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Quantitative metabolome analysis using liquid chromatography–high-resolution mass spectrometry

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ABSTRACT

In this report, we introduce a liquid chromatography single-mass spectrometry method for metabolome quantification, using the LTQ Orbitrap high-resolution mass spectrometer. Analytes were separated with hydrophilic interaction liquid chromatography. At a working resolution of 30,000 (at m/z 400), the limit of detection varied from 50 fmol to 5 pmol for 25 metabolites tested. In terms of metabolite concentration, the linearity was about 2 to 3 orders of magnitude for most compounds ($R^2 > 0.99$). To determine the accuracy of the system in complex sample matrices, the isotope dilution method was evaluated from mixtures of pure compounds and uniformly ^{13}C-labeled cell extracts. With the application of this method, quantification was possible within single runs even when the pool sizes of individual metabolites varied from 0.13 to 55.6 μM. As a case study, intracellular concentrations of central metabolites were determined for *Methylobacterium extorquens* AM1 during growth on two different carbon sources, methanol and succinate. Reproducible results from technical and biological repetitions were obtained that revealed significant variations of intracellular metabolite pool sizes, depending on the carbon source. The LTQ Obitrap offers new perspectives and strategies for metabolome quantification.

Metabolomics aims at the analysis of all small molecules in a biological sample [1]. It is a rapidly expanding field in the postgenomic era because it provides a detailed measurement of the phenotypic responses of living systems to genetic or environmental changes. A wide range of analytical methods—mainly based on mass spectrometry—have been developed recently to separate, detect, and identify the hundreds or thousands of small molecules present in a biological sample. The separation of compounds in highly complex mixtures is usually performed by combining a separation in a chromatographic dimension—either LC[1], GC, or CE—with separation in the m/z dimension [2,3]. If such qualitative—or semiquantitative—approaches still need to be improved, there is an increasing need for quantitative approaches aiming at the measurement of metabolite pool sizes, which currently represents a true challenge in terms of sample preparation and analytics. The difficulties associated with metabolite sampling have been discussed recently [4,5]. They are mainly related to the high number and diversity of the physicochemical properties of metabolites, their very high turnover rates down to the subsecond range within the biological context, and their chemical instability during the entire sampling procedure [4,5]. In addition to metabolite sampling, MS analytics is also challenging with respect to quantitative metabolomics.

For the specific purpose of MS-based quantification, quadrupoles are usually preferred over other mass detectors because this type of analyzer provides a high linearity and a high dynamic range [6–11]. Quadrupole detectors operate at a low mass resolution, resulting in significant overlapping problems in single-MS analysis of complex metabolite mixtures, especially within the low mass range (m/z <400), a spectral region that contains most metabolites of interest but also nonspecific chemical impurities (chemical noise). To compensate for the low resolution of quadrupoles, the chromatographic separation can be optimized—often at the expense of the analytical time—and MS/MS strategies can be applied, with the benefit of a higher specificity and a higher sensitivity [6–9]. However, the MS/MS strategy has several drawbacks with regard to quantitative metabolomics, since (i) fragmentation decreases the absolute signal intensity; (ii) the most intensive fragment ions often result from nonspecific losses such as water or CO_2, when analyzing small molecules; and (iii) the complexity of the sample exceeds the number of possible MS/MS experiments.

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[1] *Abbreviations used:* A_{MUL}, area of the uniformly ^{13}C-labeled mass peak; A_{M0}, area of the monoisotopic mass peak; LC, liquid chromatography; CE, capillary electrophoresis; GC, gas chromatography; HPAEC, high performance anion exchange chromatography; MS, mass spectrometry; HILIC, hydrophilic interaction liquid chromatography; HR, high resolution; U-^{13}C, uniformly ^{13}C labeled; M_0, monoisotopic mass peak, M_{UL}, uniformly ^{13}C-labeled mass peak.

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High-resolution mass spectrometry appears attractive for overcoming the main problems encountered with quadrupole instruments. It provides a high separation potential in the m/z dimension, which decreases the need for chromatographic separation and provides a potential platform for large-scale quantification of metabolites from single-MS experiments. Currently, two high-resolution mass analyzers based on Fourier transformation are available: ion cyclotron resonance instruments and the recently developed LTQ Orbitrap [12]. Although the maximum resolving power of the LTQ Orbitrap is significantly lower than that of FTICR instruments, it is a high-speed ion, high-ion-transmission instrument that results in short accumulation times, which distinguishes it for the purpose of LC–MS analysis. In this study, we have evaluated the benefit of high resolution for quantitative metabolomics, with a specific focus on central metabolites that occur in almost all organisms and are therefore of broad biological relevance. A LC, single-MS method was set up on an LTQ Orbitrap instrument in which compounds were separated within a short time scale. The proposed method was validated from the analysis of 25 compounds representative of central metabolism and was further applied to quantify the central metabolome of *Methylobacterium extorquens* AM1, a microorganism of great potential for the industrial production of chemicals and proteins [13].

Materials and methods

Chemicals

All chemicals were purchased from Sigma (St. Louis, MO, USA). Acetonitrile, formic acid, and ammonium used for HPLC solvents were of LC–MS degree.

Medium and growth conditions

Cultures were carried out on a minimal medium [14]. Methanol (120 mM) or succinic acid (30 mM) was added as a carbon source. Batch cultivations were carried out in a 500-ml Multifors bioreactor (Infors HT, Bottmingen, Switzerland) at 28 °C, with an aeration rate of 0.5 vvm and a stirring rate of 1000 rpm to avoid cell aggregation. The pH was maintained at 7.0 by the addition of 2 M ammonium hydroxide or 1 M sulfuric acid.

Sampling, quenching, and metabolite extraction

Cells were sampled during exponential growth at a biomass concentration of approximately 0.6 g L^{-1}. Uniformly ^{13}C-labeled cell extracts of *M. extorquens* AM1 grown on [^{13}C]methanol and [^{13}C]carbon dioxide were used as an internal standard for subsequent metabolite quantification according to the isotope dilution method described by Wu et al. [15]. Amounts of about 0.6 mg biomass were sampled by fast filtration [16] and washed with 2 ml medium with a 90% reduced salt concentration. The carbon source (methanol or succinate) was at 50% of the initial concentration to avoid substrate limitation during sampling [4]. The filters were directly transferred into vessels containing 8 ml boiling water, and a defined volume of the internal standard solution corresponding to a biomass equivalent of 0.6 mg was added simultaneously. Total sampling time was below 10 s, and the transfer time of the filter into the extraction solution was below 1 s. After 8 min of incubation time, the extracts were cooled on ice and filtered via a RC Sartorius Minisart filter (pore size 0.2 μm), prior to chilling with liquid nitrogen. All samples were lyophilized immediately or had been stored previously at −20 °C. The dried sample was redissolved in 100 μl double-distilled water and diluted 30/70 (v/v) with acetonitrile prior to analysis.

LC–MS analysis of metabolites

Analyses were performed with a Rheos 2200 HPLC system (Flux Instruments, Basel, Switzerland) coupled to an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an electrospray ionization probe.

Organic acids, amino acids, and phosphorylated compounds were separated on a pHILIC column (150 × 2 mm i.d., particle size 5 μm; Sequant, Umea, Sweden). Solvent A was acetonitrile and solvent B was 2 mM formic acid with 4 mM ammonium at a flow rate of 150 μl min^{-1}. Solvent B was varied as follows: 0 min, 15%; 2 min, 15%; 4 min, 35%; 6 min, 40%; 11 min, 50%; 14.5 min 80%; 19.5 min, 90%; 24.5 min, 90%. The column was then equilibrated for 10 min at the initial conditions prior to the next sample analysis. The LC was hyphenated split free to the mass spectrometer. MS analysis was performed in the negative FTMS mode at a resolution of 30,000 (at m/z 400) with the following source parameters: sheath gas flow rate was 10, auxiliary gas flow rate was 5, tube lens was −50 V, capillary voltage was −2.5 V, and ion spray voltage was −4.4 kV.

Results

LC–MS analysis of defined metabolite mixtures

A single LC–MS method based on zwitterionic stationary phase hydrophilic interaction chromatography (HILIC) was set up to evaluate the applicability of high-resolution mass spectrometry for quantitative metabolomics, with a specific focus on central metabolites that are of wide interest because they play a key role in the survival, growth, and adaptation of all living systems. Central metabolites include diverse but mainly highly polar classes of compounds such as phosphorylated sugars, nucleotides, organic acids, and amino acids. Different approaches have been proposed for the separation of these classes of compounds, including mainly LC–MS, HPAEC–MS, and GC–MS after appropriate derivatization [6,9,17–20]. The HILIC chromatography offers the advantage of allowing the utilization of nonaqueous solvents such as acetonitrile for the LC separation of polar compounds [21]. It is therefore highly compatible with the electrospray ionization usually applied for the analysis of polar metabolites [10,11].

To examine the separation capability of the method, it was applied to the analysis of a mixture of 25 metabolites representative of central metabolism, with molecular masses in the range of 70–300 amu. The objective was not to maximize the number of metabolites that could be measured, but to define the analytical conditions for use in further quantitative investigations. A mixture of commercially available compounds including seven phosphorylated compounds, nine organic acids, and nine amino acids, was prepared and analyzed in the negative mode of ionization (Table 1). All analytes were separated within 11 min, with 13 metabolites from 25 eluting within 1 min. Most peaks were not fully separated in the chromatographic dimension, but could be separated in the m/z dimension because of the high mass resolution (Fig. 1), with the exception of two pairs of two metabolites: leucine + isoleucine and glucose 6-phosphate + fructose 6-phosphate, which have the same m/z value and were not fully separated by chromatography.

The LTQ Orbitrap instrument can operate at a mass resolution of up to 100,000[2], which is useful for maximizing the number of metabolites that can be separated. However, for the purpose of

[2] Thermo Electron defines the mass resolution of the Orbitrap at m/z 400 since the mass resolution of the Orbitrap mass analyzer depends on the mass-to-charge ratio of the ion. To simplify readability, mass resolutions shown in this article always refer to a mass-to-charge ratio of m/z 400 when not specified in the text.

Table 1
LC–MS characterization (LOD and linear range) of standard mixtures of low-molecular-weight compounds occurring in central metabolism

Metabolite	$[M-H]^-$ m/z (measured)	Δ mmu	Resolution ($m/\Delta m$)	LOD standard, pure (pmol)	Linear range (pmol)	LOD standard in complex matrix (pmol)
Glucose + fructose 6-phosphate[a]	259.0223	−0.20	45,000	0.1	0.25–300	0.5
Ribose 5-phosphate	229.0121	−0.04	47,000	0.1	0.25–100	0.5
3-Phosphoglycerate	184.9857	0.04	53,000	1	7.5–50	1
Phosphoenol pyruvate	166.9753	0.33	56,000	0.25	0.5–150	0.5
Dihydroxyacetone phosphate	168.9909	−0.03	55,000	0.75	1–100	0.5
6-Phosphogluconate	275.0172	−0.16	44,000	0.75	1–140	1
Pyruvate	87.0093	0.54	79,000	0.75	1–200	1
Citrate	191.0201	−0.10	53,000	0.25	1–160	1
2-Oxoglutarate	145.0145	0.36	59,000	0.05	0.1–100	0.5
Succinate	117.0197	0.35	66,000	0.05	0.25–50	0.5
Fumarate	115.0041	0.45	67,000	0.05	0.1–50	0.1
Malate	133.0146	0.40	62,000	0.05	0.1–75	0.1
Glyoxylate	72.9937	0.59	81,000	5	10–300	50
3-Hydroxybutyrate	103.0406	0.52	71,000	0.5	1–150	1
Methylsuccinic acid	131.0353	0.34	63,000	0.05	0.25–100	0.1
Ala	88.0409	0.54	77,000	2.5	5–300	10
Leu + Ile[a]	130.0877	0.31	63,000	0.15	0.5–600	0.1
Ser	104.0357	0.40	71,000	0.5	1–200	0.1
Thr	118.0513	0.37	67,000	0.1	0.25–200	0.25
Pro	114.0565	0.48	68,000	0.25	0.5–200	0.5
Glu	146.0463	0.38	59,000	0.1	0.25–120	0.1
Gln	145.0621	0.10	60,000	0.25	0.75–200	0.1
Val	116.0721	0.43	67,000	0.1	0.25–100	0.5

The LOD was determined for pure standards and after addition to a complex sample matrix (see text). All calibration curves had correlation coefficients of $R^2 > 0.99$.
[a] Peaks were not completely separated and therefore coeluted.

quantification, the definition of peaks is a critical parameter for signal integration. Using the chromatographic conditions mentioned above, most compounds in the metabolite mixture had a peak width around 20 s. When the Orbitrap was operated with a mass resolution of 100,000, corresponding to a 2.6-s scan time, the peaks were defined with fewer than 10 points, which was not sufficient for accurate signal integration. To obtain a higher peak definition, the MS analyses were performed with a mass resolution of 30,000, corresponding to a scan time of 0.6 s. This scan rate is sufficient to operate the LC instrument with the analytical or narrow-bore columns. When the MS analysis of the metabolite mixture was carried out at this scan rate, all the peaks were defined with at least 30 points, a peak definition that was consistent with accurate peak integration. Further investigations were therefore performed using a working mass resolution of 30,000.

The limit of detection (LOD) and the linearity range of the method were determined for the various metabolites from the analysis of a dilution series of metabolite mixtures. The LOD varied from 50 fmol to 5 pmol, depending on the metabolite (Table 1), which corresponds to a range of 4–365 pg. The LOD values obtained were comparable to results recently published for the LC–MS analysis of nitrosamines in water samples with the LTQ Orbitrap, in which a LOD of 2–14 pg was reported [22].

The linearity of the proposed LC–MS method was evaluated with regard to the amount of metabolite injected and to metabolite concentrations. In general, the detection of metabolites was found to be linear (with a correlation coefficient $R^2 > 0.99$) when the intensities of the chromatogram peaks were between $2–4 \times 10^4$ and 2×10^7 cps. These boundaries were taken into account for the determination of the linearity range for each investigated metabolite. In terms of absolute amount of metabolite injected, the linearity range (Table 1) varied between 1 (2/3-phosphoglycerate) and 3 orders of magnitude (2-oxoglutarate). In the case of 2-oxoglutarate, a linear response was observed between 0.1 and 100 pmol. Remarkably, the response was also linear between 100 and 300 pmol ($R^2 = 0.99$), but with a different slope. In terms of metabolite concentration, the linearity was about 2 orders of magnitude for most compounds.

LC–MS analysis of central metabolites in complex samples

When electrospray ionization is used for the LC–MS analysis of complex samples, the ionization yield of the analyte can be strongly affected by coeluting compounds; therefore, external quantification is not applicable. This problem can be overcome by the isotopic dilution method, in which a mass isomer of the compound of interest, usually the uniformly ^{13}C (U-^{13}C)-labeled compound, is added as an internal standard. Within the linear range of the mass spectrometer, the concentration of the compound in the sample can be directly determined by comparing the area (A_{M0}) of the monoisotopic peak to the area (A_{MUL}) of the U-^{13}C mass peak (A_{M0}/A_{MUL} ratio). In metabolomics, a very large number of metabolites are considered (up to a few thousand), but most of them cannot be obtained commercially or through chemical synthesis in the appropriate ^{13}C-labeled form. An attractive alternative was proposed by Wu et al. [15], in that samples of interest are mixed with a cellular extract containing a high proportion of U-^{13}C-labeled metabolites. This ^{13}C-labeled extract is prepared from cells—or other relevant biological material—grown with U-^{13}C-labeled substrates so that the proportion of uniformly ^{13}C-labeled isotopomer is high (typically 80–99%) for almost all metabolites occurring therein. The ^{13}C-labeled extract can be mixed with samples to ^{13}C-labeled standard for almost all central metabolites, but also for all other labeled metabolites occurring in the (^{13}C-labeled) extract. The major drawback of the approach is related to the increased complexity of the sample in the m/z dimension, since a unique analyte is detected at two major m/z peaks—i.e., at $m/z = M_0$ and M_{UL} for the unlabeled and uniformly labeled forms, respectively, instead of one. Because the labeled forms are not all 100% U-^{13}C-labeled, additional m/z peaks can be detected. Therefore, the total number of m/z peaks to be detected and analyzed in this strategy is drastically higher—at least twice—than in classical approaches, and the risk of peak overlap is increased as well. High-resolution mass spectrometry is particularly attractive to deal with such complexity because it provides the highest separation potential in the m/z dimension.

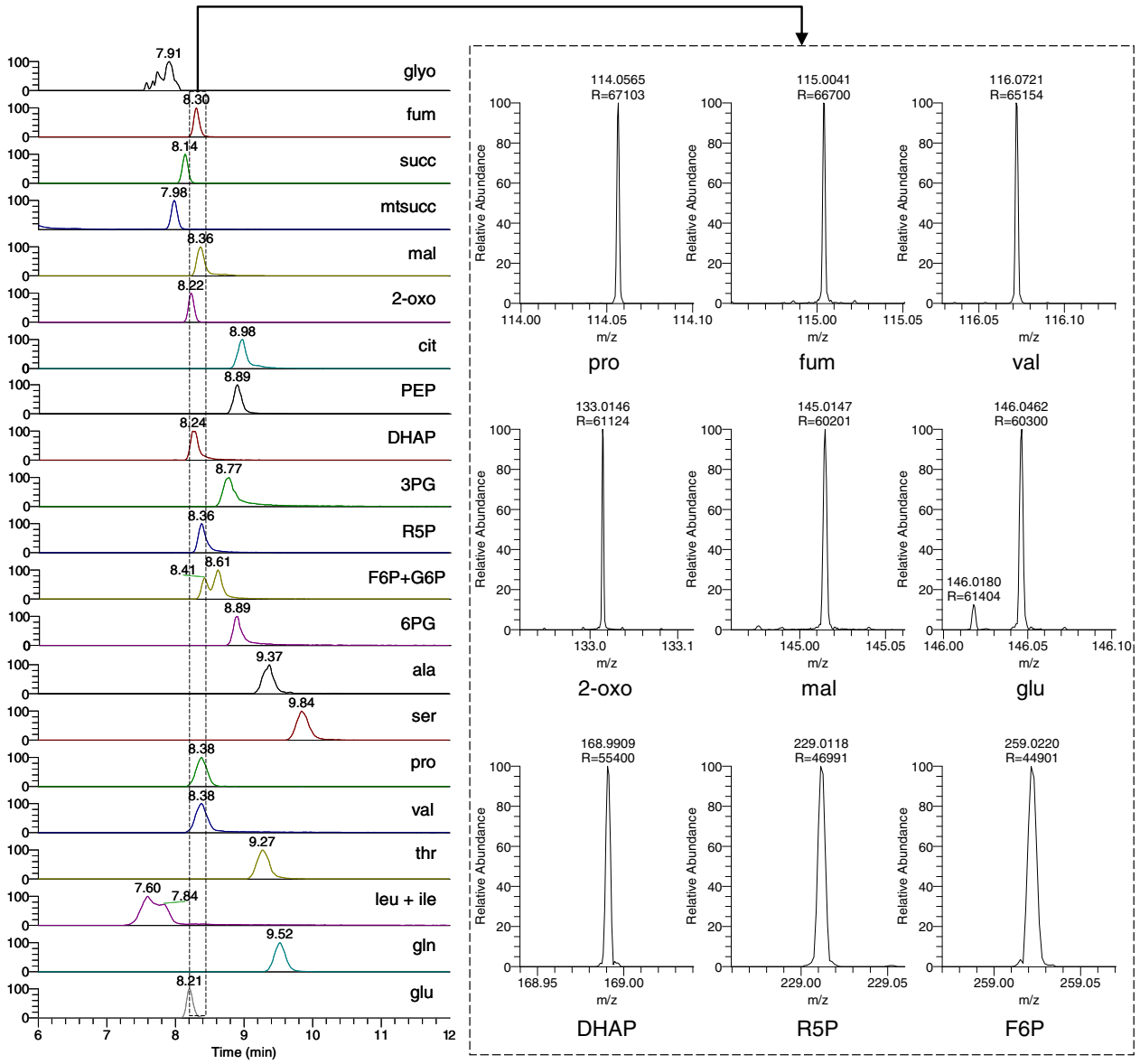

Fig. 1. Extracted ion chromatograms of standard compounds eluting between 7 and 10 min analyzed in a single run. Around 50 pmol per compound was injected. The mass tolerance of the ion peak was set to 1 millimass unit. Compounds that were not separated by liquid chromatography were completely separated by MS when having different m/z values. Corresponding mass spectra are exemplary, shown for a set of overlapping peaks upon LC (dashed rectangle). Measured $[M-H]^-$ ions: glyo, glyoxylate; fum, fumarate; succ, succinate; mtsucc, methylsuccinate; mal, malate; 2-oxo, 2-oxoglutarate; cit, citrate; PEP, phosphoenol pyruvate; DHAP, dihydroxyacetone phosphate; 3PG, 3-phosphoglycerate; R5P, ribose 5-phosphate; F6P, fructose 6-phosphate; G6P, glucose 6-phosphate; 6PG, 6-phosphogluconate; gly, glycine; ala, alanine; ser, serine; pro, proline; thr, threonine; leu, leucine; ile, isoleucine; glu, glutamate; gln, glutamine.

To evaluate the actual benefit of high-resolution mass spectrometry for the application of the isotopic dilution method, a U-^{13}C-labeled cell extract was prepared by growing *M. extorquens* on [^{13}C]methanol. The polar metabolites were extracted with boiling water, according to the protocol developed by Bolten et al. [4]. A uniformly ^{13}C-labeled mass peak was detected for the various metabolites, with the exception of dihydroxyacetone phosphate, which was not stable during boiling water extraction and was probably lost in the labeled cell extract. In total, 22 of 25 metabolites analyzed were found in the cell extract. Glyoxylate, an important metabolite of *M. extorquens* AM1 during growth on methanol, could not be detected since the LOD of glyoxylate was about 50 pmol in complex sample matrix. Only one mass peak was detected for the two mass isomers 2-phosphoglycerate and 3-phosphoglycerate, and a unique phosphoglycerate pool, named 2/3-phosphoglycerate, was further adopted in this report.

The ^{13}C-labeled cell extract was characterized with regard to the labeling state of each metabolite considered in this work (see above). The analysis of the isotopic cluster of the metabolites detected in the ^{13}C-labeled extract revealed an average proportion of U-^{13}C labeling higher than 90% (data not shown). For most metabolites, areas of the unlabeled mass peak, A_{M0}, were significantly below 1% compared to the U-^{13}C-labeled mass peak A_{MUL} or could not be detected, indicating that the amount of unlabeled molecules occurring in the ^{13}C-labeled extract was negligible in most cases. In case the amount of unlabeled metabolite in the ^{13}C-labeled extract was not negligible, the data collected for true samples were corrected to account for the amount of unlabeled metabolite in the ^{13}C-labeled extract.

To estimate the benefit of mass resolution to address the increased complexity in the m/z dimension by the addition of ^{13}C-labeled extract, unlabeled forms of phosphorylated compounds,

organic acids, and amino acids, were added to the ^{13}C-labeled cell extract. The mixture was analyzed at a low (R = 800, close to that of a quadrupole) and high (R = 30,000) mass resolution. For the two mass resolutions tested, the area of the monoisotopic peak, A_{M0}, and that of the uniformly labeled isotopomer, A_{MUL}, were measured for each metabolite. At R = 30,000, all peaks could be resolved and quantified. At R = 800, some peaks—i.e., peaks A_{M0} of pyruvate and 3-hydroxybutyrate and peaks A_{MUL} of 6-phosphogluconate, fumarate, serine, 3-hydroxybutyrate, and proline—could not be detected at all, because of overlap in the m/z dimension with other compounds or with peaks from the chemical noise. For similar reasons, the area of most peaks detected at low resolution differed significantly from that measured at high resolution (Table 2). Indeed, there were only 5 compounds of 22—namely phosphoenol pyruvate, 2/3-phosphoglycerate, alanine, threonine, and citrate—for which the same areas A_{M0} and A_{MUL} were measured at both low and high resolution.

These results emphasized that high mass resolution was critical for applying an LC–MS method with reduced chromatographical time. Indeed, at high mass resolution, the M_0 and M_{UL} peaks were well separated for the 22 investigated metabolites of the cell extract. These results clearly underscore the importance of high mass resolution for accurate quantification of compounds within a low mass range. This is also in line with previous publications pointing out that high mass resolution minimizes the requirement for chromatography [23].

The LOD in cell extracts was determined for the various metabolites by mixing different dilutions of the pure compounds with the ^{13}C-labeled extract. For each dilution, 5 μl of the pure compounds was added to 45 μl of the labeled extract, to avoid any differences in terms of matrix effects. The metabolites were injected at amounts that varied from 0.05 to 10 pmol. The results are shown in Table 1. As expected, the LOD was generally higher when the compounds were analyzed in the complex matrix. For most metabolites, the LOD in the cell extract was increased by a factor of 2 or less compared to the pure solution. From the 25 metabolites tested, the LOD increased by a factor of 5 for 3 metabolites and by a factor 10 for 3 other metabolites (succinate, 2-oxoglutarate, and alanine). A closer look at the spectra revealed that the latter compounds were found in the same region of the chromatogram, where a coeluting peak of phosphate was also detected. Phosphate was used to buffer the cell growth medium. Although cells were washed with a solution containing 10% of the original phosphate concentration of the medium, significant amounts of phosphate still remained in the samples and resulted in increased LOD for coeluting compounds (see Table 1). However, these problems are related to the LC separation and the electrospray ionization, but not to the LTQ Orbitrap instrument per se. To reduce such problems in future analyses, the quality of the ^{13}C-labeled extract as regards the salt residuals can be optimized by reducing the amount of phosphate in the growth medium and by reducing the total content of salts in the washing solutions even further.

Quantitative metabolite analysis in complex mixtures was evaluated from the analysis of different mixtures of pure compound solutions and ^{13}C-labeled cell extracts. Since the concentrations of the investigated metabolites present in the cell extract varied greatly, the amounts of cell extracts as well as the natural labeled metabolite standards were varied to maximize the evaluable linear range. The mixtures were than lyophilized and redissolved in the same final volume prior to analysis.

Table 3 shows the linear ranges (R^2 > 0.99) and concentrations observed for the 22 metabolites detected in the U-^{13}C-labeled cell extract. The boundaries of the linear range varied significantly among the investigated metabolites. This may be due to differences in the concentrations of the metabolites and to matrix effects. The significance of the latter was demonstrated with calibration curves using higher amounts of cell extracts. The linearity range was significantly altered when the amount of cell extract was increased. This result could be explained by enhanced ionization suppression, as mentioned above.

The concentrations of U-^{13}C-labeled metabolites were calculated from the slopes of the calibration curves. The lowest metabolite concentration was determined for methylsuccinate (0.13 μM) and the highest for glutamate (55.6 μM). Although the concentrations varied by a factor of up to 500 between the measured metab-

Table 2
Influence of mass resolution on peak area

Metabolite	A_{M0} (10^6 count seconds)		A_{MUL} (10^6 count seconds)	
	HR	LR	HR	LR
Glc/Fru 6-phosphate	11.4	14.6	10.5	13.4
2/3-Phosphoglycerate	8.3	8.2	6.8	7.1
Phosphoenol pyruvate	4.9	4.5	3.3	3.3
6-Phosphogluconate	3.1	3.3	2.6	nd[a]
Pyruvate	5.1	nd[a]	1.1	1.0
Citrate	34	35	44.	45
2-Oxoglutarate	32	31	24	52
Succinate	31	21	25	31
Fumarate	11	4.6	5.8	120
Malate	31	34	31	110
3-Hydroxybutyrate	15	nd	3.6	nd
Methylsuccinic acid	20	28	3.5	52
Ala	6.4	6.3	9.8	10
Leu/Ile	11	42	9	19
Ser	3.7	3.8	2.4	nd
Thr	7.0	6.9	11	11
Pro	3.4	3.1	2.8	120
Glu	400	430	260	320
Gln	140	150	40	52
Val	3.7	11	7.9	36

Peak areas of $[M-H]^-$ ions of unlabeled peak A_{M0} and U-^{13}C-labeled peak A_{MUL} were determined at low mass resolution (LR, R = 800) and at high mass resolution (HR, R > 30,000), respectively.
[a] Not detectable.

Table 3
Evaluation of the isotope dilution method for LC–MS analysis of low-molecular-weight metabolites with an Orbitrap mass spectrometer at a mass resolution of 30,000

Metabolite	Observed linearity (A_{M0}:A_{MUL} ratio)	Concentration (μM)
Glucose + fructose 6-phosphate	0.25–10	2.6 ± 0.2
2/3-Phosphoglycerate	0.25–5	1.4 ± 0.1
Phosphoenol pyruvate	0.25–10	0.4 ± 0.1
6-Phosphogluconate	0.5–3	0.7 ± 0.1
Pyruvate	0.2–5	0.40 ± 0.02
Citrate	0.2–30	0.72 ± 0.03
2-Oxoglutarate	0.2–25	1.4 ± 0.1
Succinate	0.25–3	2.1 ± 0.2
Fumarate	0.04–5	0.7 ± 0.1
Malate	0.2–25	0.8 ± 0.1
3-Hydroxybutyrate	0.1–10	0.26 ± 0.03
Methylsuccinic acid	0.33–30	0.13 ± 0.01
Ala	0.5–5	7.7 ± 0.1
Leu + Ile	0.06–25	1.7 ± 0.1
Ser	0.04–8	0.8 ± 0.1
Thr	0.11–4	2.4 ± 0.3
Pro	0.2–8	1.5 ± 0.2
Glu	0.07–3	55.6 ± 1.2
Gln	0.1–50	11.8 ± 0.2
Val	0.25–3	1.0 ± 0.1

Cell extracts of *M. extorquens* AM1 grown on a uniformly labeled carbon source were mixed with naturally labeled standard compounds at various volume ratios. For each standard compound the corresponding concentration of the U-^{13}C-labeled compound was calculated by the role of proportion. A_{MUL}, area of U-^{13}C-labeled mass peak of the cell extract; A_{M0}, peak area of the monoisotopic mass peak of the standard compound.

olites, it was possible to measure all metabolites in the same run. These results show that liquid chromatography–high-resolution mass spectrometry with the LTQ Orbitrap is well suited to metabolite quantification in complex samples.

Application: comparison of pool sizes of central metabolites of M. extorquens AM1 under methylotrophic and nonmethylotrophic growth conditions

A quantitative metabolite analysis was applied to the model methylotrophic strain, *M. extorquens* AM1, after cultivation on the monocarbon source methanol and on the multicarbon source succinate. Growth on these carbon sources requires very different metabolic activities [24,25]; hence, different metabolic pool sizes can be expected [26]. Two independent cultures were grown in a bioreactor, one on each carbon source. Metabolites were extracted during midexponential growth at a biomass concentration of about 0.6 g L^{-1}. For each cultivation, at least five samples were taken consecutively, and for each sample the metabolic pools sizes were determined. The peak areas A_{M0} and A_{MUL}, as well as the A_{M0}:A_{MUL} area ratios of analyzed metabolites, were determined and validated to be in line with the requirements for adequate quantification. Fig. 2 shows the intracellular concentrations of organic acids and phosphorylated compounds involved in central metabolic pathways of *M. extorquens* AM1 during growth on methanol and on succinate. The two biological repetitions resulted in very similar intracellular concentrations, and the standard deviations obtained from at least five different samples were generally low. All metabolites could be quantified with high accuracy for growth on methanol. Since the ^{13}C-labeled cell extract was obtained from *M. extorquens* cells grown on methanol, peak areas from the samples and the U-^{13}C-labeled metabolites were very similar, as expected. During growth on succinate, intracellular succinate and fumarate could not be quantified since succinate was also present as a carbon source in the supernatant during the sampling procedure. As a consequence, the ionization of metabolites eluting at similar retention times was reduced, with some compounds (fumarate, methylsuccinate) being below the quantification limit. The quantification of malate was also critical during growth on succinate because its area A_{M0} was just above the upper boundary of the linear intensity range determined for this compound. The quantification of free intracellular amino acids revealed similar accuracy for cells grown on both carbon substrates, and the results showed precision and reproducibility similar to those obtained for organic acids and phosphorylated metabolites.

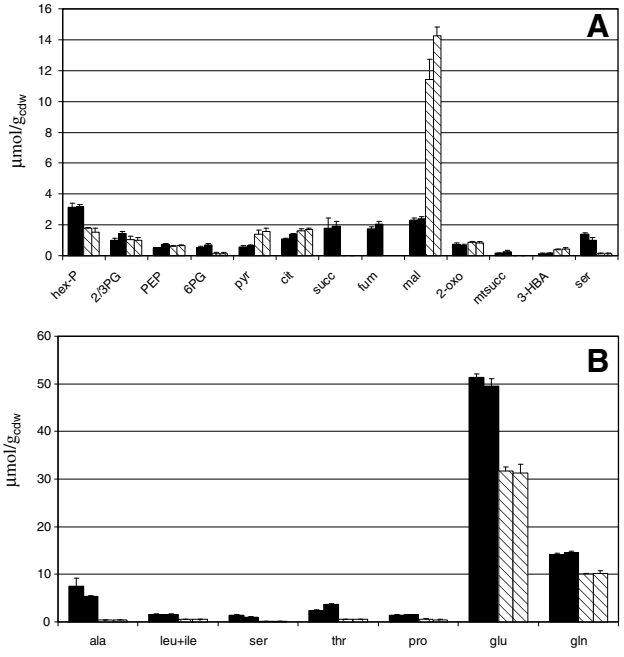

Fig. 2. Intracellular concentrations of central metabolites extracted from *M. extorquens* AM1 during growth on methanol (dark bars) and on succinate (hatched bars). For each carbon source two biological repetitions were carried out. Intracellular concentrations are given in micromoles per gram cell dry weight (cdw). To determine average and standard deviation for each metabolite five different samples per culture were analyzed. (A) Organic acids and phosphorylated compounds of central metabolism. (B) Amino acids. hex-P, hexose phosphate; 2/3PG, 2-phosphoglycerate and 3-phosphoglycerate; PEP, phosphoenol pyruvate; 6PG, 6-phosphogluconate; pyr, pyruvate; cit, citrate; succ, succinate; fum, fumarate; mal, malate; 2-oxo, 2-oxoglutarate; mtsucc, methylsuccinate; 3-HBA, 3-hydroxybutyrate.

Discussion

The first objective of this work was to assess the applicability of HR-MS for quantitative metabolomics. Metabolite determination using the LTQ Orbitrap was shown to be linear over 2–3 orders of magnitude for the metabolites considered in this investigation, which qualifies the LTQ Orbitrap for accurate quantification of intracellular metabolites, within a broad concentration range. Quantification of metabolites could be performed at trace levels (down to 4 pg), illustrating the high potential of the LTQ Orbitrap for quantitative metabolomics with high sensitivity.

The increased separation potential in the *m*/*z* dimension provided by HR-MS has three main benefits as regards quantitative metabolomics, including (i) application of single-MS quantification approaches, (ii) decreased chromatographic requirements, and (iii) increased potential for the application of the isotope dilution mass spectrometry method. Single LC–MS analysis at high mass resolution provides a number of advantages compared to quantification strategies based on tandem MS. The latter approach is inherently restricted to compounds for which specific fragments can be generated, which is not the case for a relatively large number of compounds of interest in metabolomics, including numerous central metabolites. With the Orbitrap, the selectivity is ensured by the high mass resolution, and quantification can be extended to compounds that generate no or nonspecific fragmentation. More specifically, the high resolution proved to be crucial to the smallest metabolites, for which tandem MS strategies cannot be easily applied. For instance, the analysis of organic acids could be performed with both specificity and sensitivity, since the limit of quantification was in the subpicomolar range.

A second advantage of HR-MS for quantitative metabolomics is the reduction in the chromatographic requirements, which can be profitably employed to reduce the analytical time. Basically, the high resolution power overcomes typical overlapping problems of low mass resolution instruments and minimizes also the risk of false identification. This is because the elemental composition can be assigned owing to high mass accuracy, limiting the requirement for LC method development. A clear benefit is the reduction in the analytical time, which can be profitably used to develop high-throughput quantitative methods. However, even with the limited number of metabolites that were investigated in this study, some overlapping problems remained between mass isomers. Such problems can be addressed uniquely in the chromatographic dimension, either by improving the method or by choosing alternative column chemistry. Therefore, for the purpose of quantitative metabolomics, a compromise has to be made between the chromatographic separation and the run time to optimize the number of metabolites that can be resolved and quantified properly.

Another important benefit of the LTQ Orbitrap originates from its mode of acquisition, which allows the analysis of a multitude of analytes over a broad *m*/*z* range without losing acquisition

speed. This characteristic proved to be useful for the application of the isotope dilution method [15]. The method was introduced to overcome the negative consequences of ionization suppression, since the U-^{13}C-labeled form of a metabolite is expected to behave exactly like its unlabeled form in the ionization source. In addition, the mixing of samples of interest with an extract that contains ^{13}C-labeled forms of a large number of metabolites drastically increases the number of m/z peaks to be detected and exploited. With the LTQ Orbitrap, high-resolution spectra can be collected at very high speed over the complete m/z range of the instrument, making possible the detection of both the labeled and the unlabeled forms of the analyte and the quantification of a very large number of compounds within the same run.

The present report shows that, even if the absolute quantitative performances are a little lower than those obtained with MS/MS strategies, high mass resolution analysis is promising for metabolite quantification on the metabolome scale based on single LC–MS strategies.

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References

[1] O. Fiehn, Metabolomics—the link between genotypes and phenotypes, Plant Mol. Biol. 48 (2002) 155–171.
[2] W.B. Dunn, Current trends and future requirements for the mass spectrometric investigation of microbial, mammalian and plant metabolomes, Phys. Biol. 5 (2008) 11001.
[3] K. Dettmer, P.A. Aronov, B.D. Hammock, Mass spectrometry-based metabolomics, Mass Spectrom. Rev. 26 (2007) 51–78.
[4] C.J. Bolten, P. Kiefer, F. Letisse, J.C. Portais, C. Wittmann, Sampling for metabolome analysis of microorganisms, Anal. Chem. 79 (2007) 3843–3849.
[5] J.D. Rabinowitz, E. Kimball, Acidic acetonitrile for cellular metabolome extraction from *Escherichia coli*, Anal. Chem. 79 (2007) 6167–6173.
[6] S.U. Bajad, W. Lu, E.H. Kimball, J. Yuan, C. Peterson, J.D. Rabinowitz, Separation and quantitation of water soluble cellular metabolites by hydrophilic interaction chromatography–tandem mass spectrometry, J. Chromatogr. A 1125 (2006) 76–88.
[7] L. Gu, A.D. Jones, R.L. Last, LC-MS/MS assay for protein amino acids and metabolically related compounds for large-scale screening of metabolic phenotypes, Anal. Chem. 79 (2007) 8067–8075.
[8] K.L. Ross, T.T. Tu, S. Smith, J.J. Dalluge, Profiling of organic acids during fermentation by ultraperformance liquid chromatography–tandem mass spectrometry, Anal. Chem. 79 (2007) 4840–4844.
[9] J.C. van Dam, M.R. Eman, J. Frank, H.C. Lange, G.W.K. van Dedem, S.J. Heijnen, Analysis of glycolytic intermediates in *Saccharomyces cerevisiae* using anion exchange chromatography and electrospray ionization with tandem mass spectrometric detection, Anal. Chim. Acta 460 (2002) 209–218.
[10] H. Schlichtherle-Cerny, M. Affolter, C. Cerny, Hydrophilic interaction liquid chromatography coupled to electrospray mass spectrometry of small polar compounds in food analysis, Anal. Chem. 75 (2003) 2349–2354.
[11] V.V. Tolstikov, O. Fiehn, Analysis of highly polar compounds of plant origin: combination of hydrophilic interaction chromatography and electrospray ion trap mass spectrometry, Anal. Biochem. 301 (2002) 298–307.
[12] A. Makarov, E. Denisov, A. Kholomeev, W. Balschun, O. Lange, K. Strupat, S. Horning, Performance evaluation of a hybrid linear ion trap/orbitrap mass spectrometer, Anal. Chem. 78 (2006) 2113–2120.
[13] S.J. Van Dien, M.E. Lidstrom, Stoichiometric model for evaluating the metabolic capabilities of the facultative methylotroph *Methylobacterium extorquens* AM1, with application to reconstruction of C(3) and C(4) metabolism, Biotechnol. Bioeng. 78 (2002) 296–312.
[14] M.M. Attwood, W. Harder, A rapid and specific enrichment procedure for *Hyphomicrobium spp*, Antonie Van Leeuwenhoek 38 (1972) 369–377.
[15] L. Wu, M.R. Mashego, J.C. van Dam, A.M. Proell, J.L. Vinke, C. Ras, W.A. van Winden, W.M. van Gulik, J.J. Heijnen, Quantitative analysis of the microbial metabolome by isotope dilution mass spectrometry using uniformly ^{13}C-labeled cell extracts as internal standards, Anal. Biochem. 336 (2005) 164–171.
[16] C. Wittmann, J.O. Kromer, P. Kiefer, T. Binz, E. Heinzle, Impact of the cold shock phenomenon on quantification of intracellular metabolites in bacteria, Anal. Biochem. 327 (2004) 135–139.
[17] C. Zorb, G. Langenkamper, T. Betsche, K. Niehaus, A. Barsch, Metabolite profiling of wheat grains (Triticum aestivum L.) from organic and conventional agriculture, J. Agric. Food Chem. 54 (2006) 8301–8306.
[18] X. Huang, F.E. Regnier, Differential metabolomics using stable isotope labeling and two-dimensional gas chromatography with time-of-flight mass spectrometry, Anal. Chem. 80 (2008) 107–114.
[19] P. Kiefer, C. Nicolas, F. Letisse, J.C. Portais, Determination of carbon labeling distribution of intracellular metabolites from single fragment ions by ion chromatography tandem mass spectrometry, Anal. Biochem. 360 (2007) 182–188.
[20] A. Kamleh, M.P. Barrett, D. Wildridge, R.J. Burchmore, R.A. Scheltema, D.G. Watson, Metabolomic profiling using Orbitrap Fourier transform mass spectrometry with hydrophilic interaction chromatography: a method with wide applicability to analysis of biomolecules, Rapid Commun. Mass Spectrom. 22 (2008) 1912–1918.
[21] A.J. Alpert, Hydrophilic-interaction chromatography for the separation of peptides, nucleic-acids and other polar compounds, J. Chromatogr. 499 (1990) 177–196.
[22] M. Krauss, J. Hollender, Analysis of nitrosamines in wastewater: exploring the trace level quantification capabilities of a hybrid linear ion trap/orbitrap mass spectrometer, Anal. Chem. 80 (2008) 834–842.
[23] S.C. Brown, G. Kruppa, J.L. Dasseux, Metabolomics applications of FT-ICR mass spectrometry, Mass Spectrom. Rev. 24 (2005) 223–231.
[24] M. Laukel, M. Rossignol, G. Borderies, U. Völker, J.A. Vorholt, Comparison of the proteome of *Methylobacterium extorquens* AM1 grown under methylotrophic and nonmethylotrophic conditions, Proteomics 4 (2004) 1247–1264.
[25] Y. Okubo, E. Skovran, X. Guo, D. Sivam, M.E. Lidstrom, Implementation of microarrays for *Methylobacterium extorquens* AM1, OMICS 11 (2007) 325–340.
[26] X. Guo, M.E. Lidstrom, Metabolite profiling analysis of *Methylobacterium extorquens* AM1 by comprehensive two-dimensional gas chromatography coupled with time-of-flight mass spectrometry, Biotechnol. Bioeng. 99 (2008) 929–940.