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Ionic Liquids as Matrices in Microfluidic Sample Deposition for High-mass Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry

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3 Ionic Liquids as Matrices in Microfluidic
4 Sample Deposition for High-mass Matrix-
5 Assisted Laser Desorption/Ionization Mass
6 Spectrometry

7

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17

1 **Abstract**

2

3 Sample preparation for MALDI-MS via a microfluidic deposition device using ionic liquid matrices
4 addresses several problems of standard protocols with crystalline matrices, such as the
5 heterogeneity of sample spots due to the co-crystallization of sample and matrix, and the limited
6 capability for high-throughput analysis. Since ionic liquid matrices do not solidify during the
7 measurement, the resulting sample spots are homogeneous. The use of these matrices is also
8 beneficial for automated sample preparation, since crystallization of the matrix is avoided, and thus
9 no clogging of the spotting device can occur. The applicability of ionic liquids to the analysis of
10 biomolecules with high molecular weights, up to ≈ 1 MDa is shown, as well as a good sensitivity
11 (5 fmol) for recombinant human fibronectin, a protein with a molecular weight of 226 kDa.
12 Microfluidic sample deposition of proteins with high molecular weights will in the future allow
13 parallel sample preparation for MALDI-MS and for electron microscopy.

14 **Keywords**

15 MALDI mass spectrometry; ionic liquids; high-mass protein analysis; microfluidic sample preparation

16 **Introduction**

17

18 Since its invention in the early 1980s by Karas and Hillenkamp¹, matrix-assisted laser
19 desorption/ionization mass spectrometry (MALDI-MS) has developed into a very powerful analytical
20 method, especially for the analysis of biomolecules such as oligonucleotides,^{2,3} peptides and
21 proteins.^{2,4} One of the main features of MALDI is the incorporation of the sample into a suitable
22 matrix. The matrix normally co-crystallizes with the sample, and also serves to absorb the laser
23 wavelength that is used to desorb and ionize the sample/matrix mixture.⁵⁻⁷ However, the

1 crystallization process is not homogeneous when using solid matrices and the sample incorporation
2 is therefore not homogeneous, either. This often leads to the formation of regions within a sample
3 where the signal intensity is much larger than elsewhere. This well-documented “sweet spot” effect
4 obscures the relation between the measured signal intensity and the sample concentration present.
5 A homogeneous sample preparation is one approach to overcome this problem. For example, special
6 crystallization conditions that lead to smaller and therefore more homogeneous crystals can be
7 applied.^{4,8,9} Another possibility is the use of liquid matrices, e.g., ionic liquids where the acidic proton
8 of the matrix is replaced by another cation such as an ammonium or a phosphonium moiety.¹⁰
9 Depending on the cation used, the matrix can remain liquid or become solid, but in any case will
10 present a much more homogeneous environment,¹⁰ in which the sample is more evenly distributed.
11 It has already been shown that ionic liquids can be used as matrices in MALDI.¹⁰⁻¹⁵ Due to the
12 homogeneous sample distribution, the mass spectra obtained are more reproducible compared to
13 the ones using solid matrices.^{12,15}

14 Besides determining the chemical identity of components within a sample, it is also important to
15 analyze the associated spatial distribution pattern. Since its introduction by Caprioli et al.¹⁶ MALDI-
16 MS imaging became an important tool to investigate proteins and peptides¹⁷⁻²⁰ or lipids^{21,22} in tissue
17 sections. One of the crucial points in MS imaging is a fast and reproducible deposition of matrix over
18 large sample areas. Several different approaches for automation, such as acoustic matrix
19 deposition,²³ electric field mediated deposition,²⁴ piezo-electric dispensing,²⁵ ink-jet printing,^{26,27} or
20 spotting robots^{28,29} have been published, although all of the deposition methods mentioned are
21 applied to low-mass samples only. The analysis of the intact proteome of tissue sections has several
22 advantages compared to the commonly used approach, where the proteins are digested before MS
23 imaging: the information on the protein is directly available without database search of the
24 fragments, and since no waiting time for digestion is needed, and spreading or leaking of compounds
25 into neighboring compartments is minimized. Only few examples of imaging of proteins with
26 molecular weights exceeding 50 kDa have been published.^{30,31} The samples for these experiments

1 were prepared either manually^{30,31} or using an automated sprayer.³¹ Development of a matrix
2 spotting method suitable for high-mass MALDI would allow the profiling and imaging of compounds
3 such as proteins or protein complexes with masses higher than 100 kDa.

4 Among the methods mentioned above, the simplest way to prepare such samples is by the use of a
5 robotic spotting device. However, plugging of the capillary or the spotting nozzle due to
6 crystallization of the matrix, or sample loss inside components of the spotting device due to adhesion
7 of the sample to the tubing/capillaries (if delivered via the spotting device) are known to cause
8 problems.²³ The use of ionic liquid matrices in such a spotting device would therefore be beneficial;
9 however, this has never been shown.

10 In this paper, a microfluidic sample delivery set-up for spotting liquid MALDI matrices is presented. It
11 has also been developed for compatibility with automated deposition on electron microscopy (EM)
12 grids.³² In contrast to the work published by Benesch and coworkers, where deposition is achieved in
13 vacuo after mass separation and soft landing on an EM grid,³³ the whole proteome is deposited
14 without prior mass separation in our approach. In conjunction with cell lysis and loss-less sample
15 transport,³² “visual proteomics” is one of the long-term goals:³⁴ complete lysis of few cells allows for
16 the preparation of minute amounts of sample, either by “smearing” of the whole proteome onto an
17 EM grid, or by aliquoting the lysate onto MALDI targets. Since the very same sample could be
18 analyzed in parallel by electron microscopy and high-mass MALDI, information about the shape and
19 the mass of the proteome of cells could be obtained, and individual proteins could be identified
20 according to these parameters. If a sufficiently large number of EM images can be recorded, 3D
21 reconstruction of the protein shape should also become possible.

22 Until now, the highest molecular weight molecules that has been measured by MALDI using ionic
23 liquid matrices is immunoglobulin G (molecular weight of 146 kDa);¹² the heaviest ion ever detected
24 in this fashion was the nonspecific trimer of urease (molecular weight of 270 kDa).¹¹ The goal of this
25 work is to show the capability of ionic liquids as matrices for the analysis of biomolecules with even

1 higher molecular weights (up to 1 MDa), and to prove that the use of ionic liquid matrices does not
2 compromise sensitivity, as demonstrated by determining the limit of detection for samples with
3 masses well above 100 kDa with manual sample preparation, as well as in combination with
4 microfluidic sample deposition. The use of such a combination would potentially allow for extending
5 MALDI imaging or profiling of biomolecules with higher masses and therefore provide insight into
6 biological pathways within tissue. The development of a spotting device, which can be used to
7 prepare electron microscopy grids as well as MALDI plates, is useful for future investigations of the
8 cell content, e.g. fast identification of proteins by both methods.

9 The suitability of ionic liquids for the analysis of biomolecules with high molecular weights is shown
10 here. Sensitivity restriction is not compromised, and improved homogeneity of the sample spots is
11 observed. In conjunction with a microfluidic spotting device, this rapid, sensitive and reproducible
12 sample preparation method paves the road towards visual proteomics.

13 **Experimental**

14 **Materials**

15 Sinapinic acid (SA), α -cyano-4-hydroxycinnamic acid (CHCA), trimethylamine solution (43%),
16 triethylamine, pyridine, and bovine serum albumin (BSA) were purchased from Fluka (Buchs,
17 Switzerland). Trifluoroacetic acid (TFA) was purchased from Acros Organics (Geel, Belgium). N-
18 methyl-N-isopropyl-N-tertbutyl-amine (MIT-amine), phosphate buffered saline tablets (pH 7.4),
19 acetonitrile, immunoglobulin G (IgG) and immunoglobulin M (IgM) were obtained from Sigma-Aldrich
20 (Buchs, Switzerland). Bovine thyroglobulin (Tg bov) was purchased from Morphosys AbD (Düsseldorf,
21 Germany), and recombinant human fibronectin (rh Fn) from R&D Systems Europe (Abingdon, UK).
22 The water used was of nanopure quality ($18.2 \text{ M}\Omega \text{ cm}^{-1}$) and prepared using a NANOpure water
23 purification system (Barnstead, IA, USA).

1 **Sample preparation**

2 Lyophilized rh Fn was reconstituted in PBS at a concentration of $100 \mu\text{g mL}^{-1}$. Tg bov was used as
3 delivered. The ionic liquid matrices were synthesized by a procedure similar to that published by
4 Zabet-Moghaddam et al.¹⁵ After addition of an equimolar amount of amine to a 10 mg mL^{-1} solution
5 of sinapinic acid in acetonitrile/water (2:1, v:v) containing 0.1% TFA, and subsequent sonication for
6 10 minutes, the sample was mixed with the freshly prepared ionic liquid matrix in a 1:1 (v:v) ratio and
7 vortexed. 0.5 - 1 μL of the sample mixture was deposited on a 384 spot stainless steel MALDI plate
8 and the solvents were allowed to evaporate.

9 **Spotting device**

10 Besides manual sample deposition, automated deposition with a self-built spotting device³² was also
11 performed. The spotting device (figure 1) consists of a syringe pump (KDS210, Ismatec SA,
12 Switzerland), equipped with a gas-tight syringe (Vici AG International, Schenk, Switzerland)
13 containing nanopure water, a ten-port valve for sample injection (Valvo 10-port 2-position valve; BGB
14 Analytik AG, Bockten, Switzerland) and a fused silica capillary as nozzle for the deposition of the
15 sample. For accurate deposition of the sample onto the MALDI plate, a plate holder was constructed
16 and mounted on a xyz translation stage. A commercial MALDI plate was cut in half and positioned on
17 the sample holder, where it was held in place by two magnets. To place the nozzle correctly with
18 respect to the plate, its position was observed with two cameras positioned at different angles for
19 precise alignment in all three directions. The coordinates of the spot positions were stored in a list
20 before the deposition, for a faster access during the deposition. The pump, the valve, and the stage
21 were controlled via software programmed in LabView (National Instruments, Austin TX, USA). The
22 protein samples were mixed with an ionic liquid matrix and injected into a 5 μL PEEK-loop attached
23 to the ten-port valve. From another PEEK-loop with a volume of 10 μL , 5 μL of air were injected into
24 the spotting capillary before and after the sample. This procedure allows the separation of the
25 sample into several aliquots and protects the samples from dilution by surrounding water and cross
26 contamination of different samples.³⁵ In the future, different samples might be injected sequentially

1 into different bubbles. A flow rate of $5 \mu\text{L min}^{-1}$ was chosen for all steps that were necessary to
2 create the sample bubble. To deposit the spot on the plate, a droplet was “grown” on the nozzle to a
3 volume of $0.5 \mu\text{L}$ with a flow rate of $1 \mu\text{L min}^{-1}$. The plate was then lifted close to the nozzle (but
4 without making contact), such that just the drop made contact to the plate, where it remained after
5 retraction of the plate. To avoid carry-over of sample or matrix solution, the capillaries, the sample
6 loops and the nozzle were rinsed with water at a flow rate of $5 \mu\text{L min}^{-1}$ after every series of
7 deposition. The nozzle was also dipped into a water reservoir to get rid of possible contaminations on
8 the outer part of the capillary.

9 **Mass Spectrometry**

10 A MALDI TOF/TOF mass spectrometer (4800 Plus, AB SCIEX, Darmstadt, Germany) equipped with a
11 frequency-tripled Nd:YAG laser (355 nm) was used. The laser power is reported in arbitrary units
12 ranging from 0 to 7000. An acceleration voltage of 20 kV was applied. In one run, the same sample
13 spot was measured several times with identical conditions. One of these measurements consisted of
14 200 to 500 shots that were accumulated and averaged to obtain a mass spectrum. The laser shots
15 were distributed in a randomized pattern over the whole sample spot. To allow for measurements in
16 the high mass region, the system was equipped with a special high mass detector (HM2tuvo, CovalX,
17 Zürich, Switzerland) based on ion-to-secondary ion-to-electron conversion. The detector voltages for
18 the first and second conversion dynodes were -2.5 kV and -20 kV , respectively. Calibration was
19 performed using proteins with known masses such as BSA or IgG and extrapolated to a higher m/z
20 range. The data was recorded using software from the manufacturer (4000 Series Explorer V.3.5.3,
21 AB SCIEX, Darmstadt, Germany) and exported as a text file to OriginPro 8.5.0 (OriginLab Corporation,
22 Northampton MA, USA), where the data were smoothed with the provided Savitzky-Golay algorithm.

23

1 Results and discussion

2

3 Ionic liquids have been successfully used as MALDI matrices for a broad range of analytes such as
4 amino acids¹⁵, DNA oligomers¹³, peptides^{10,11} and proteins with molecular weights up to
5 approximately 146 kDa per monomer.¹⁰⁻¹² Since sinapinic acid usually shows the best performance
6 for the analysis of high-mass proteins, especially in the mass range over 100 kDa, we chose
7 sinapinate as the anion for the ionic liquids. Different amines were tested as counter cations. For
8 comparison, an ionic liquid consisting of N-methyl-N-isopropyl-N-tertbutyl amine in combination with
9 CHCA was also used. Using this matrix, the trimer of urease with a total molecular weight of 270 kDa
10 has previously been detected.¹¹

11 When investigating complex samples such as lysates or mixtures with unknown compounds with
12 MALDI-MS, sensitivity is a key aspect. To determine the sensitivity, a dilution series with rh Fn was
13 generated with either PBS buffer or water as the solvent. These freshly prepared diluted samples
14 were mixed in a 1:1 (v:v) ratio with the matrix under investigation and analyzed by MALDI-MS. The
15 sample is detected mainly as protonated species, but sodiated species cannot be excluded entirely.
16 Due to the limited resolution of the instrument in this m/z range the different species cannot be
17 distinguished. The limit of detection was taken to be the lowest concentration where the signal of
18 the singly charged monomer (226 kDa) was still visible with a signal-to-noise ratio of 3:1 (figure 2).
19 The observed limit of detection was found to depend on the solvent used for dilution as well as on
20 the matrix. The limits of detection for solid sinapinic acid as matrix were determined as 15 fmol or
21 7.5 fmol of rh Fn diluted in water or PBS, respectively (data not shown). When using an ionic liquid
22 matrix consisting of trimethylammonium and sinapinate, the limit of detection was 5 fmol when
23 using either water or PBS as solvent. This is in the same range, or even slightly lower, compared to
24 using a solid matrix. Therefore, no drawbacks arise concerning the sensitivity when ionic liquids are
25 used as matrix instead of sinapinic acid.

1 Besides the sensitivity, the homogeneity of the sample spot also plays an important role. Measuring
2 a sample spot many times generally leads to decreasing signal intensity in MALDI, since the sample is
3 gradually depleted by laser ablation. If the sample is distributed evenly and the laser shots are
4 distributed randomly over the whole spot, the signal intensity should decrease monotonically. If
5 sweet spots exist, the signal decrease might not be monotonic but more random. For example, if in
6 the first measurements of a run the sweet spots are missed, but hit in a later measurement, the
7 signal intensity will increase again. To compare the behavior of an ionic liquid and a solid matrix, IgG
8 was measured using an MIT-amine ionic liquid matrix as well as solid sinapinic acid. Both matrices
9 were prepared at a concentration of 10 mg mL⁻¹ in 2:1 acetonitrile:water (v/v). An equal volume of
10 matrix and sample was mixed and spotted eight times with a volume of 0.5 µL per spot. Each of the
11 eight spots was analyzed by ten series of 500 shots in a randomized pattern with a laser power of
12 4600 a.u. Eight replicates of the ten series were averaged and plotted. Figure 3 shows that the signal
13 intensity of the experiment with the solid matrix does not decrease steadily, but reaches a maximum
14 intensity in the fourth run. This somewhat unexpected finding is probably due inhomogeneity in
15 crystallization, that may lead to accumulation of salts or other impurities in different layers of the
16 crystallized sample, and exposure of the highest sample concentration once the laser has removed
17 some of these layers. In contrast, the use of ionic liquids leads to a steadily decreasing signal. This
18 observation allows the design of an automated run with a reduced number of laser shots and thus
19 shorter overall measurement time, since the signal intensity is monotonically decaying. When
20 performing a series of measurements manually, it is also possible to search for a sweet spot to obtain
21 the best signal possible. However, if the laser beam is “parked” on such a sweet spot it will soon
22 become depleted and yield no signal anymore. If a large number of measurements is needed, for
23 example in MALDI imaging, manual acquisition of spectra is very time consuming or impossible and
24 therefore, automated acquisition is highly beneficial.¹⁸

25 In table 1, results for all four proteins measured with different cations are summarized. This table
26 shows that the choice of the amine cation is crucial for the success of a measurement. Some proteins

1 seem to possess properties that render them rather easy to ionize (e.g. IgG) while others are only
2 ionizable with a certain combination of cation and anion of the liquid matrix. Moreover, ammonium
3 cations which work for a certain organic acid as counterion, such as MIT-amine in combination with
4 CHCA,¹¹ do not necessarily work well with others. The reason for these findings might be that the size
5 of the cation, which decreases from left to right in table 1, hampers the protonation of the amine by
6 the sinapinic acid molecule and this leads to a mixture of ionic liquid matrix and sinapinic acid. The
7 amine might get protonated during the MALDI process and therefore reduce the ionization yield of
8 the sample. In a study published by Crank and Armstrong¹¹, the pK_a and the proton affinities were
9 identified as two major factors determining the ability of a cation to form ionic liquid matrices that
10 work well. They observed a trend that the cation needs a $pK_a \geq 11$ and a proton affinity $> 930 \text{ kJ mol}^{-1}$
11 to form a matrix that performs well in MALDI. We found that in addition, the sample itself influences
12 the success of the experiment: the two cations that worked well for the analysis of IgM, pyridine and
13 trimethylamine, do not fulfill the requirements concerning pK_a . Similar results were obtained by
14 Mank et al.¹² where IgG was detected using tributylamine as cation ($pK_a = 9.9$).¹¹ However, all these
15 cations have a proton affinity $\geq 930 \text{ kJ mol}^{-1}$ and thus fulfill the second requirement.³⁶ In general it
16 could be shown that ionic matrices are applicable for the analysis of biomolecules with very high
17 molecular weights, and present a suitable alternative to conventional solid matrices.

18 To allow for the analysis of high-mass biomolecules, e.g. in a cell lysate, the suitability of a spotting
19 device, such as the one shown in figure 1, in combination with ionic liquids as matrices needs to be
20 verified. The spotting device used in this work is compatible with commercially available 384 spot
21 MALDI plates that were cut in half. For reasons of the limited travel range of the xyz stage, only
22 eleven rows with eight spots each were accessible. Typically, 0.5 μL of sample were deposited with a
23 flow rate of $1 \mu\text{L min}^{-1}$. If the time needed for movements of the sample plate is taken into account,
24 an overall spotting time of only ca. 45 s per spot is currently required. The accessible range of the
25 MALDI plate can be spotted in about 1 h. The accessible range could easily be increased by replacing

1 the currently used stage motors with motors with greater travel distances, large enough to cover the
2 whole 384-spot plate.

3 Two proteins that are considered to be “difficult” due to their high masses were chosen as test
4 samples. They were deposited in the same way as described above, using the spotting device. In case
5 of rh Fn, a matrix made from trimethylamine solution and sinapinic acid was used, for IgM the matrix
6 consisted of SA and pyridine. 10 μ L of the sample/matrix mixture was injected with the gas-tight
7 syringe into the spotting device (figure 1). High-mass protein samples were then spotted onto the
8 stainless steel MALDI plate and analyzed (figure 4) with 200 shots at a laser power of 5100 a.u. The
9 fused silica nozzle could be used for 48 consecutive runs without any signs of plugging. This shows
10 the advantages of ionic liquids over conventional matrices, where plugging was observed.²³ The
11 protein peaks were easily detected and assigned. The width of the signal of IgM is caused by the
12 microheterogeneity in the primary amino acid sequence and the glycosylations that are present. The
13 spotting device is able to handle samples with such high masses without losing the sample in the
14 tubing due to adhesion. Furthermore, no cross contamination was observed. Although the use of
15 ionic liquids for MALDI imaging has already been shown,²² this was never extended to biomolecules
16 with high masses. With the approach introduced in this work, imaging of these compounds might
17 become feasible and allow further insight into the distribution of proteins and similar samples within
18 tissues.

19 The setup presented here enables the parallel preparation of EM grids³² and MALDI-MS targets using
20 a microfluidic platform. Since the handover of the sample is lossless, even the preparation of tiny
21 sample amounts becomes possible. In a next step, the sample preparation will become online, which
22 means that the sample and the matrix is mixed within the microfluidic device and therefore the
23 sample amount needed is reduced even further. During this step, other sample pretreatment, such
24 as desalting, dilution, or buffer exchange can be performed as well. In the future, this workflow could
25 allow for the analysis of cell lysates, where the samples of interest are only present in limited
26 quantity. Complete automation of the whole spotting procedure allows rapid preparation of the

1 targets. Therefore, information about the content of cells concerning the shape and the mass of the
2 compounds could be gained and probably changes in the expression of certain proteins could be
3 monitored.

4

5 **Conclusions**

6

7 In this work, we could show that ionic liquids are useful for the analysis of high mass biomolecules
8 and that the deposition of ionic liquid matrices with a spotting device proves to be useful for the
9 preparation of MALDI samples. The process of sample deposition can either be automated or is
10 controlled manually. Since the same spotting device can be used for preparation of both, MALDI
11 samples and EM grids, analysis of the same sample with respect to mass and shape is possible.

12 Furthermore, it was possible to show the suitability of these matrices for high-throughput MALDI-
13 MS, especially considering the fact that the benefits do not lead to disadvantages such as lower
14 sensitivity. This fact is indeed crucial for the success of the presented combination, since a low limit
15 of detection allows extending the range of applications and might in the future allow for the survey
16 of reactions or events along a time axis. To achieve this goal, it is necessary to continue the
17 developments of the existing system and combine it, for example, with a continuous feed of matrix.
18 This would allow skipping the sample preparation in vials and enabling online sample processing.

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20

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8

9 Captions

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11 Figure 1: Schematic drawing of the spotting device. The sample and the air bubble were introduced
12 via the injection port (1) into the 5 μL (2) and the 10 μL loop (3), respectively. The sample was
13 separated from the surrounding solvent with a 5 μL air bubble on both sides in the capillary (4). The
14 droplet of the sample was formed at the tip of the nozzle (5) to a final volume of 0.5 μL and
15 deposited by lifting the MALDI plate close to the nozzle.

16

17 Figure 2: Determination of the limit of detection for rh Fn. The limit of detection is defined to be the
18 concentration where the singly charged fibronectin monomer (226 kDa) is still detectable with a
19 signal-to-noise ratio of 1:3, which is in this example 7.5 fmol rh Fn per spot, whereas the next lower
20 amount of 3.8 fmol did not yield a signal.

21

22 Figure 3: Comparison between the signal intensity of the rh Fn monomer measured either in solid
23 sinapinic acid (grey) or in ionic liquid consisting of sinapinate and trimethylammonium (black). The
24 steady signal decay when using the ionic matrix shows the homogeneous distribution of the sample
25 within the spot. The decrease is caused by depletion of sample during the measurement. The grey
26 curve shows much more fluctuations in signal intensity, therefore leading to the conclusion that the
27 sample was not distributed homogeneously but forming sweet spots.

28

29 Figure 4: The spectra of recombinant human fibronectin (a) and Immunoglobulin M (b) were
30 recorded from spots deposited using an automated spotting device. For rh Fn an ionic matrix
31 produced from trimethylamine and sinapinic acid was used. The spectrum of IgM was recorded using
32 sinapinic acid as anion as well, but pyridine as counterion.

1

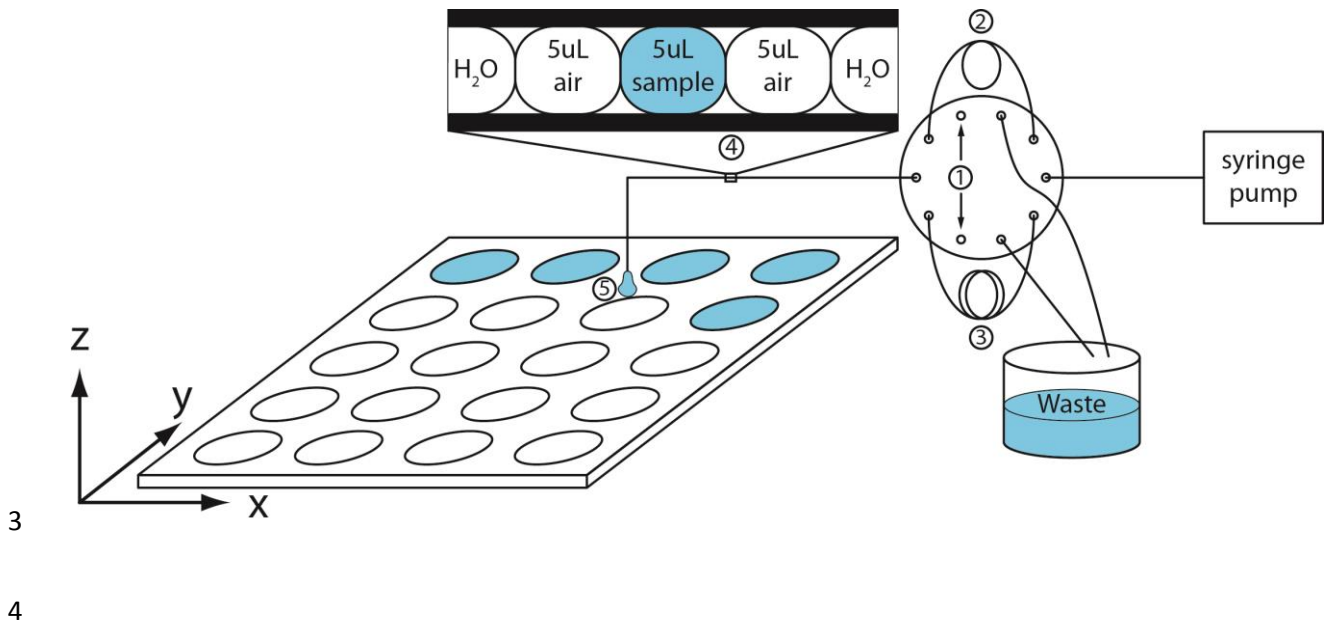
2 Table 1: Summary of the different proteins and ionic liquids that were investigated. The pK_a and the
3 proton affinity (PA) of the used amines are specified, where available. The quality of the obtained
4 spectra is indicated with a three star ranking system, where three stars indicate the optimum. No
5 signal obtained is indicated with a "0". As can be seen, there are proteins that seem to work with
6 every matrix (IgG) and matrices which are able to ionize all the proteins (trimethylamine).

7

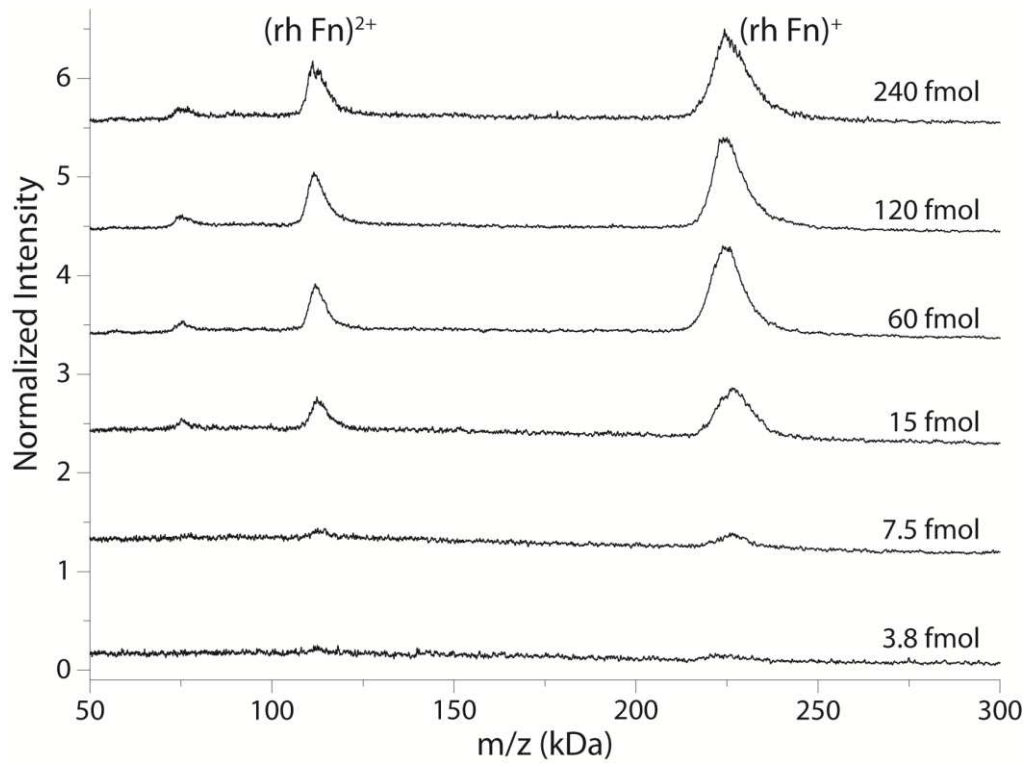
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1 **Figures**

2 Figure 1:



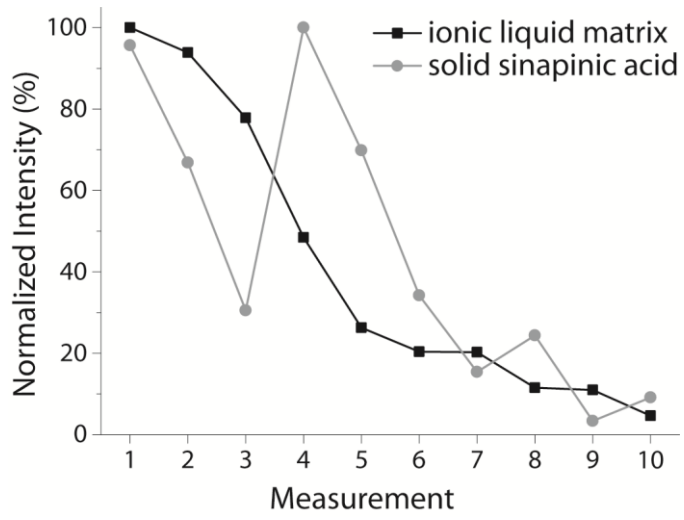
1 Figure 2:



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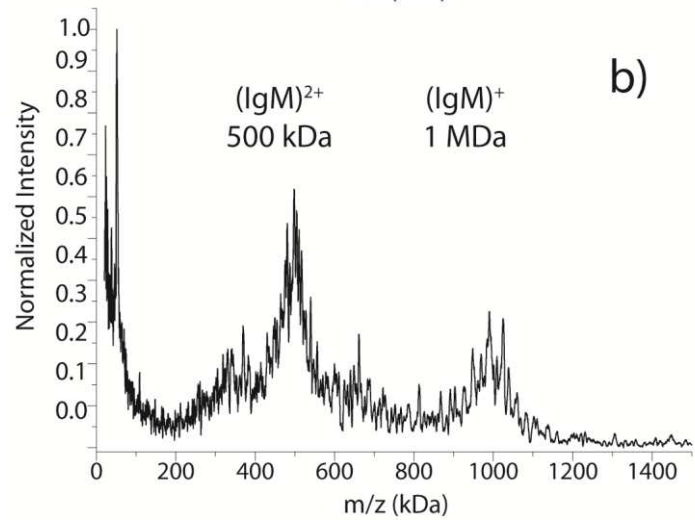
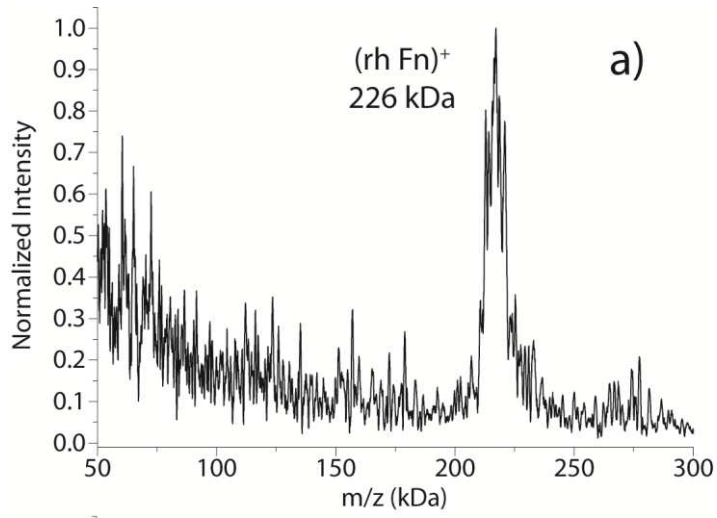
1 Figure 3:



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1 Figure 4:



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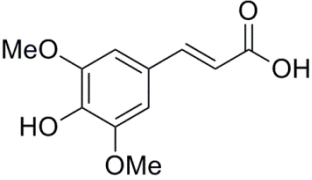
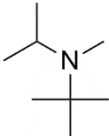

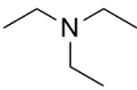

1 **Tables**

2

3

4 Table 1:

5

 <p>Sinapinic Acid</p> <p>pK_a^{36}</p> <p>PA (kJ mol^{-1})³⁶</p>	 <p>MIT-amine</p> <p>10.9¹²</p> <p>NA</p>	 <p>Pyridine</p> <p>5.23</p> <p>930</p>	 <p>Triethylamine</p> <p>10.75</p> <p>981.8</p>	 <p>Trimethylamine</p> <p>9.80</p> <p>948.9</p>
<p>Protein</p> <p>MW (kDa)</p>				
<p>Immunoglobulin M</p> <p>1000</p>	<p>0</p>	<p>**</p>	<p>0</p>	<p>*</p>
<p>Bovine Thyroglobulin</p> <p>660</p>	<p>0</p>	<p>0</p>	<p>0</p>	<p>*</p>
<p>Recombinant human Fibronectin</p> <p>226</p>	<p>0</p>	<p>*</p>	<p>0</p>	<p>**</p>
<p>Immunoglobulin G</p> <p>148</p>	<p>*</p>	<p>**</p>	<p>**</p>	<p>***</p>

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