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DOI: https://doi.org/10.1021/ac102445r

Posted at the Zurich Open Repository and Archive, University of Zurich
ZORA URL: https://doi.org/10.5167/uzh-81056
Journal Article
Accepted Version

Originally published at:
Kiefer, Patrick; Delmotte, Nathanaël; Vorholt, Julia A (2011). Nanoscale Ion-Pair Reversed-Phase HPLC−MS for Sensitive Metabolome Analysis. Analytical Chemistry, 83(3):850-855.
DOI: https://doi.org/10.1021/ac102445r

Nanoscale ion-pair reversed-phase HPLC-MS for sensitive metabolome analysis

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ABSTRACT

In this report, we introduce a method using nano-scale ion-pair reversed-phase high-performance liquid chromatography (nano-IP-RP-HPLC), hyphenated to nano-electrospray ionization high-resolution mass spectrometry (nano-ESI-HRMS) to separate and identify metabolites in cell extracts. Separation of metabolites was performed on a 100 µm i.d. C18 column with tributylamine (TBA) as ion-pairing reagent and methanol as eluent. Basic pH (9.4) of the mobile phase was critical to achieve sufficient retention and sharp metabolite elution at low concentration of TBA (1.7 mM). Limits of detection were determined for 54 standards with an LTQ-Orbitrap mass spectrometer to be in the upper attomol to low femtomol range for key metabolites such as nucleotides, phosphorylated sugars, organic acids, and coenzyme A thioesters in solvent as well as in complex matrix. To further evaluate the method, metabolome analysis was performed injecting different amounts of biomass of the methylotroph model organism *Methylobacterium extorquens* AM1. A $^{12}C/^{13}C$ labeling strategy was implemented to improve metabolite identification Analysis of three biological replicates performed with 1.5 ng cell dry weight biomass equivalents resulted in the identification of 24 ± 4 metabolites and analysis of 150 ng allowed identifying 157 ± 5 metabolites from a large spectrum of metabolite classes.

INTRODUCTION

Mass spectrometry (MS) is a key technology and driving force for the fast prospering field of metabolomics. In the last decade, great progress has been made, both with respect to mass spectrometry as well as to chromatographic methods coupled to mass spectrometry to analyze complex biological samples. In particular, high-performance liquid-chromatography (HPLC) technology underwent rapid development. Miniaturization of column diameters and concomitant reduction in flow rates below 1 µL/min (typically 20-500 nL/min) allowed nano-electrospray ionization (nano-ESI). The latter generally improves the ionization process, reduces ion suppression effects,[1] and increases tolerance towards salt contaminations in the sample.[2, 3] In LC-MS based proteomics, nano-flow reversed phase HPLC coupled to nano-ESI mass spectrometry is the method of choice.[4-7] Due to miniaturization sensitivities at low zeptomol level could be achieved for individual proteins upon detection of proteolytic peptides.[8] Usually, peptides are separated using C18 stationary phases combined with aqueous mobile phases acidified with formic acid, or trifluoroacetic acid and using acetonitrile or methanol as organic modifier. In contrast, metabolites comprise a large range of compounds with various physico-chemical properties demanding different separation mechanisms. Central metabolites such as sugar phosphates, amino acids or small organic acids are highly polar. They can therefore not be retained on reversed-phase stationary phases. Good chromatographic separation of central metabolite classes are obtained by ion chromatography (IC),[9, 10] or by hydrophilic interaction liquid chromatography (HILIC).[11-13] However, both separation techniques are currently not well suited for nano-flow application. To our knowledge, nanobore IC columns and corresponding ion suppressors are not yet commercially available. In case of HILIC, loading capacity is rather low which reduces its applicability in nano-scale dimension.[14] So far only two nano-HPLC-MS methods were reported for analysis of highly polar metabolites on self-packed columns (anion exchanger or mixed functional RP with cation exchanger) achieving limits of detection in the sub picomol to low picomol range.[14, 15]

Ion-pair reversed-phase (IP-RP) HPLC offers an attractive principle for nano-ESI based metabolome analysis. Amphiphilic amines are generally used to retain negatively charged compounds.[16-18] In two recently published studies, tributylamine (TBA) was used as ion-pairing reagent for HPLC-ESI-MS analysis in the negative ionization mode allowing the analysis of more than 100 core metabolites.[19, 20] Ion-pairing with hexylamine was investigated by Coulier et *al.* for the analysis of various metabolite classes such as nucleotides or sugar bisphosphates.[21] Gu *et al.* used perfluoroheptanoic acid for ion-pair reversed-phase analysis of amino acids and metabolically related compounds in the positive ionization mode, obtaining limits of detections in the sub-picomol range for most of analytes tested when using a 2 mm i.d. C18 column.[22] Nanobore columns based on C18 stationary phases have high robustness, sufficient loading capacities, and they are used routinely in nano-scale HPLC-MS based proteomics. Since ion-pairing is mainly based on C18 stationary phases, the IP-RP approach is promising for nano-scale HPLC-MS linked metabolomics. In the present study, we developed a nano-HPLC-ESI-MS method for central metabolome analysis in the negative ionization mode using TBA as ion-pairing reagent. Limits of detection obtained were in the low femtomol down to hundreds of attomol range for all metabolites tested. Compared to a previous HPLC-MS based metabolome analysis of a model bacterium[12], sample amount per injection could be reduced by a factor of more than 1'000. The study demonstrates the potential of downscaling HPLC-MS methods for metabolome analysis. The introduced method thus opens the perspective to pioneer investigation of biological samples for which only limited amounts are available.

EXPERIMENTAL SECTION

Chemicals. Reference standards of the various metabolites as well as acetonitrile (LC-MS grade), formic acid (≥ 88%), acetic acid (≥ 99.0%), and tributylamine (≥ 99.5%) were purchased by Sigma-Aldrich Chemie GmbH (Buchs, Switzerland). Uniformly ^{13}C labeled

methanol was obtained through Cambridge Isotope Labs Inc. (Innerberg, Switzerland). Ammonia solution (25%) was purchased by Merck KGaA (Darmstadt, Germany) and methanol (LC-MS grade) was supplied by Fisher Scientific UK Limited (Loughborough, United Kingdom).

Medium and growth conditions. *Methylobacterium extorquens* AM1 was grown on minimal medium using 120 mM methanol as carbon source.[23] 400 mL batch culture was performed in 500 mL Multifors bioreactor (Infors HT, Switzerland) (temperature 28 °C, stirring rate 1000 rpm, aeration rate 0.5 L/min). The pH was maintained at pH 7 by addition of 1 M ammonium hydroxide.

Sample preparation. Cells were sampled during mid-exponential growth. Optical density of the culture was determined at 595 nm prior to sampling. 1 mL of culture was directly injected into 4.5 mL of -20°C cold acidified acetonitrile with 0.1 M formic acid.[24] Prior to extraction, uniformly ^{13}C labeled cell extract (*M. extorquens* AM1 grown on uniformly ^{13}C labeled methanol obtained by the same protocol) was added to the sample. For extraction samples were incubated 15 min on ice and subsequently freeze dried and stored at -20°C until analysis. Samples were re-suspended in deionized water adjusting cell dry weight concentration to 10 µg/µL. Cell debris and other insoluble parts were separated by centrifugation (10 min, 20'000 *g*, 4°C). Prior to injection, samples were diluted with solvent A as indicated below. The correlation of the optical-density and cell-dry-weight was determined to be 0.27 mg/mL.

HPLC-MS analysis. Nanoflow high-performance liquid chromatography nanospray high-resolution mass spectrometry analysis was carried out with a split-free nanoLC Ultra system (Eksigent, Dublin, CA) hyphenated to an LTQ-Orbitrap mass spectrometer (ThermoFisher Scientific, San Jose, CA). For HPLC separation, a C18 column was used as stationary phase (Thermo Hypersil gold 150 x 0.1 mm i.d., 5 µm particle size, pH range 1-11). For nanospray ionization the column was connected via liquid junction to a silica tip emitter (20 µm i.d., tip 10 ± 1 µm, New Objective, Woburn, MA). Tributylamine (TBA) was employed as reagent for ion-pair

reversed-phase (IP-RP) separation. Solvent A was obtained by dissolving 1.7 mM TBA in 1.5 mM acetic acid and solvent pH was subsequently adjusted to pH 9.4 with 6 M ammonium hydroxide. Eluent B was methanol and varied as follows: 0 min, 3 %; 30 min, 90 %; 35 min, 90 %; 36 min, 3 %; 45 min, 3%. Injection volume was 1 μL. Mass acquisition was carried out in the negative FTMS mode at unit resolution of 60'000 (at *m/z* 400) applying ion spray voltage of -1.9 kV. Capillary temperature was 150 °C and capillary voltage was set at -10 V. To increase sensitivity of the mass spectrometer, acquisition was performed in single ion monitoring mode (SIM) for three different scan ranges. For high mass ranges ($202 \leq m/z \leq 650$, and $600 \leq m/z \leq 1'000$) tube lens voltage was -100 V. Tube lens voltage was reduced to -60 V for the scan range $100 \leq m/z \leq 350$.

Limit of detection (LOD). To determine LOD, dilution series of standard mixtures were prepared using solvent A. To determine LOD in complex matrix, cell extract from *M. extorquens* AM1 grown on ^{13}C methanol was diluted 10-fold with solvent A to a final concentration of 1 μg/μL biomass cell dry weight equivalent, and this solution was used to prepare dilution series of standard mixtures. Mass isomers were injected separately to avoid peak overlapping. Prior to sample analysis blanks (solvent) were injected to examine for eventual presence of analytes in solvent or in the analytical system. Dilution series of standard mixtures were carried out starting with the lowest concentration. For several compounds (i.e. citric acid and succinic acid), monoisotopic peaks M_0 were always present in the blank. Therefore, concentrations of uniformly ^{13}C labeled peaks M_{UL} were determined in ^{13}C cell extract using the isotope dilution method [25, 26] and LOD of M_{UL} in ^{13}C cell extract dilution series was determined instead.

Identification of potential metabolites in cell extracts. Potential metabolites were identified by stable ^{13}C isotope assignment.[27, 28] For attribution of molecular formulas to detected mass peaks, a list of 14'943 molecular formulas was extracted from PubChem compound database (http://pubchem.ncbi.nlm.nih.gov/) from all compounds of category metabolic pathways composed of C and H, N, O, P, or S. The list was used to generate mass

lists for assignment of molecular formulas. A molecular formula of the database was assigned to a mass peak when i. the differences between theoretical and measured *m/z* values of the monoisotopic peak M_0, of the corresponding U-^{13}C labeled peak M_{UL} as well as the delta value M_{UL}-M_0 were below 1 mmu, and ii. peaks of M_0 and of M_{UL} had the same retention time. Chromatographic peaks were considered for identification when signal to noise ratio of monoisotopic peak and uniformly ^{13}C labeled peak were at least three. As mass drifts occurred during data acquisition, identification was improved by correcting measured *m/z* values using mass peaks with known *m/z* values present in the U-^{13}C labeled cell extract.

RESULTS AND DISCUSSION

Evaluation of nano-scale IP-RP HPLC-MS with standards. For IP-RP based HPLC separation, tributylamine (TBA) was chosen as ion pairing reagent. To achieve efficient pairing between analytes and TBA (pKa 10.4), analytes should be negatively charged and tributylamine positively charged. As many metabolites have a pKa ~6 (e.g. phosphorylated sugars) or even pKa ~9 (e.g. nucleotides), pH was set to pH 9.4 and a C18 stationary phase stable under basic conditions was used. Since ion-pairing reagents cause ion suppression effects and thus significantly reduce sensitivity of HPLC-MS[29], the minimal concentration of TBA in the mobile phase providing retention of metabolites was added (1.7 mM). The method was evaluated using a 100 µm i.d. column and a flow rate of 500 nL/min. Under these conditions, retention times of standard compounds from various metabolite classes such as organic acids, phosphorylated compounds, sugar derivates, or coenzyme A (CoA) thioesters on the column were measured (**Table 1**). Chemically related compounds are generally eluting in the same separation window, indicating similar retention behavior. Organic acids eluted between 16 and 20 min, whereas CoA thioesters were detected in the 21-23 min time range. Nucleotide monophosphates eluted first (15-16 min), followed by nucleotide diphosphates (18-19 min) and nucleotide triphosphates

(19.3 min). The additional charges in nucleotide triphosphates compared to nucleotide monophosphates provide a better ion-pairing with TBA cations and retention was increased. The chromatographic separation resulted in sharp elution profiles (see also **Figure 1**), separating most of the metabolites (**Table 1**). Notably however, incomplete chromatographic separation of metabolites with different *m/z* values is principally not of concern, since data acquisition with a high accuracy mass spectrometer allows the display of extracted ion chromatograms for the compound of interest. Separation of mass isomers such as glucose-6-phosphate and fructose-6-phosphate was generally not achieved and only one peak was observed in the corresponding extracted ion chromatogram (see **Figure 1**, hexose-6-phosphate). In some cases, MS/MS experiments might allow distinguishing between co-eluting mass isomers by compound specific fragment ions. For such purpose, hyphenation of the nano-IP-RP-HPLC setup with a triple quadrupole instrument can be envisaged in order to perform multiple reaction monitoring experiments (MRM). For nucleotides (e.g. ADP), two peaks were generally observed (**Figure 1**). The first peak is assigned to adenosine diphosphate (ADP), whereas the second one is due to in-source fragmentation of adenosine triphosphate (ATP). Reduction of the transfer tube temperature reduced ATP fragmentation; however, the phenomenon could not be completely eliminated. This is not of concern, since ATP and ADP are chromatographically almost baseline separated and fragmentation did not hamper determination of the nucleotides. This is of major importance when applying the method for quantitative analysis, as ATP/ADP ratios are often determined to evaluate energetic states of cells. Similarly, ADP fragmention results in AMP detection (**Figure 1**).

Limits of detection were determined for 54 commercially available metabolites (**Table 1**). Most of these were detected in the upper attomol range. Compared to state of the art HPLC-MS methods based on hydrophilic liquid chromatography (HILIC) or ion-pair reversed-phase liquid chromatography employing 2 mm i.d. columns,[12, 19, 20] sensitivity is thus drastically increased with nanoscale HPLC-MS. Except for fumarate, all investigated compounds had limits of

detection significantly lower than those determined with other analytical setups (**Table 2**). Detection sensitivity was generally several hundred times higher and for some metabolites (e.g. ADP) LOD was even increased by a factor of several thousands. As mass spectrometers employed in the previous studies are either equivalent (Orbitrap and Exactive, ThermoFisher Scientific, San Jose, CA) or even more detection sensitive (TSQ Quantum, ThermoFisher Scientific), increase in detection sensitivity achieved in the present study is not due to the MS instrument itself but to preceding nano-IP-RP-HPLC coupled to nanospray ionization.

In a second step, limits of detection were investigated in a complex matrix. To this end, metabolite standards were diluted with cell extract of *Methylobacterium extorquens* AM1 grown on ^{13}C labeled methanol. As expected, a decrease in sensitivity was observed but limits of detection were still in the 0.1-10 fmol range for most metabolites; CoA thioesters, however, which play a central role in the assimilatory metabolism during growth of one carbon compounds of *M. extorquens* AM1[24] were even detected below 1 fmol. Exemplarily, linearity was investigated for 15 compounds in complex matrix revealing a linear concentration range between 2-3 orders of magnitude when using the LTQ Orbitrap instrument (see **Table S1**).

Table 1. Limits of detection for 54 standard metabolites in alphabetical order. LOD's were determined in aqueous solution without and with complex matrix (uniformly 13C labeled cell extract from 500 ng CDW of *M. extorquens* AM1).

metabolite	formula	RT [min]	LOD [amol]		MF detected in cell extract [a]			
			solvent A	complex matrix	0.5 ng/inj.	5 ng/inj	50 ng/inj.	250 ng/inj.
2-deoxyribose-5-phosphate	$C_5H_{11}O_7P$	15.0	500	2'500	-	3	3	3
2-oxoglutaric acid	$C_5H_6O_5$	16.3	2'500	5'000	-	-	-	-
3-hydroxybutyric acid	$C_4H_8O_3$	16.8	25'000 [b]		-	-	-	3
3-hydroxybutyryl-CoA	$C_{25}H_{42}N_7O_{18}P_3S$	21.1	100	250	-	3	3	3
3-phosphoglycerate	$C_3H_7O_7P$	18.2	7'500	2'500	-	2	3	3
6-phosphogluconate	$C_6H_{13}O_{10}P$	18.9	750	2'500	-	3	3	3
acetyl-CoA	$C_{23}H_{38}N_7O_{17}P_3S$	21.2	100	250	3	3	3	3
acetoacetyl-CoA	$C_{25}H_{40}N_7O_{18}P_3S$	21.0	250	1'000	-	-	-	-
adenine di-phosphate	$C_{10}H_{15}N_5O_{10}P_2$	19.0	250	100	-	1	3	3
ADP-ribose	$C_{15}H_{23}N_5O_{14}P_2$	17.1	250	500	-	-	3	3
adenine mono-phosphate	$C_{10}H_{14}N_5O_7P$	16.5	100	250	1	3	3	3
adenine tri-phosphate	$C_{10}H_{16}N_5O_{13}P_3$	19.4	750	2500	3	3	3	3
aspartic acid	$C_4H_7NO_4$	8.1	25'000		-	-	1	-
butyryl-CoA	$C_{25}H_{42}N_7O_{17}P_3S$	22.5	100	100	-	-	3[c]	3[c]
biotin	$C_{10}H_{16}N_2O_3S$	15.9	500	750	-	-	-	-
cADP-ribose	$C_{15}H_{21}N_5O_{13}P_2$	12.4	5'000	5'000	-	-	3	3
cAMP	$C_{10}H_{12}N_5O_6P$	16.0	250	500	3	3	3	3
cGMP	$C_{10}H_{12}N_5O_7P$	14.1	250	500	-	2	3	3
cis-aconic acid	$C_6H_6O_6$	18.6	5'000 [b]		-	3	3	3
citric acid/ isocitric acid	$C_6H_8O_7$	19.8	5'000 [b]		-	3	3	3
crotonyl-CoA	$C_{25}H_{40}N_7O_{17}P_3S$	22.1	250	250	-	-	3	3
cytidine di-phosphate	$C_9H_{15}N_3O_{11}P_2$	18.0	5'000	2'500	-	-	3	3
cytidine mono-phosphate	$C_9H_{14}N_3O_8P$	15.3	500	2'500	-	3	3	3
cytidine tri-phosphate	$C_9H_{16}N_3O_{14}P_3$	19.3	500	1'000	-	3	3	3
dihydroxyacetone phosphate	$C_3H_7O_6P$	19.3	2'500	7'500	-	-	3	3
ethylmalonic acid	$C_5H_8O_4$	16.7	5'000 [b]		3	3	3	3
erythrose-4-phosphate	$C_4H_9O_7P$	15.4	7'500		-	-	-	-
FADH	$C_{27}H_{33}N_9O_{15}P_2$	19.3	100	250	-	3	3	3
fructose-6-phosphate	$C_6H_{13}O_9P$	15.1	500	1'000	3[c]	3[c]	3[c]	3[c]
fructose-1,6-bisphosphate	$C_6H_{14}O_{12}P_2$	19.7	500	750	-	-	3	3
fumaric acid	$C_4H_4O_4$	17.0	40'000 [b]		-	-	-	-
glucose-6-phosphate	$C_6H_{13}O_9P$	15.0	500	500	3[c]	3[c]	3[c]	3[c]
glutamic acid	$C_5H_9NO_4$	8.0	25'000		-	-	3	3
guanidine di-phosphate	$C_{10}H_{15}N_5O_{11}P_2$	18.4	500	750	-	3	3	3
guanidine mono-phosphate	$C_{10}H_{14}N_5O_8P$	15.4	500	2'500	-	-	3	2
guanidine tri-phosphate	$C_{10}H_{16}N_5O_{14}P_3$	19.2	750	2'500	1	3	3	3
isobutyryl-CoA	$C_{25}H_{42}N_7O_{17}P_3S$	22.5	200	100	-	-	3[c]	3[c]
malic acid	$C_4H_6O_5$	17.5	10'000 [b]		-	3	3	3
methylmalonyl-CoA	$C_{25}H_{40}N_7O_{19}P_3S$	21.4	300	100	-	3[c]	3	3[c]
NAD	$C_{21}H_{27}N_7O_{14}P_2$	12.3	250	500	2	3	3	3
oxoglutathione	$C_{20}H_{32}N_6O_{12}S_2$	16.5	250	500	3	3	3	3
pantothenic acid	$C_9H_{17}NO_5$	14.1	100	250	3	3	3	3
phosphoenol pyruvate	$C_3H_5O_6P$	18.3	5'000	5'000	-	-	3	3
phosphoserine	$C_3H_8NO_6P$	16.0	7'500	5'000	-	-	-	
propionyl-CoA	$C_{24}H_{40}N_7O_{17}P_3S$	21.7	50	100	-	3	3	3
pyridoxal phosphate	$C_8H_{10}NO_6P$	18.3	5'000		-	1	3	1
ribose-5-phosphate	$C_5H_{11}O_8P$	15.9	500	1'000	-	3[c]	3[c]	3[c]
ribulose-5-phosphate	$C_5H_{11}O_8P$	15.3	500	2'500	-	3[c]	3[c]	3[c]
succinyl-CoA	$C_{25}H_{40}N_7O_{19}P_3S$	21.3	300	750	-	3[c]	3	3[c]
trehalose-6-phosphate	$C_{12}H_{23}O_{14}P$	15.0	250	750	3	3	3	3
uridine-di-phosphate	$C_9H_{14}N_2O_{12}P_2$	18.1	250	500	-	3	3	3
uridine-mono-phosphate	$C_9H_{13}N_2O_9P$	15.4	100	2'500	-	1	3	3
uridine-tri-phosphate	$C_9H_{15}N_2O_{15}P_3$	19.3	250	750	1	3	3	3
xylulose-5-phosphate	$C_5H_{11}O_8P$	15.6	500	2'500	-	3[c]	3[c]	3[c]

[a] How often a MF was detected in 3 analyses performed with a given amount of biomass per injection (ng/inj.). [b] LOD determined by U-13C mass peak of 13C cell extract using isotope dilution for quantification. [c] Co-eluting mass isomers that cannot be distinguished by the method.

Table 2. Comparing limits of detection by HPLC-ESI-MS and by nano-HPLC-ESI-MS. Values are expressed in fmol.

metabolite	separation with IP-RP or HILIC on 2 mm i.d. columns[12, 19, 20]	separation with IP-RP on 0.1 mm i.d. column (this study)
2-oxoglutarate	250-4'100	1
3-phosphoglycerate	1'000	2.5
6-phosphogluconate	460-750	0.25
acetyl-CoA	124-12'300	0.1
adenosine diphosphate (ADP)	460-2'940	0.1
adenosine monophosphate (AMP)	51-342	0.1
adenosine triphosphate (ATP)	3'950-4'100	0.5
aspartate	17-151	25
citrate	250-10'400	5
dihydroxyacetone phosphate	750-2'940	2.5
fructose 1,6-bisphosphate	150-589	0.25
glucose 6-phosphate	38-100	0.1
glutamate	100-1'400	25
phosphoenol pyruvate	51-595	2.5
ribose 5-phosphate	43-100	0.1

Application of the method to metabolome analysis of a model bacterium. To investigate the potential of the developed method, cell extracts of the model strain *M. extorquens* AM1 were analyzed. A critical question was the sensitivity of the method for true biological samples. Since the LTQ-Orbitrap instrument with its high mass resolution (up to 100'000 at *m/z* 400) is particularly suited for compound identification,[30] we investigated the suitability of the above-described method and correlated the number of detected metabolites with the amount of the analyzed biomass. Stable carbon isotope labeling was combined with high-resolution mass spectrometry to assign molecular formulas (MF) to detected mass peaks (see **experimental section**). To this end *M. extorquens* AM1 was grown on methanol as sole carbon source. Three samples were taken during exponential growth, quenched and extracted. Prior to extraction, 99 % ^{13}C labeled cell extract from similar biomass amount was added to the samples. All samples were diluted to final cell dry weight concentrations of 0.5 ng/µL, 5 ng/µL, 50 ng/µL, and 250 ng/µL, respectively. Dilution series of each sample were analyzed by the introduced HPLC-MS method and numbers of detected molecular formulas were determined for each sample (**Table 3**).

Table 3. Metabolome analysis of *M. extorquens* AM1. Number of identified molecular formulas (MF) in dilution series of cell extracts of three different samples. CDW/inj., cell dry weight equivalent of injected sample amount; 1 (2, 3) out of 3, number of samples in which MF was detected; Σ, total of MF identified at least in one out of three analyzed samples.

CDW/inj. [ng]	average ± SD	3 out of 3	2 out of 3	1 out of 3	Σ
0.5	20 ± 4	17	1	6	24
5	67 ± 2	65	2	6	73
50	157 ± 5	142	14	17	173
250	149 ± 4	136	15	7	158

Details concerning the identified MF in analyzed dilution series are available as **supporting information (TablesS2-S5)**. Since databases play a key role in metabolite identification, the structure of the database used in the present study is described in the **experimental section**. With 0.5 ng CDW per injection an average of 20 MF was identified and 17 MF were detected in all 3 analyzed samples. The number of identified MF increased with injected biomass amount until 50 ng CDW per injection where average number of identified molecular formulas was 157 ± 5. Further increase of injected biomass amount did not increase the total number of detected metabolites (149 ± 4) and the number of MF identified in all three samples slightly decreased from 142 to 136. This suggests, negative effects of sample matrix and increasing sample complexity had a more significant influence on HPLC-MS analysis than increased metabolite amount on the column resulting in column overload. Examination of the identifications in the cell extracts of metabolites for which LOD's were determined (standards), a similar correlation was found (see **Table 1**). In at least two out of three analyzed samples, 7 standard compounds were found at 0.5 ng, 23 at 5 ng, 41 at 50 ng, and 40 at 250 ng CDW per injection. However, several standards were not detected at injection of the highest biomass concentration despite being known to be present in *M. extorquens* AM1. Various reasons such as low intracellular concentrations, the method of extraction, or the stability of the compounds may explain this absence of detection. The results suggest that 50 ng CDW per injection are sufficient to identify a maximum number of metabolites. Extracted ion chromatograms from metabolites of various classes detected at 50 ng CDW per injection are depicted in **Figure 1**. Narrow peaks were obtained and almost no fronting or tailing was observed. Retention times among all samples and across all biomass concentrations was below 5%, i.e. between 0.1 and 4.3 %. Since three analyses per sample were carried out for different scan ranges to increase detection sensitivity, total biomass amount was 150 ng corresponding to about 400'000 bacterial cells.

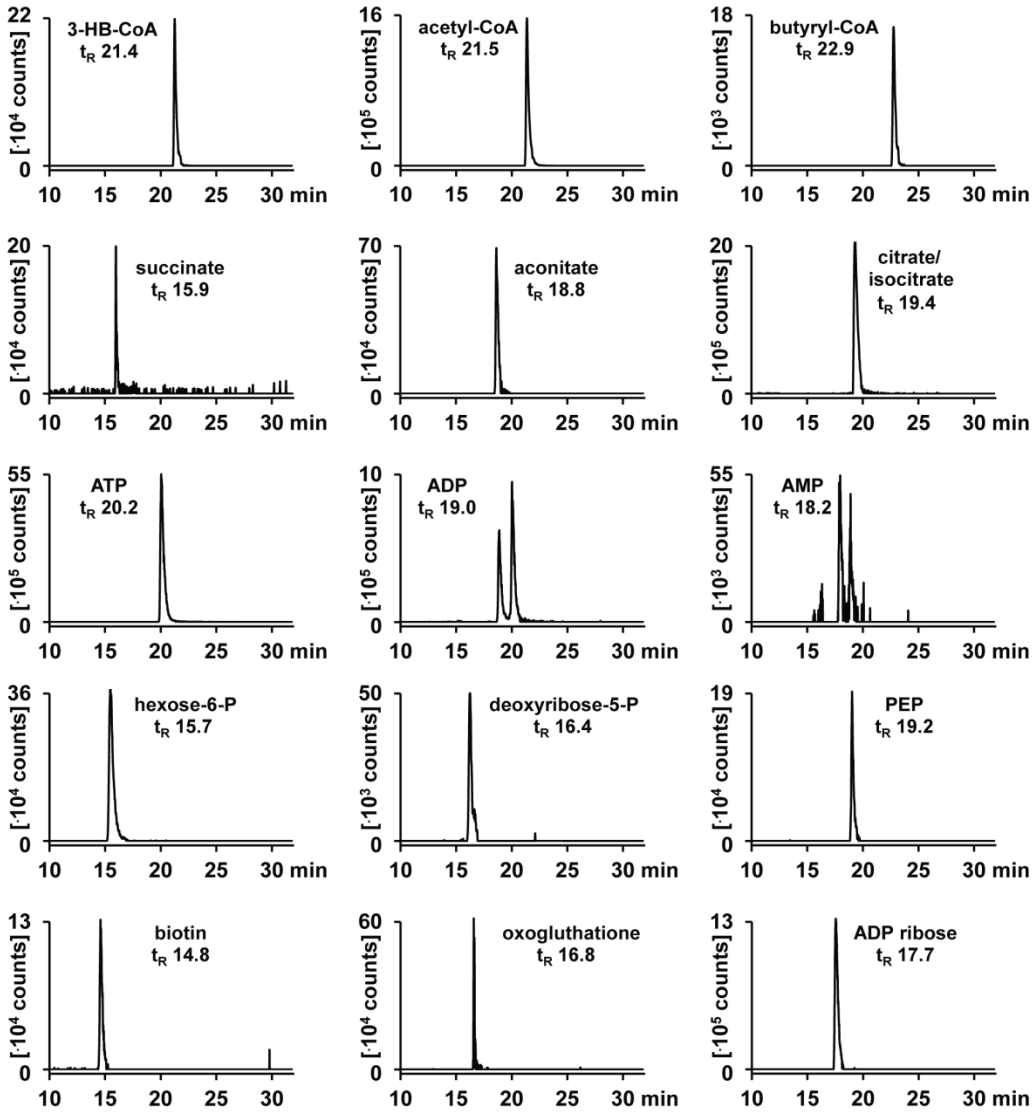

Figure 1. Extracted ion chromatograms of selected metabolites detected in cell extract of *M. extorquens* AM1 during exponential growth on methanol. Metabolites were extracted with cold acidified acetonitrile. Injected amount corresponded to 50 ng biomass cell dry weight.

CONCLUSION

The introduced method, which combines nano-IP-RP-HPLC with nano-ESI-HRMS, demonstrates that very high sensitivities can be achieved in metabolome analyses. A few hundreds nanograms of cell extracts are enough to get a comprehensive coverage of central metabolism and cellular energy status. The present work was carried out with an LTQ-Orbitrap mass spectrometer. By hyphenating the developed nano-IP-RP-HPLC setup with more sensitive instruments such as latest generation triple quadrupoles, sample amounts needed for analysis will be further reduced. This reduction in sample need will permit for instance to miniaturize bacterial culture volumes or enable the analysis of high valuable cells (i.e. stem cells). The perspective of *in situ* metabolomics or even meta-metabolomics is now wide open.

ACKNOWLEDGMENT

This work was supported by BattleX within the Swiss Initiative in Systems Biology (SystemsX.ch).

SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is free of charge via the Internet at http://pubs.acs.org.

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