ELECTRONIC SUPPLEMENTARY INFORMATION (ESI)

Mass spectrometric method incorporating enzymatic amplification for attomole-level analysis of target metabolites in biological samples

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Experimental details

Materials

9-Aminoacridine (9-AA) and β-nicotinamide adenine dinucleotide (reduced form) disodium salt hydrate were purchased from Acros Organics (Geel, Belgium); acetone, adenosine 5'-monophosphate monohydrate (AMP), adenosine 5'-triphosphate disodium salt (ATP), ethylenediaminetetraacetic acid tetrasodium salt dihydrate (EDTA), lithium potassium acetyl phosphate and potassium chloride from Sigma-Aldrich (Buchs, Switzerland); phosphoenol pyruvate (PEP) monopotassium salt from ABCR (Karlsruhe, Germany); coenzyme A (CoA) free acid from AppliChem (Darmstadt, Germany); oxaloacetic acid from AlfaAesar (Karlsruhe, Germany); ammonium acetate and magnesium sulfate heptahydrate from Merck (Dietikon, Switzerland).

All the reactant solutions (typically, 1.0 mg mL^{-1}; EDTA, potassium chloride, magnesium sulfate, 100 mM; ammonium acetate, 1 M) were prepared in HPLC-MS grade water (Fisher Scientific, Wohlen, Switzerland). The 9-AA solution was prepared by dissolving 9.0 mg of 9-AA in 1 mL of acetone.

For the cycling reactions, the following enzymes supplied by Sigma-Aldrich were used: pyruvate kinase from rabbit muscle (type III, lyophilized powder, 350-600 units mg^{-1} protein; P9136), myokinase from chicken muscle (adenylate kinase; essentially salt-free, lyophilized powder, 1500-3000 units mg^{-1} protein; M5520), phosphotransacetylase from *Methanosarcina thermophila* (recombinant, expressed in *Escherichia coli*, lyophilized powder, ≥ 5000 units mg^{-1} protein; P1329), citrate synthase from porcine heart (ammonium sulfate suspension, ≥ 100 units mg^{-1} protein; C3260). Apyrase from potato (ATPase, ≥ 200 units mg^{-1} protein,

lyophilized powder; A6535), used for decomposition of contaminant ADP and ATP, was also from Sigma-Aldrich.

For assay development, solutions of the enzymes were prepared in the following way: the commercial pyruvate kinase preparation was dissolved in 20 mM ammonium acetate to the final concentration of 1 mg mL^{-1} (350-600 U mL^{-1}); 1000 units of the myokinase preparation were dissolved in 500 µL of 20 mM ammonium acetate (2000 U mL^{-1}); 250 units of the phosphotransacetylase preparation were dissolved in 500 µL of 20 mM ammonium acetate (500 U mL^{-1}); 200 units of the citrate synthase preparation (suspension) were mixed with 500 µL of 10 mM ammonium acetate (< 400 U mL^{-1}); 100 units of the apyrase preparation were dissolved in 500 µL of 20 mM ammonium acetate (200 U mL^{-1}). In the case of citrate synthase, the buffer was subsequently exchanged for pure 10 mM ammonium acetate solution using Micro Bio-Spin chromatography columns filled with Bio-Gel P-6 (Bio-Rad Laboratories, Reinach, Switzerland); four initial column washes were applied.

For the final testing of the developed method, a crude sample rich in biological matrix was prepared; this was made by cooking ~ 1 g of dry yeast (*Saccharomyces cerevisiae*, type I; YSC1; Sigma-Aldrich) with 20 mL of water in a microwave oven at 700 W for total time of 3 min. The turbid suspension obtained was then filtered with a polyvinylidene fluoride syringe filter (pore size 0.45 µm; Whatman, Clifton, NJ, USA). The resulting pale-brown filtrate was then regarded as a stock extract (100%). It was subsequently added to the standard test solutions in order to emulate a ‘dirty’ biological sample; containing either 5 or 20% (v/v) of the stock extract.

Mass spectrometry

Initial method development was carried out using two MALDI-MS instruments: MALDI-TOF/TOF-MS 4700 and MALDI-TOF/TOF-MS 4800 (Applied Biosystems, Foster City, CA, USA). For the final demonstration of the *in-situ* protocol, a MALDI-TOF-MS instrument (Axima; Shimadzu/Kratos Analytical, Manchester, UK) equipped with a 337 nm nitrogen laser was used. The mass spectra were acquired using the standard acquisition software packages supplied by the instrument manufacturers: Applied Biosystems and Shimadzu/Kratos Analytical; *i.e.* Launchpad/Kompact software (ver. 2.4.1; Shimadzu/Kratos Analytical) in the latter case. Normally, a rectangular laser raster (2 × 2 mm) with 81 equally spaced sampling points was used for collecting negative ion mode spectra. Laser power was optimized on regular basis; only spectra acquired during the same measurement sessions and with the same laser power were used for comparisons.

Protocol development

At the beginning of this study, a series of preliminary experiments, involving reactant incubation in PCR-type test tubes (Thermo) with varied concentrations of reactants and enzymes, incubation temperatures and times, was carried out. Following this optimization stage, the method was transferred onto the lab-on-plate format. Then, the incubation times, applied with a given analyte concentration range, had to be adjusted, so that the reaction was not finished when quenched. Too short incubation times and insufficient enzymatic activities led to poor outcome of the procedure. As a result of this optimization, the incubation times were set to ~ 30 and ~ 60 min in the case of ADP/ATP and CoA/Acetyl-CoA amplification, respectively. Following incubation, the reaction was quenched by addition of 9-AA solution in acetone. After drying, the plate was directly analyzed in the MALDI-MS instrument.

For the *in-situ* amplification, a standard MALDI plate with large sample pods (∅ = 5 mm; DE1487TA; Shimadzu/Kratos Analytical) was used. 3 µL aliquots of the reaction mixture were pipetted onto the samples, followed by addition of 3 µL of the analyzed samples (dilution series of ATP or CoA standards, either in water or spiked into a diluted crude yeast extract). The resulting 6 µL droplets were then covered with caps from 2 mL microcentrifuge tubes (Vaudaux-Eppendorf, Schönenbuch/Basel, Switzerland) to prevent evaporation of the liquid. In the case of long incubations (> 10 min), the plate was subsequently placed in a hermetically sealed polyethylene box, next to a beaker filled with ~ 100 mL of water. This precaution was taken in order to saturate the incubation environment with water vapor. The box was incubated inside a Peltier-thermostatted chamber, at ~ 30°C in the case of ADP/ATP amplification, and ~ 37°C for CoA/Acetyl-CoA amplification.

For the ATP amplification, 1 mL of the following aqueous reaction mixture was initially prepared: 10 mM ammonium acetate, 274 µM AMP, 485 µM PEP, 276 µM NADH (internal standard), 1 mM EDTA, 1 mM potassium chloride, 1 mM magnesium sulfate. Then, 10 µL of apyrase solution (200 U ml^{-1}) was added and the mixture was incubated at 37°C to decompose residue ADP and ATP to AMP. Following 30 min incubation, the vial was microwaved at 700 W for 2 min to denature apyrase. The solution was cooled down to room temperature and an aliquote of 100 µL was trasferred to a separate microcentrifuge test tube. It was then merged with 5 µL of the pyruvate kinase solution and 3 µL of myokinase (10× diluted stock solution; *i.e.* 200 U mL^{-1}).

For the CoA amplification, the following aqueous reaction mixture was used: 10 mM ammonium acetate, 1.09 mM acetyl phosphate, 227 µM oxaloacetic acid, 97.0 µM PEP (internal standard), 1 mM EDTA. The enzymes were diluted and dispensed in the following

manner: 1 µL of the citrate synthase solution (after buffer exchange) and 3 µL of the phosphotransacetylase solution were used per 100 µL of the reaction mixture.

Semi-quantitative aspects of the assay

Although quantitative information could be obtained for picomole range in the amplification assay of ADP/ATP coupled with spectrophotometric detection (Valero *et al.*, *Biochem. J.*, 2000, **350**, 237; Valero *et al.*, *Biophys. J.*, 2004, **86**, 3598), obtaining quantitative results in MALDI-MS is not straightforward (Vaidyanathan and Goodacre, *Rapid Commun. Mass Spectrom.*, 2007, **21**, 2072). Therefore, in its present version, the MALDI-based *in-situ* amplification assay shall be considered as a qualitative or semi-quantitative analytical method. However, an attempt has been made to correlate the relative signal responses, obtained in the experiments presented in the main text (**Figures 1B and 2B**), with initial amounts of analytes (ATP or CoA). The results are shown in bar plots (**Figures S2-A and S2-B**).

Additional figures

Figure S1. MALDI-MS spectra obtained during direct analysis (without amplification) of standard solutions of ATP (no amplification). All spectra have been scaled to the highest peak within the *m/z* range.

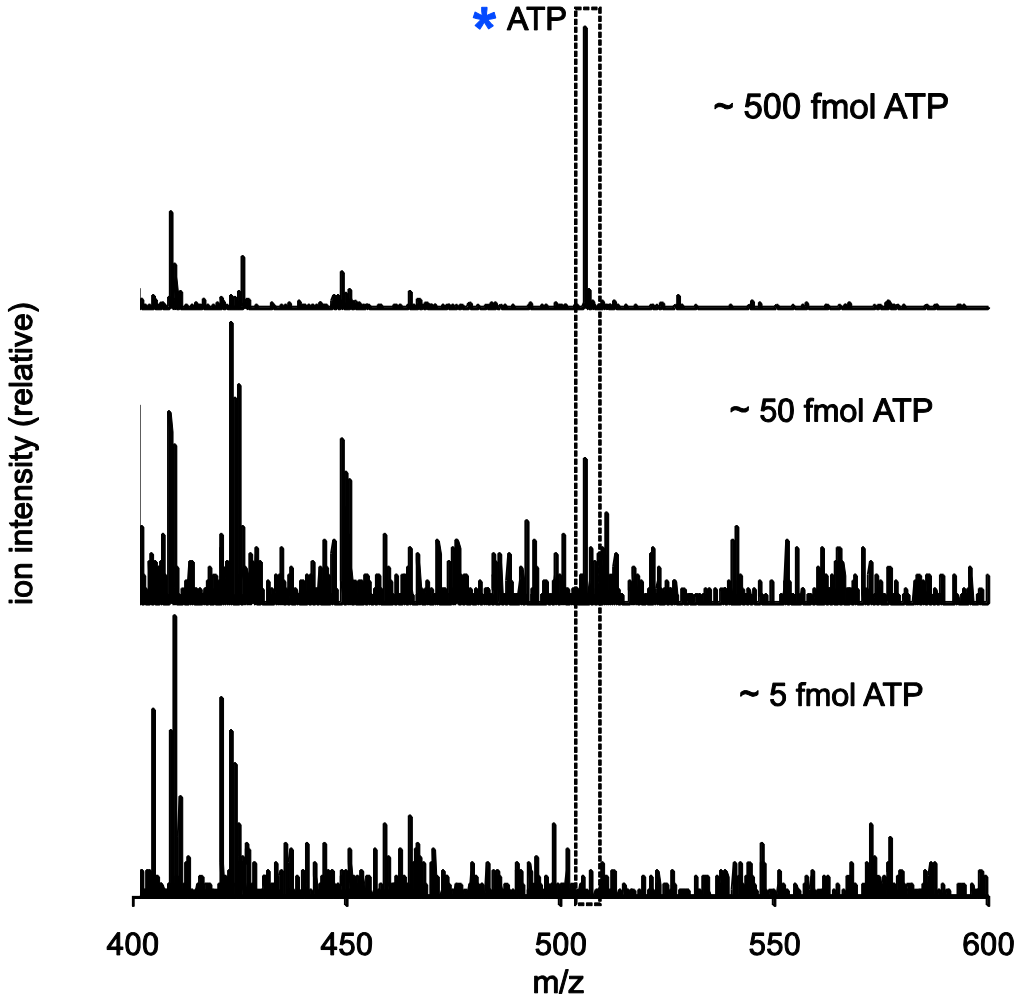

Figure S2. Dependence of signal response after the *in-situ* enzymatic amplification on the initial amounts (*) of (A) ATP, and (B) CoA.