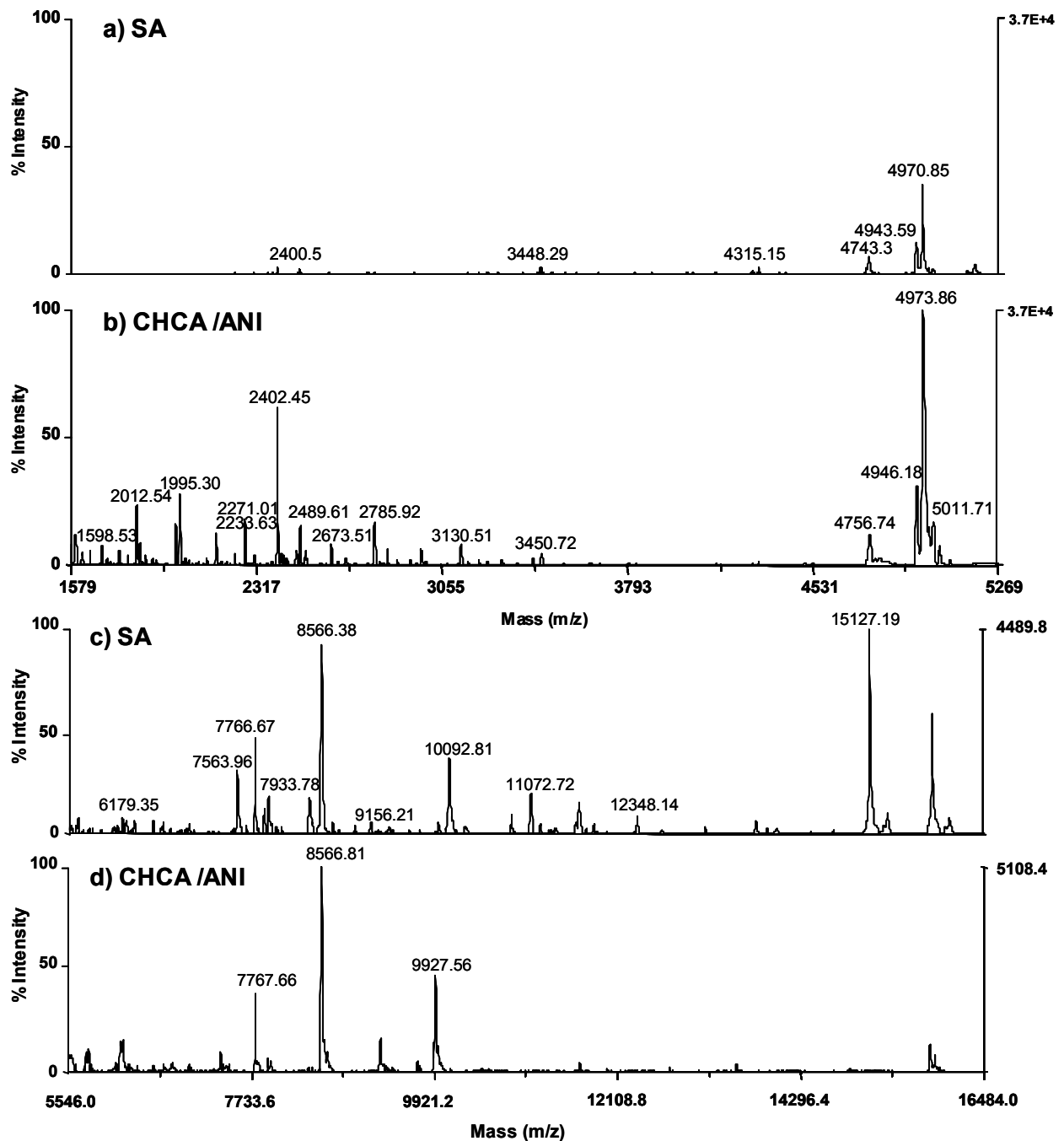
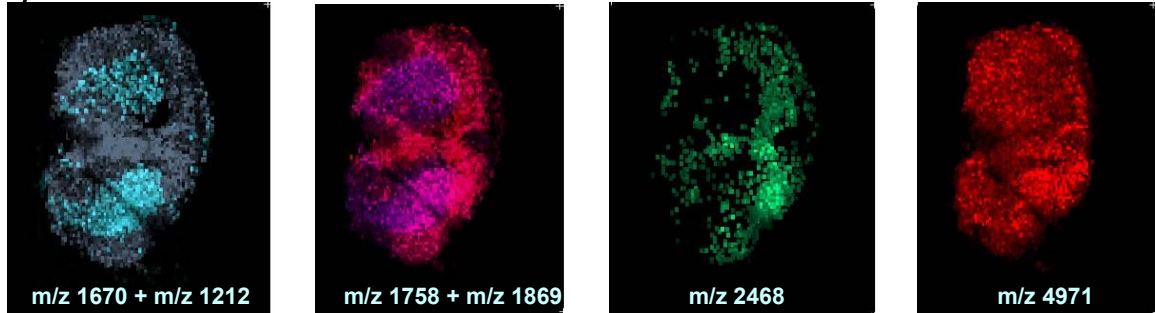
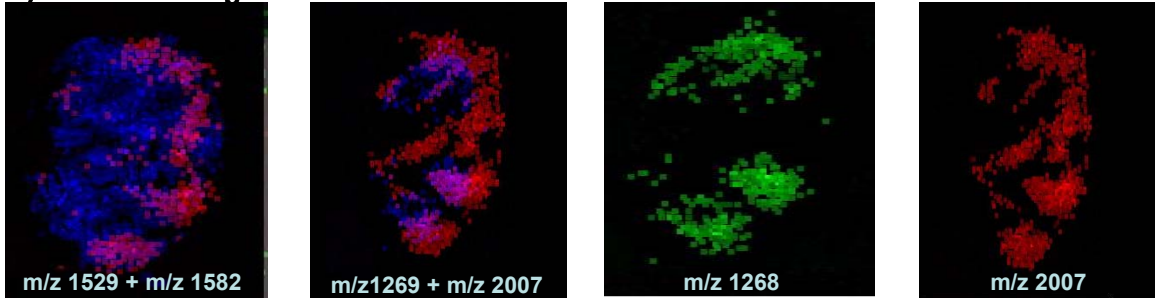


Figure 6. Direct analysis MALDI mass spectra of tissue using SA (a and c) and CHCA/ANI (b and d) in linear positive mode, for mass range mass m/z 1580-5270 (a and b) and m/z 5546-16484 (c and d).

a) CHCA/ANI Positive mode



b) CHCA/ANI Negative mode



c) CHCA Positive mode

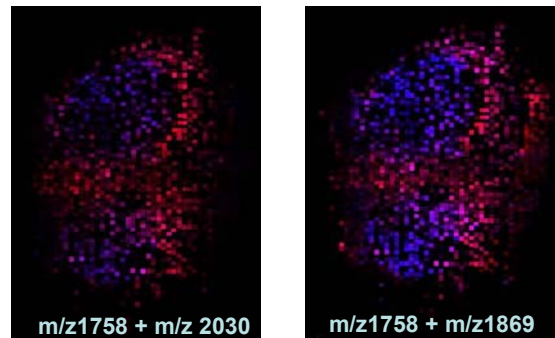


Figure 7. MALDI-IMS using MALDI LIFT/TOF in reflector mode at 50 Hz repetition rate with ionic matrix CHCA/ANI (a) in positive and (b) negative mode and with CHCA in positive mode (c). For CHCA/ANI, acquisitions in both polarities were performed on the same rat brain cut. Images have been reconstructed using FlexImaging software and represent the repartition of a m/z in the tissue slice. Images with two colours correspond to the superposition of two m/z images.