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# Rapid estimation of the energy charge from cell lysates using matrix-assisted laser desorption/ionization mass spectrometry: Role of in-source fragmentation

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ABSTRACT

Nucleotides are key players in the central energy metabolism of cells. Here we show how to estimate the energy charge from cell lysates by direct negative ion matrix-assisted laser desorption/ionization mass spectrometry (MALDI–MS) using 9-aminoacridine as matrix. We found a high level of in-source decay of all the phosphorylated nucleotides, with some of them producing considerable amounts of adenosine-5′-diphosphate (ADP) fragment ions. We investigated the behavior of adenosine-5′-monophosphate (AMP), ADP, and adenosine-5′-triphosphate (ATP) as well as the cofactors coenzyme A (CoA) and acetyl-coenzyme A (ACoA) and nicotinamide adenine dinucleotides ($NAD^+$ and NADH) in detail. In-source decay of these compounds depends strongly on the applied laser power and on the extraction pulse delay. At standard instrument settings, the 9-aminoacridine (9-AA) matrix resulted in a much higher in-source decay compared with 2,4,6-trihydroxyacetophenone (2,4,6-THAP). By adding $^{13}C$-labeled ATP to a cell lysate, we were able to determine the degree of in-source decay during an experiment. Analyzing a cell extract of the monocytic cell line THP-1 with [$^{13}C$]ATP as internal standard, we were able to obtain values for the energy charge that were similar to those determined by a reference liquid chromatography electrospray ionization coupled to mass spectrometry (LC–ESI–MS) method.



Typically, metabolites are low-molecular-weight compounds (<1000 Da) [1] whose detection and characterization are challenging due to structural similarities and their propensity to degrade during sample preparation and the analytical measurement process. Specifically, the class of nucleotides is prone to degradation. Nucleotides, which play a key role in cellular metabolism, are able to transport reaction energy in living organisms using the energy-rich phosphate bonds. During the early 1960s, Atkinson described for the first time the key role of nucleotides and expected these to have regulatory functions at all branching points between anabolism and catabolism [2]. This discovery, which included the equilibrium of the three nucleotides adenosine-5′-triphosphate (ATP), adenosine-5′-diphosphate (ADP), and adenosine-5′-monophosphate (AMP), is known today as the *energy charge hypothesis.* Moreover, investigating the organization and regulation of the nucleotide metabolism, in particular in the central metabolic pathway, provides insight into both catabolic and anabolic regulatory functions in a biological system [3]. If a measurement distorts the ratio of the three phosphorylated nucleosides ATP, ADP, and AMP (adenosine-5′*x*-phosphates), an erroneous value for the energy charge will result and their regulatory function might be misinterpreted.

Commonly, adenosine-5′*x*-phosphates can be analyzed optically by immunohistochemical tagging or chemical staining or, alternatively, by radiolabeling [4,5]. However, most optical methods are currently limited in sensitivity, selectivity for the reactive compounds, and their ability to simultaneously detect several target compounds [6]. Investigations of the metabolome require the simultaneous detection of a wide range of metabolites in a quantitative manner. Consequently, improvement of existing analytical technologies and methodologies, as well as development of new ones, is thought to be an important subject in the context of metabolomics. State-of-the-art methods for metabolite analysis include liquid chromatography electrospray ionization coupled to mass spectrometry (LC–ESI–MS) [7–9], gas chromatography in combination with mass spectrometry (GC/MS) [10], enzymatic

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[1] *Abbreviations used:* ATP, adenosine-5′-triphosphate; ADP, adenosine-5′-diphosphate; AMP, adenosine-5′-monophosphate; adenosine-5′*x*-phosphates, ATP, ADP, and AMP; LC–ESI–MS, liquid chromatography electrospray ionization coupled to mass spectrometry; MALDI–MS, matrix-assisted laser desorption/ionization mass spectrometry; 9-AA, 9-aminoacridine; ISD, in-source decay; PSD, post-source decay; 2,4,6-THAP, 2,4,6-trihydroxyacetophenone; FAD, flavin adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; $NAD^+$, β-nicotinamide adenine dinucleotide (oxidized form); NADH, β-nicotinamide adenine dinucleotide (oxidized form) disodium hydrate; ACoA, acetyl-coenzyme A; CoA, coenzyme A; THAP, 2′,4′,6′-trihydroxyacetophenone monohydrate; TOF, time-of-flight; FT–ICR, Fourier transform ion cyclotron resonance; CID, collision-induced dissociation.

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assays [11], nuclear magnetic resonance (NMR) [12], and (on the single-cell level) live single-cell mass spectrometry [13]. The list of techniques is extended by biological sensors. Sensors enable online detection, but they are generally limited in their chemical versatility. Furthermore, matrix-assisted laser desorption/ionization mass spectrometry (MALDI–MS) has been shown to be a powerful tool for the investigation of metabolites in biological systems [14–16]. MALDI–MS as a tool for the analysis of small molecules has gained more and more interest during the past decade [17–20]. The high sensitivity, selectivity, and throughput of MALDI–MS allow experiments even at the single-cell level [21,22]. A high-density microarray for mass spectrometry that enables high-throughput experiments was recently designed by Urban and coworkers [23]. Negative ion mode MALDI–MS has been demonstrated to be a suitable approach for selective and sensitive detection of phosphorylated analytes in a complex biological matrix. Especially the use of 9-aminoacridine (9-AA), a matrix with low chemical background in the low-mass region, enables the analysis of phosphorylated nucleosides with outstanding sensitivity [15,24,25]. Some strategies, including the use of isotopically labeled standards, have been developed to use MALDI–MS as a quantitative analytical technique [26–29].

A well-documented property of MALDI–MS analysis is the fragmentation of fragile analytes via either in-source decay (ISD) or post-source decay (PSD) [30–32]. Hillenkamp and coworkers categorized possible fragmentations occurring in MALDI–MS, according to their time scale, into four categories: prompt, fast, early metastable, and metastable [31]. The most prominent decays are the prompt and fast ones, which take place in the source. These decays are a result of three individual processes: (i) the direct absorption of the laser energy by the analyte, which results in thermally excited molecules; (ii) collisional activation by the acceleration through the matrix plume; and (iii) exoergic chemical processes such as proton transfer [33,34]. In 1995, Brown and Lennon described ISD for the first time as promptly formed fragment ions on ion generation [32]. ISD and PSD are both used, for example, in top-down sequencing of intact proteins and are powerful tools in the identification of small and moderately sized peptides [30,35]. However, ISD and PSD can be hurdles in the quantification of phosphorylated nucleosides because the fragmentation can prevent their correct identification in MALDI–MS analysis of living organisms. Thus, the aim of this study was to characterize fragmentation and to minimize it by optimizing the conditions while maintaining the high sensitivity of our experiments.

In the current study, we investigated the in-source fragmentation behavior of selected adenosine-containing nucleotides and cofactors in MALDI–MS experiments using 9-AA as matrix. Validation of the metabolite standards was performed with ESI and an additional MALDI source from a different supplier. Potential fragmentation sources affecting the quantitation performance of our approach were identified and evaluated in view of estimating the energy charge of cells. Finally, the MALDI matrix 9-AA is compared to 2,4,6-trihydroxyacetophenone (2,4,6-THAP), another commonly used matrix in negative ion mode. By adding [$^{13}$C] ATP as internal standard to cell lysates, we could estimate the degree of in-source fragmentation during analysis and perform a correction of the determined ratios. Finally, the direct MALDI–MS analysis delivered similar energy charge values as a standard reference LC–ESI–MS method.

## Materials and methods

### *Metabolites*

ATP, ADP, AMP, flavin adenine dinucleotide (FAD), and nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Sigma–Aldrich (Switzerland). β-Nicotinamide adenine dinucleotide (oxidized form) ($NAD^+$), and β-nicotinamide adenine dinucleotide (oxidized form) disodium hydrate (NADH) were purchased from Acros Chemicals (Belgium). Acetyl-coenzyme A (ACoA) and coenzyme A (CoA) were purchased from AppliChem (Germany). All metabolites were used without further purification and were diluted to a concentration of 100 fmol/μl in 50% MeCN. To avoid measuring hydrolysis artifacts of the standards, all standards were freshly prepared directly before each experiment and validated by direct infusion to ESI–MS.

### *Matrices*

9-AA hydrochloride monohydrate (99%) was purchased from ABCR (Germany). 9-AA (10 mg/ml) was dissolved in 90% ethanol. 2′,4′,6′-Trihydroxyacetophenone monohydrate (THAP) was purchased from Acros Organics. THAP (10 mg/ml) was dissolved in 50% aq. acetonitrile. Dibasic $NH_4$ citrate (50 mg/ml, aq.) was added to the matrix solution at a ratio of 9:1. Experiments with 2′,3′,4′-trihydroxacetophenone in MeCN/$H_2O$ (1:1, v/v) with dibasic $NH_4$ citrate were also conducted, but did not yield good results.

### *Sample preparation*

Prior to analysis, a 2-μl aliquot of the matrix solution (as described above) was mixed with 2 μl of the analyte solution. An aliquot of 0.5 μl of the mix was spotted onto a MALDI plate (384 spots, AB Sciex, stainless steel). Nucleotide extracts were obtained as described elsewhere [36]. Briefly, 1 ml of cells ($1 \times 10^6$ cells/ml) was washed with cold water. Nucleotides were extracted with 350 μl of cold 80% MeCN and subsequently with 150 μl of cold 50% MeCN. Then 2 μl of the combined cell extract was mixed with 1 μl of a 50-μmol [$^{13}$C]ATP standard solution and further with 3 μl of matrix. Next 0.5 μl of this analyte–matrix mix was spotted onto a standard stainless-steel MALDI plate. The cell extract was vacuum dried and redissolved in 200 μl of 30 mM $NH_4CO_3$ for LC–MS analysis.

### *Instrumentation*

A MALDI–TOF (time-of-flight) mass spectrometer (AB Sciex 4800) equipped with an Nd:YAG laser was used in negative ion reflector mode for data acquisition. The Nd:YAG laser operates at 355 nm and has a repetition rate of 200 Hz. A laser attenuator is installed to vary the intensity of the laser beam reaching the sample. As a standard laser energy setting, approximately $5.5 \times 10^7$ W/cm$^2$ was used. The optimal extraction time delay was found to be 400 ns. For experiments on the in-plume acceleration, the extraction pulse delay was varied from 100 to 400 ns. Ten shots per subspectrum were used, and 40 subspectra were recorded.

A Bruker Solarix FT–ICR (Fourier transform ion cyclotron resonance) mass spectrometer equipped with a 9.4 Tesla cryomagnet and an ESI and MALDI source was used for complementary fragmentation and sample validation experiments. The MALDI source was equipped with a smartbeam-II laser and operated with a laser power of 27.00% (arbitrary units) and 150 laser shots. The ESI capillary voltage was operated at 4500 V, and the drying gas flow was set to 3.7 L/min, 1.3 bar, 200 °C. The FT–ICR was measured in broadband mode with a source accumulation time of 1 ms, an ion accumulation time of 20 ms, and an ion cooling time of 1 ms.

## Results and discussion

### *In-source decay versus post-source decay*

ATP was measured by MALDI–MS using 9-AA (90% EtOH) and, using standard instrument conditions, generated approximately