

Negative mode nanostructure-initiator mass spectrometry for detection of phosphorylated metabolites

Andrea Amantonico · Luca Flamigni ·
Reto Glaus · Renato Zenobi

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Abstract The chemical complexity of the metabolome requires the development of new detection methods to enlarge the range of compounds detectable in a biological sample. Recently, a novel matrix-free laser desorption/ionization method called nanostructure-initiator mass spectrometry (NIMS) [Northen et al., *Nature* 449(7165):1033–1036, 2007] was reported. Here we investigate NIMS in negative ion mode for the detection of endogenous metabolites, namely small phosphorylated molecules. 3-Aminopropyltrimethylethoxysilane was found to be suitable as initiator for the analytes studied and a limit of detection in the tens of femtomoles was reached. The detection of different endogenous cell metabolites in a yeast cell extract is demonstrated.

Keywords

Laser desorption/ionization mass spectrometry ·
Phosphorylated metabolites · Nucleotide detection ·
Nanostructure initiator mass spectrometry · Porous silicon

1 Introduction

In contrast to the proteome and transcriptome, the large chemical heterogeneity of the metabolome poses additional analytical challenges (Hollywood et al. 2006; Weckwerth 2003). The metabolome includes a broad variety of compounds (amino acids, other organic acids, other organic amines, nucleotides, lipids, sugars, etc.) that possess quite diverging physical and chemical properties and are present

in a wide concentration range. Thus, the analysis of the metabolome can realistically only be addressed with a multi-diagnostic approach to cover different classes of analytes (Dunn et al. 2005; Fiehn 2002). Consequently, new analytical methods able to expand the range of detectable metabolites are needed. Today several detection techniques are already employed for metabolite analysis (Dunn et al. 2005). Among them, mass spectrometry (MS) is one of the most widespread (Dettmer et al. 2007; Villas-Boas et al. 2005). Different MS methods are available for the analysis of cellular metabolites, but it is becoming clear that even a versatile technique like MS will require multiple ionization strategies to obtain a detailed knowledge of the metabolome (Nordstrom et al. 2008). For example, electron impact (EI) coupled with gas-chromatographic separations (Fiehn and Kind 2007), and electrospray ionization in direct infusion studies, or coupled with liquid chromatography (Tomita and Nishioka 2005) or with capillary electrophoresis (Soga et al. 2002; Edwards et al. 2006) are popular methods in metabolomics.

Recently, MS methods based on laser desorption/ionization, such as MALDI, have been gaining significance in metabolome analysis (Nordstrom et al. 2008). While MALDI is an extremely powerful tool for proteomics, its application to the analysis of small molecules like the ones constituting the metabolome is still difficult. The use of a chemical matrix to assist the laser desorption process creates background signals that can interfere with the detection of small molecules and can complicate quantification. In spite of these obstacles, Kennedy and coworkers, for example, developed an extremely useful MALDI method for metabolome analysis by choosing an appropriate chemical matrix (Edwards and Kennedy 2005; Sun et al. 2007). This method was further optimized to reach a sensitivity capable even of detecting the metabolites of a

A. Amantonico · L. Flamigni · R. Glaus · R. Zenobi (✉)
Department of Chemistry and Applied Biosciences,
ETH Zurich, CH-8093 Zurich, Switzerland
e-mail: zenobi@org.chem.ethz.ch

single yeast cell (Amantonico et al. 2008). Several other MALDI methods tailored to specific analytes classes have been developed through careful optimization of the sample preparation and proper choice of the matrix (Cohen and Gusev 2002). A recent study exploits the infrared adsorption properties of water and uses the natural water content in a biological sample as matrix for IR-MALDI (Shrestha et al. 2008).

An interesting approach for LDI-MS of small molecules is to avoid the use of a chemical matrix altogether and to employ a matrix-free technique (Peterson 2007). Desorption ionization on porous silicon (DIOS) (Wei et al. 1999) has found much interest in the area of small molecules analysis, and has also been applied to metabolite analysis (Vaidyanathan et al. 2007). In DIOS, the analyte is deposited onto a porous silicon target plate. The light adsorption properties of the porous silicon allow one to use the plate directly for laser desorption–ionization without the interference of other substances. DIOS has been shown to be capable of reaching extremely low limits of detection for particular analytes, such as peptides, when on-surface sample pre-concentration was achieved by surface derivatization (Trauger et al. 2004). On the other hand, DIOS generally showed a lower ion yield compared to traditional MALDI (Hillenkamp and Karas 2007) and its widespread use has been hampered by a lack of robustness and reproducibility (Northen et al. 2007). Recently a related method called nanostructures-initiator mass spectrometry (NIMS) was introduced by Siuzdak and coworkers (Northen et al. 2007; Woo et al. 2008). In NIMS a chemical substance, the “initiator”, is trapped within the pores of a nanostructured silicon surface. Using a surface with a pore size of about 10 nm, different initiators were studied; the target primed in this fashion was then used for laser desorption–ionization MS of small amounts of peptides (tryptic digest), small molecules such as verapamil, and even of the content of a single cancer cell (Northen et al. 2007). NIMS also showed a big potential for small molecule detection in complex biological mixtures, creating hopes that it could become a powerful detection method for metabolome analysis. Yet the efficacy of NIMS for the detection of endogenous metabolites has so far not been studied and it remains to be established which class of metabolites is detectable with it.

Here we investigate the use of NIMS for the detection of small molecules belonging to the central cellular metabolism (such as phosphoenolpyruvate and nucleotides like ATP). Different initiators were tested in order to develop a sensitive method for endogenous metabolites. The detection in negative ion mode and the use of 3-aminopropyltrimethoxysilane (APDMES) as initiator showed to be a combination particularly suitable for the analysis of the phosphorylated species examined, and a LOD in the

femtomole range was reached. The applicability of this method was then finally tested on a biological sample: the detection of different endogenous metabolites in a yeast cell extract was achieved.

2 Materials and methods

2.1 NIMS target preparation

The preparation of a NIMS target has been accurately described elsewhere (Woo et al. 2008). We only present a brief description of the materials and experimental conditions used in this study. NIMS substrates were made by electrochemical etching of p-type silicon wafers in HF solution via anodization (Halimaoui 1997). The silicon wafers employed (boron doped, diameter 50.8 mm, resistivity 0.001–0.02 Ω cm, <100> orientation, one side polished) were purchased from Semiconductor Wafer, Inc. (Hsinchu, Taiwan). Hydrofluoric acid (puriss. p.a., $\geq 40\%$) was from Fluka (Buchs, Switzerland) and was diluted to 25% (v) with methanol for the etching. HF is extremely toxic and corrosive, avoid inhalation, skin or eye contact, wear protecting clothing and use suitable material for handling. The whole wafer was etched in a custom made Teflon cell keeping a constant current density of 48 mA/cm² for approximately 30 min. In order to minimize the spectral background the silicon wafers were cleaned with piranha solution before the etching. Piranha solution is highly corrosive and potentially explosive, extreme caution and appropriate protective clothing are necessary.

Figure 1 shows the etched silicon chip (b) in a Petri dish and a SEM image of the surface with the typical 10–20 nm size pores (a). The surface obtained can be used directly for LDI-MS, can be derivatized, or an initiator can be loaded for NIMS. If derivatization is desired, a layer of silane is pipetted onto the surface, with the wafer placed in a Petri dish, and warmed to 100°C in an oven for 30 min. The etched silicon is then ready for loading of the initiator.

2.2 Loading initiators

Loading the initiator onto the surface was found to be a crucial step for the LDI performance of the NIMS target. The initiator is added onto the etched wafer with a pipette (about 20 μ l/cm²) and distributed over the whole surface. To minimize hydrolysis of the initiator, the operation was carried on under a stream of nitrogen. After soaking at room temperature in a Petri dish for 30 min, excess initiator is gently removed with a nitrogen flow until only a thin film is left. Then, the NIMS target is placed on a heating plate (100°C) for about 3 s and the initiator excess is again blown off with a nitrogen jet. The last step is repeated at least three

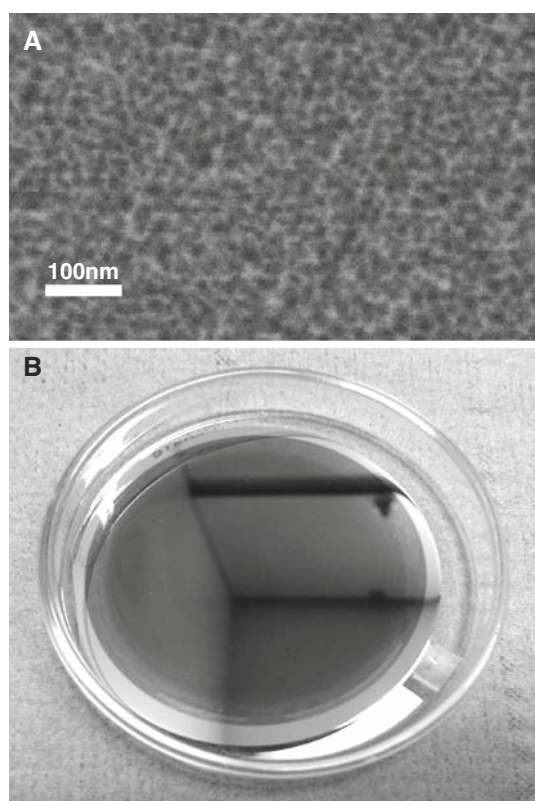


Fig. 1 **a** Preparation of the silicon substrate for NIMS. SEM image of the surface shows 10–20 nm size pores. The surface was prepared by anodic etching in HF of a 2 in. silicon wafer. **b** After the treatment, the etched part of the chip appears black

times until the chip appears dry. The chip should not contain an excess of initiator but on the other hand should not lose it completely; in both cases the LDI capabilities will be compromised. The initiators tested in this study were (i) perfluorinated silanes including 1H,1H,2H,2H-perfluorooctyldimethylchlorosilane (PFOS) from ABCR (Karlsruhe, Germany), (ii) aminosilanes like 3-aminopropyltrimethoxysilane (APDMES) and 3-(2-aminoethylamino)propyltrimethoxysilane (AEDMS) from Sigma Aldrich (Buchs, Switzerland) and Alfa Aesar (Karlsruhe, Germany), respectively, (iii) perfluorinated amines such as 1H,1H,2H,2H,3H,3H-perfluoroundecylamine (PFUA) and perfluorotributylamine (PFTBA) from Fluka (Buchs, Switzerland), and (iv) organic amines like cyclohexylethylamine, dimethylethylenediamine and hexanamine from Acros (Geel, Belgium). Some of these compounds are listed in Table 1. The initiator loading required specific optimization for each compound; for example during the PFUA loading a solid white layer is formed, which at the end is removed with the nitrogen stream. The procedure described above proved to be suitable for loading the aminosilanes and perfluorinated silanes and perfluorinated amine used in this study. The organic amines tested turned out to be too volatile to be used as initiator.

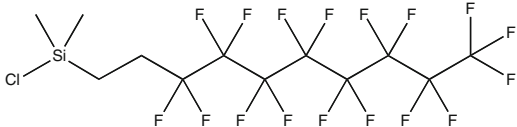
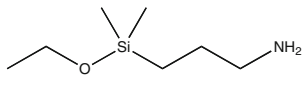
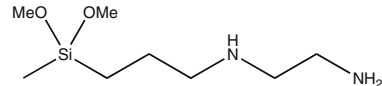
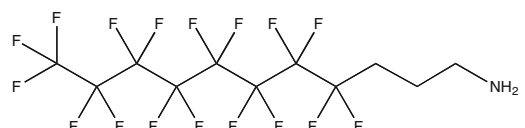
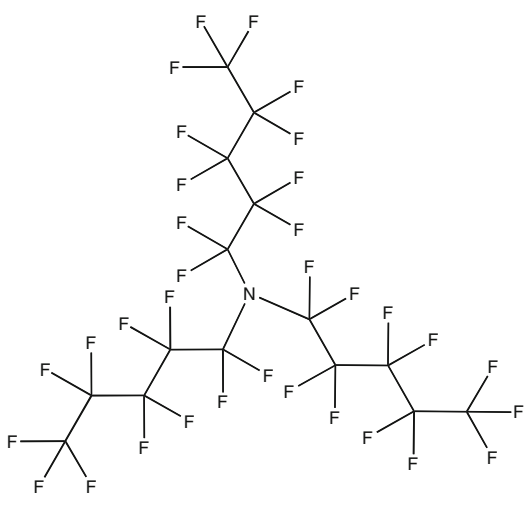
2.3 Mass spectrometry

All the metabolites used to create the model mixture were commercially available and used without further purification. Phosphoenolpyruvate (PEP), 5'-guanylate diphosphate (GDP), adenosine 5'-triphosphate (ATP), and uridine 5'-diphosphate were purchased from Sigma Aldrich. Adenosine 5'-diphosphate (ADP) was purchased from ACROS Organics, and guanosine 5'-triphosphate (GTP) was from Fluka. A total of 1- μ l samples were deposited directly on the NIMS targets using a pipette, and the position of the samples was marked using a white pen (Edding, Germany). All MS measurements were performed on a commercial MALDI time-of-flight mass spectrometer (Axima-CFR, Shimadzu/Kratos Analytical, Manchester, UK). Samples were ionized using a nitrogen laser (337 nm, 4 ns pulse width) and ions were accelerated with 20 kV. All mass spectra on the model mixture were measured in linear mode, while the cell extract samples were measured in reflectron mode to allow the identification of selected peaks in a more complex sample. The silicon targets were attached to a standard stainless steel MALDI target using a double-sided conducting copper adhesive tape (3M[®], Code 1182). The MALDI target plate was slightly modified for the silicon surface to be flush with that of the plate.

2.4 Yeast cells: culture, quenching of the metabolism, and extraction of metabolites

The yeast *Saccharomyces cerevisiae* CEN.PK 113-7D was cultivated under fully aerobic conditions. At optical density at 600 nm (OD₆₀₀) of 1.18, a sample of 0.9 ml was taken from the culture and, in order to stop any metabolic activity, transferred immediately into a 60% methanol solution precooled to -40°C (buffered to pH 7.5). The resulting solution was mixed and cooled in a cryostat for 30 s, then centrifuged at -9°C for 1 min at 10000 g using a pre-cooled rotor. The supernatant was completely removed and the remaining sample was shock-frozen in liquid nitrogen and stored at -80°C . For extraction of the metabolites from the cells, the sample was thawed to -40°C , after which 1 ml of boiling ethanol (75%, buffered to pH 7.5) was added. This solution was incubated at 90°C for 3 min. Then it was cooled, centrifuged and the supernatant was collected and stored at -80°C . For NIMS analysis, the sample was completely dried in a vacuum centrifuge (cooled to 10°C) and re-suspended in 50 μ l of nanopure water; 0.5 μ l was then dispensed onto the silicon surface. Previous cytometric measurements allowed to correlate the OD₆₀₀ with the number of cells in the initial suspension; it is estimated that the sample analyzed corresponds to approximately 160,000 cells.

Table 1 Some of the initiators tested in this study and their structure

Name (short form)	Structure
1H,1H,2H,2H-perfluorooctyldimethylchlorosilane (PFOS)	
3-Aminopropyldimethylethoxysilane (APDMES)	
3-(2-Aminoethylamino)-propylmethyldimethoxysilane (AEDMS)	
1H,1H,2H,2H,3H,3H-perfluoroundecylamine (PFUA)	
Perfluorotributylamine (PFTBA)	

3 Results and discussion

3.1 Negative ion mode NIMS

The objective of this study was to evaluate the possibility to use NIMS to detect metabolites belonging to the central cellular metabolism. First, an aqueous mixture of four synthetic phosphorylated metabolites (ADP, GDP, ATP,

and GTP) was used as test sample to optimize the method for this class of analytes. The mixture was analyzed using different silicon targets to test the influence of the initiator loading for the detection of the studied substances (Fig. 2). The spectra show the peaks corresponding to the analytes only when the nanoporous silicon surface was previously treated with APDMES and in negative ion mode. In fact, the analysis using either a monocrystalline Si wafer with a

polished surface (Fig. 2A) or an electrochemically etched porous Si surface (Fig. 2B) as target plate did not show any interesting peaks.

On the contrary, when the porous surface is derivatized using APDMES (Fig. 2C) or when APDMES, according to the NIMS protocol (Woo et al. 2008), is loaded as initiator (Fig. 2D), strong peaks corresponding to the analytes are found. Arguably, the presence of the amino groups onto the surface promotes the negative ion formation. Indeed in a DIOS experiment, i.e., without the addition of an initiator, the porous silicon is effective to detect phosphorylated metabolites only when the surface is derivatized with amino silanes (Fig. 2C). In any case, the NIMS method shows superior sensitivity: the addition of the initiator, which in NIMS is trapped as a liquid in the porous silicon surface, seems to effectively boost the negative ion yield (Fig. 2D). Conversely, the spectra obtained in positive mode did not show the peaks corresponding to the analytes with any of the surfaces tested. Limits of detections (LODs) in the femtomole range were obtained for negative mode NIMS (Fig. 3c). The LODs were obtained by calculating the signal-to-background ratio for selected peaks through serial dilution of the test sample mixture (Fig. 3b), where the signal was measured as the peak intensity and the background as the maximum intensity value in the spectrum in the peak-free range between m/z 496 and 498. Due to the very low chemical background as apparent from the blank measurements (Fig. 3a), NIMS allows to detect low molecular weight analytes. Therefore the metabolite

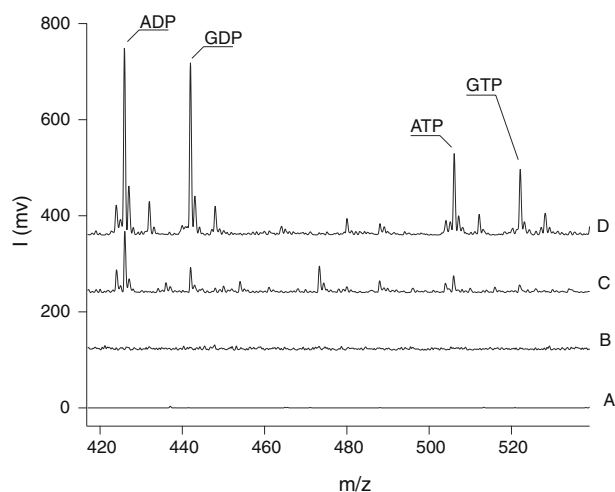


Fig. 2 Comparison of matrix-free LDI-MS spectra of the model mixture. A total of 1 μ l of the mixture solution 1 μ g/ml (1 ng of each analyte) was deposited on four different silicon surfaces and then analyzed in a MALDI mass spectrometer. The targets are (A) a polished silicon wafer, (B) a porous black silicon after anodic etching of the wafer in HF, (C) a porous silicon after derivatization with APDMES, and (D) porous silicon after loading of APDMES as initiator according to NIMS protocol. The spectra are reported as stacked traces with an offset for easy visualization

model mixture was increased adding to the previous mixture, also low molecular weight phosphorylated metabolite like: UDP, AMP and PEP, that were efficiently detected with the method here presented (Fig. 3a). In order to obtain an optimum signal-to-background ratio, a laser pulse energy as low as 50 μ J was used. The NIMS target surface loaded with APDMES led to a hydrophilic surface, therefore aqueous sample solutions tended to spread on the surface. We believe that this behavior of the surface is responsible for the “saturation behavior” of the signal-to-background ratio at higher analyte concentration (Fig. 3b). Also, this prevented us from establishing an accurate calibration curve. This problem could be overcome by surface patterning with different functional domains, for example with different wettability, to confine the sample. We are conducting further studies in this direction to improve the reproducibility of the sample deposition onto the NIMS target.

3.2 Choice of the initiator

Various initiators can be loaded onto the nanostructured surface to specifically detect a particular kind of analyte (Woo et al. 2008). The choice of initiator is a crucial step for performing NIMS successfully. A compound has to fulfill different requirements to be a good initiator: it has to be a low viscosity liquid at room temperature to be easily immobilized onto the surface. It has to have a low vapor pressure to be compatible with the high vacuum in a mass spectrometer. Moreover, it should not strongly adsorb the UV radiation to avoid chemical background that is one of the main problems in standard MALDI of small molecules. Here, different compounds were tested in order to choose the most suitable initiator for the analysis of phosphorylated metabolites. According to the firsts data of Siuzdak and coworkers (Northen et al. 2007) the best results in terms of sensitivity were obtained using perfluorinated silanes. Thus a similar compound, PFOS, was tested. While it proved to be a good initiator for MS analysis in positive ion mode (data not shown), this initiator turned out to be unsuitable for the detection of our analytes, which are rather acidic due to the phosphorylation, i.e., more likely to be detectable in negative mode. Although the mechanism of action of the initiator in NIMS still needs to be clarified, the specificity of an initiator for a particular class of analytes suggests some involvement of this compound in the ionization process. Thus we tested a range of compounds containing amino groups as initiators, because their basicity could help the formation of negative ions. As already shown in Fig. 2 we found that an aminosilane such as APDMES is the ideal initiator for small phosphorylated and hydrophilic molecules. APDMES also has the low volatility required to “survive” inside the vacuum of the

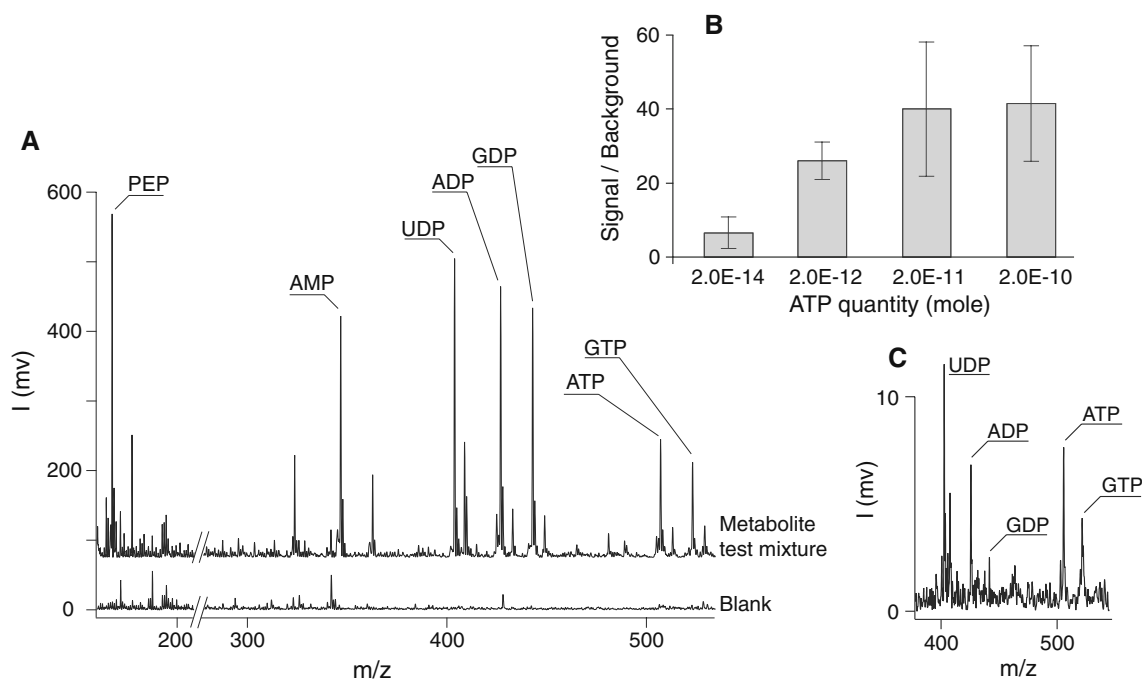


Fig. 3 NIMS of a test mixture of metabolites using APDMES as initiator. The low chemical background and the resulting capability to detect low molecular weight compounds such as PEP are shown. **a** NIMS data from 1 μ l of the four-component test mixture with PEP, AMP and UDP added, corresponding to deposition of 1 ng of each

metabolite. The blank was generated by analyzing 1 μ l of water. **b** Signal-to-background ratio for different amounts of ATP. **c** NIMS spectrum corresponding to only 10 pg (20 fmol of ATP, close to its LOD) of each analyte

TOF mass spectrometer. Other organic amines like cyclohexenylethylamine, dimethylethylenediamine and hexanamine were tested but were found to be too volatile to be successfully trapped in the porous Si surface. In order to avoid the sample spreading on the hydrophilic silicon surface treated with APDMES, some perfluorinated amines were also tested as initiators. While PFTBA was again too volatile, the loading of PFUA was successful. PFUA proved to be usable as initiator for the analysis of phosphorylated metabolites, showing that for the detection of this kind of analytes in negative mode, the use of an initiator containing an amino group is beneficial. Overall, however, APDMES proved to be more sensitive (Fig. 4), with a limit of detection more than an order of magnitude lower compared to NIMS with other initiators.

3.3 NIMS of a yeast cell extract

Finally the utility of the NIMS method here presented was tested for the analysis of a biological sample such as a yeast cell extract. Due to its the superior performance for metabolites, APDMES was chosen as initiator. The results show that several peaks are detectable with virtually no background ions (Fig. 5). The spectrum recorded in reflectron mode allows one to tentatively identify the peaks belonging to the class of analytes studied in the model mixture (i.e., nucleotides). Identifying each peak is beyond

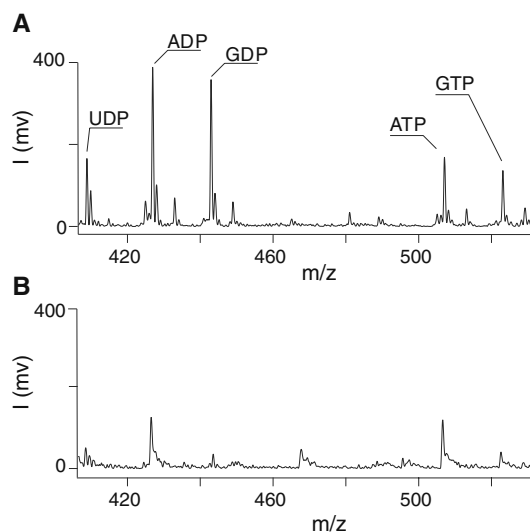
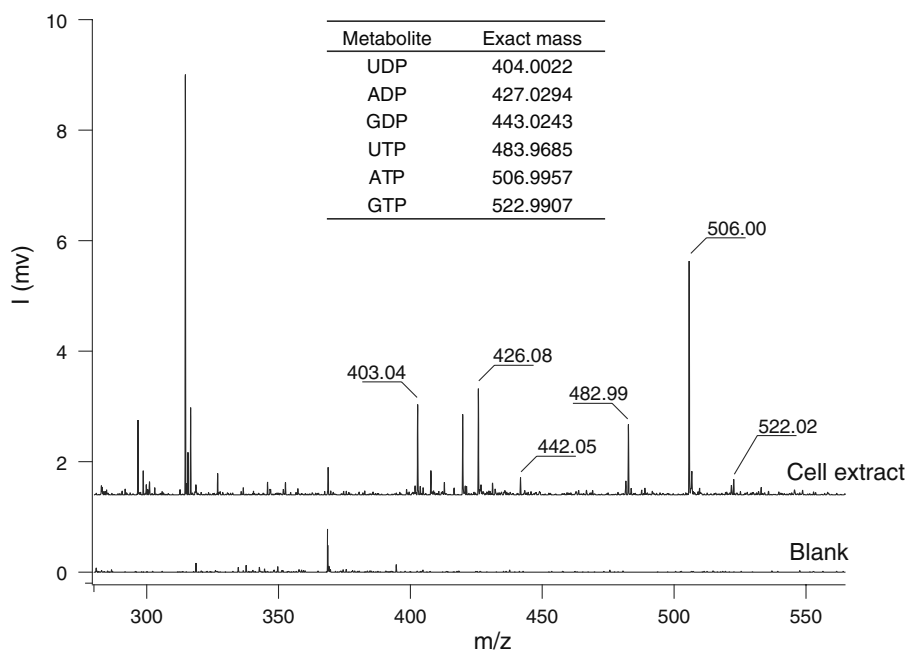


Fig. 4 Comparison of initiators. The NIMS method was applied to the test mixture of metabolites (1 μ g/ml of each compound; 1 μ l applied to the target) using **a** APDMES and **b** PFUA as initiators. The APDMES is better suited for the detection of the analytes

the scope of this paper; a systematic study can in principle be carried with tandem MS and or with accurate mass determination. Figure 5, however, shows that the NIMS can be a powerful tool to directly analyze a biological sample without previous separations.

Fig. 5 Analysis of a yeast cell extract. The spectra of 1 μ l of a yeast cell extract and of water (blank) are shown. The spectra are recorded in negative reflectron mode, showing deprotonated ions of the analytes $(M-H)^-$, and the identification of some peaks is suggested in the table; the exact masses of the corresponding neutral compounds are given



4 Concluding remarks

In this work we presented a matrix-free laser desorption-ionization for the detection of endogenous cell metabolites. The method was optimized by testing different initiators; APDMES as initiator and negative ion mode detection showed to be particularly suitable for the analytes studied. Furthermore the application to a biological sample was demonstrated analyzing a yeast cell extract. Further studies, based on surface patterning for sample confinement, are planned to reduce the spreading of the sample. This should decrease the variability related to the sample deposition and can be a step forwards in order to obtain quantitative information with the method presented. In conclusion, NIMS with a well-chosen initiator compound is a useful method for metabolite detection that can complement other methods available to achieve an extremely powerful suite of tools for comprehensive studies of the cellular metabolism.

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References

Amantonico, A., Oh, J. Y., Sobek, J., Heinemann, M., & Zenobi, R. (2008). Mass spectrometric method for analyzing metabolites in

yeast with single cell sensitivity. *Angewandte Chemie International Edition*, 47, 5382–5385. doi:10.1002/anie.200705923.

Cohen, L. H., & Gusev, A. I. (2002). Small molecule analysis by MALDI mass spectrometry. *Analytical and Bioanalytical Chemistry*, 373, 571–586. doi:10.1007/s00216-002-1321-z.

Dettmer, K., Aronov, P. A., & Hammock, B. D. (2007). Mass spectrometry-based metabolomics. *Mass Spectrometry Reviews*, 26, 51–78. doi:10.1002/mas.20108.

Dunn, W. B., Bailey, N. J., & Johnson, H. E. (2005). Measuring the metabolome: Current analytical technologies. *Analyst (London)*, 130, 606–625. doi:10.1039/b418288j.

Edwards, J. L., Chisolm, C. N., Shackman, J. G., & Kennedy, R. T. (2006). Negative mode sheathless capillary electrophoresis electrospray ionization-mass spectrometry for metabolite analysis of prokaryotes. *Journal of Chromatography A*, 1106, 80–88. doi:10.1016/j.chroma.2005.08.082.

Edwards, J. L., & Kennedy, R. T. (2005). Metabolomic analysis of eukaryotic tissue and prokaryotes using negative mode MALDI time-of-flight mass spectrometry. *Analytical Chemistry*, 77, 2201–2209. doi:10.1021/ac048323r.

Fiehn, O. (2002). Metabolomics—the link between genotypes and phenotypes. *Plant Molecular Biology*, 48, 155–171. doi:10.1023/A:1013713905833.

Fiehn, O., & Kind, T. (2007). Metabolite profiling in blood plasma. In W. Weckwerth (Ed.), *Metabolomics, methods and protocols* (pp. 3–18). Totowa, NJ: Humana Press, Inc.

Halimaoui, A. (1997). Porous silicon formation by anodisation. In L. Canham (Ed.), *Properties of porous silicon* (pp. 11–27). London: INSPEC.

Hillenkamp, F., & Karas, M. (2007). The MALDI process and method. In F. Hillenkamp & J. Peter-Katalinić (Eds.), *MALDI MS: A practical guide to instrumentation, methods and applications* (p. 3). Weinheim: WILEY-VCH.

Hollywood, K., Brison, D. R., & Goodacre, R. (2006). Metabolomics: Current technologies and future trends. *Proteomics*, 6, 4716–4723. doi:10.1002/pmic.200600106.

Nordstrom, A., Want, E., Northen, T., Lehtio, J., & Siuzdak, G. (2008). Multiple ionization mass spectrometry strategy used to

- reveal the complexity of metabolomics. *Analytical Chemistry*, 80, 421–429. doi:[10.1021/ac701982e](https://doi.org/10.1021/ac701982e).
- Northen, T. R., Yanes, O., Northen, M. T., Marrinucci, D., Uritboonthai, W., Apon, J., et al. (2007). Clathrate nanostructures for mass spectrometry. *Nature*, 449, 1033–1036. doi:[10.1038/nature06195](https://doi.org/10.1038/nature06195).
- Peterson, D. S. (2007). Matrix-free methods for laser desorption/ionization mass spectrometry. *Mass Spectrometry Reviews*, 26, 19–34. doi:[10.1002/mas.20104](https://doi.org/10.1002/mas.20104).
- Shrestha, B., Li, Y., & Vertes, A. (2008). Rapid analysis of pharmaceuticals and excreted xenobiotic and endogenous metabolites with atmospheric pressure infrared MALDI mass spectrometry. *Metabolomics*, 4, 297–311. doi:[10.1007/s11306-008-0120-8](https://doi.org/10.1007/s11306-008-0120-8).
- Soga, T., Ueno, Y., Naraoka, H., Ohashi, Y., Tomita, M., & Nishioka, T. (2002). Simultaneous determination of anionic intermediates for *Bacillus subtilis* metabolic pathways by capillary electrophoresis electrospray ionization mass spectrometry. *Analytical Chemistry*, 74, 2233–2239. doi:[10.1021/ac020064n](https://doi.org/10.1021/ac020064n).
- Sun, G., Yang, K., Zhao, Z. D., Guan, S. P., Han, X. L., & Gross, R. W. (2007). Shotgun metabolomics approach for the analysis of negatively charged water-soluble cellular metabolites from mouse heart tissue. *Analytical Chemistry*, 79, 6629–6640. doi:[10.1021/ac070843+](https://doi.org/10.1021/ac070843+).
- Tomita, M., & Nishioka, T. (2005). *Metabolomics: The frontier of systems biology*. Tokyo: Springer-Verlag.
- Trauger, S. A., Go, E. P., Shen, Z. X., Apon, J. V., Compton, B. J., Bouvier, E. S. P., et al. (2004). High sensitivity and analyte capture with desorption/ionization mass spectrometry on silylated porous silicon. *Analytical Chemistry*, 76, 4484–4489. doi:[10.1021/ac049657j](https://doi.org/10.1021/ac049657j).
- Vaidyanathan, S., Jones, D., Ellis, J., Jenkins, T., Chong, C., Anderson, M., et al. (2007). Laser desorption/ionization mass spectrometry on porous silicon for metabolome analyses: Influence of surface oxidation. *Rapid Communications in Mass Spectrometry*, 21, 2157–2166. doi:[10.1002/rcm.3078](https://doi.org/10.1002/rcm.3078).
- Villas-Boas, S. G., Mas, S., Akesson, M., Smedsgaard, J., & Nielsen, J. (2005). Mass spectrometry in metabolome analysis. *Mass Spectrometry Reviews*, 24, 613–646. doi:[10.1002/mas.20032](https://doi.org/10.1002/mas.20032).
- Weckwerth, W. (2003). Metabolomics in systems biology. *Annual Review of Plant Biology*, 54, 669–689. doi:[10.1146/annurev.arplant.54.031902.135014](https://doi.org/10.1146/annurev.arplant.54.031902.135014).
- Wei, J., Buriak, J. M., & Siuzdak, G. (1999). Desorption–ionization mass spectrometry on porous silicon. *Nature*, 399, 243–246. doi:[10.1038/20400](https://doi.org/10.1038/20400).
- Woo, H. K., Northen, T. R., Yanes, O., & Siuzdak, G. (2008). Nanostructure-initiator mass spectrometry: A protocol for preparing and applying NIMS surfaces for high-sensitivity mass analysis. *Nature Protocols*, 3, 1341–1349. doi:[10.1038/nprot.2008.110](https://doi.org/10.1038/nprot.2008.110).