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Quantitative Evaluation of Intracellular Metabolite Extraction Techniques for Yeast Metabolomics

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Accurate determination of intracellular metabolite levels requires well-validated procedures for sampling and sample treatment. Several methods exist for metabolite extraction, but the literature is contradictory regarding the adequacy and performance of each technique. Using a strictly quantitative approach, we have re-evaluated five methods (hot water, HW; boiling ethanol, BE; chloroform−methanol, CM; freezing-thawing in methanol, FTM; acidic acetonitrile−methanol, AANM) for the extraction of 44 intracellular metabolites (phosphorylated intermediates, amino acids, organic acids, nucleotides) from *S. cerevisiae* cells. Two culture modes were investigated (batch and chemostat) to check for growth condition dependency, and three targeted platforms were employed (two LC-MS and one GC/MS) to exclude analytical bias. Additionally, for the determination of metabolite recoveries, we applied a novel approach based on addition of ^{13}C-labeled internal standards at different stages of sample processing. We found that the choice of extraction method can drastically affect measured metabolite levels, to an extent that for some metabolites even the direction of changes between growth conditions can be inverted. The best performances, in terms of efficacy and metabolite recoveries, were achieved with BE and CM, which yielded nearly identical levels for the metabolites analyzed. According to our results, AANM performs poorly in yeast and FTM cannot be considered adequate as an extraction method, as it does not ensure inactivation of enzymatic activity.

The ability to accurately determine intracellular concentrations of metabolites is of key importance in studying signaling and metabolic reaction networks and their regulation in vivo. With the dawn of the metabolomics era, mainly fueled by the development of sensitive high-throughput MS-based analytics, metabolome-wide analysis is set to become reality. However, despite half a century of experience in the measurement of intracellular metabolites, the procedures used for preparation of the biological samples remain an issue.[1−5] It is increasingly recognized that, whether for semiquantitative or for quantitative purposes, the accuracy and reliability of the results are to a large extent determined by the first steps of sample treatment, namely rapid sampling, quenching of metabolic activity, separation of extracellular medium (when applicable), and metabolite extraction. We have recently addressed the problem of metabolite leakage from yeast cells during cold methanol quenching.[1] Here, we approach the main issues regarding the performance of extraction methodologies for microbial metabolomics, with a focus on *S. cerevisiae*.

There are three essential requirements that an ideal metabolite extraction method must fulfill:[6,7] (i) completeness of extraction, to ensure all intracellular pools are made entirely accessible for analysis, (ii) prevention of metabolite conversion during the extraction or subsequent steps, mainly by ensuring effective inactivation of enzymes, and (iii) absence of extensive degradation of metabolites by the procedure itself. With respect to this last requirement, partial losses may be acceptable if the results can be corrected by means of metabolite-specific recovery factors[6,7] or through the use of adequate internal standards (e.g., isotope based).[8−10] Theoretically, completeness of extraction cannot be directly verified, since one does not know a priori how much of each metabolite is initially present in the cells. Instead, completeness can be evaluated by comparing the ability of different methods to release metabolites from identical biological samples, which we shall refer to as efficacy. The absence of enzyme activity and the extent of metabolite degradation can both be tested by determining metabolite recoveries, traditionally by means of spiking or standard additions. Metabolite-specific recoveries much below 100% typically indicate degradation, while mixed recoveries above and below 100% for different metabolites would indicate interconversion.

Extraction can be achieved using high temperature, extreme pH, organic solvents, mechanical stress, or combinations of these.

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Some of the most well-known extraction methods, such as perchloric acid (PCA),[11,12] hot water (HW),[13,14] and boiling ethanol (BE),[15,16] have been employed at least since the 1950s. Eventually, the perceived need to adopt less aggressive procedures to prevent metabolite degradation seems to have steered researchers into the development of milder extraction methods. In the 1990s, a neutral low-temperature technique based on a two-phase liquid extraction with chloroform−methanol (CM) was introduced,[6] presumably adapted from a method for lipid extraction from tissues.[17] More recently, two other methods have been proposed, one based on freezing-thawing cycles in methanol (FTM),[18] another using acidic acetonitrile−methanol (AANM).[19]

Given the variety of extraction methods available, how does one decide which method to use? To demonstrate the complexity of this choice, we have assembled a complete (to the best of our knowledge) survey of method comparisons in the literature, which we present in Table 1. As this overview shows, each of the methods is considered very good, or best, by some studies and poor, or inadequate, by others (with the exception of AANM which was evaluated only once). Since method comparisons have been performed in different species, when comparing them, one should keep in mind that some aspects of extraction performance can be organism dependent. In particular, completeness of extraction may be microbe specific, since some types of cells are more susceptible to lysis than others (e.g., eukaryotes vs prokaryotes, gram-positive vs gram-negative). Enzyme resistance to inactivation may also be organism dependent; although among mesophile species, one would expect it to be comparable. As for metabolite stability, it should be nearly independent of the type of cell extracted. Although these factors must be considered, they seem unlikely to change the performance of a particular method from bad to best, nor can they explain the discrepancies found within the same or closely related species. Another observation from the overview in Table 1 is the prominence of mild extractions in recent literature. Five out of the last eight studies consider FTM the best extraction method. Although cold methanol is known to release intracellular metabolites,[1,5,20] it is less clear whether enzyme activity can be effectively inactivated, especially if one considers that enzyme activities can be assayed at temperatures below 0 °C in 70% methanol.[21,22] Furthermore, of the 15 studies examined, 5 did not determine metabolite recoveries, without which it is not possible to check for metabolite conversion or degradation, and 8 investigated only 2−3 extraction methods, which limits the conclusions that can be drawn in terms of efficacy. In addition, all but 3 studies examined relatively small sets of compounds of very similar molecular properties. Finally, 5 of the last 8 studies based their evaluation on qualitative or semiquantitative measures. Qualitative measures (e.g., number of GC peaks) bear little or no information on the fulfillment of the essential requirements, while the use of semiquantitative measures (e.g., peak area or height, normalized intensities) generally implies the assumption of linearity of response and absence of matrix effects, which often is not valid for MS-based analysis of complex samples such as cell extracts.[10]

The purpose of this study was to carry out an objective and quantitative re-evaluation of available extraction methods. The model organism *S. cerevisiae* was used, for which we have previously validated sampling and quenching procedures.[1,23] Of

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Table 1. Survey of Comparisons of Extraction Procedures for Intracellular Metabolite Analysis in Microorganisms

		authors' evaluation, per extraction method[a]							
source	microrganism(s)	PCA	KOH	HW	BE	CM	FTM	AANM	notes
Bagnara and Finch, 1972[55]	*E. coli*	+++		+					Quantitative, TLC, 6 metabolites (nucleotides), no recoveries. Tested 1 other method (TCA).
Lundin and Thore, 1975[45]	5 bacteria species	++	+++	+++	+++				Quantitative, enzymatic analysis, 3 metabolites (ATP, ADP, AMP), recoveries by spiking. Tested 6 other methods.
Larsson and Olsson, 1979[46]	4 algae species	+++		++	++				Quantitative, enzymatic analysis, 3 metabolites (ATP, ADP, AMP), no recoveries. Tested 3 other methods.
de Koning and van Dam, 1992[6]	*S. cerevisiae*	+++				+++			Quantitative, enzymatic analysis, up to 13 metabolites (data not shown), recoveries by spiking (only CM).
Gonzalez et al., 1997[7]	*S. cerevisiae*	++			+++				Quantitative, enzymatic analysis, 6 metabolites, recoveries by spiking.
Hajjaj et al., 1998[25]	*Monascus ruber*	++	++		+++				Quantitative, enzymatic analysis, 2−6 metabolites, recoveries by spiking.
Hans et al., 2001[31]	*S. cerevisiae*			+++	+++				Quantitative, HPLC, 17 metabolites (amino acids), recoveries by spiking (only BE).
Maharjan and Ferenci, 2003[18]	*E. coli*	+	+		++	+	++		2D-TLC, semiquantitative for efficacies (total extract intensity and relative intensities for 13 metabolites), no recoveries. Tested 1 other method (hot methanol).
Jernejc, 2004[56]	*A. niger*	+++	+++		++				Quantitative, enzymatic analysis, 3 metabolites (organic acids), recoveries by stability in solution.
Villas-Boas et al., 2005[24]	*S. cerevisiae*	+	+		+	++	++		GC/MS, qualitative for efficacies (number of peaks detected), quantitative for recoveries, 27 metabolites, by spiking.
Wang et al., 2006[44]	*E. coli*		++		+		+++		Qualitative, ESI-MS (richness and reproducibility of mass spectra), no recoveries.
Hiller et al., 2007[47]	*E. coli*	+		+++	++				Quantitative, enzymatic analysis, 7 metabolites, recoveries by standard additions.
Rabinowitz and Kimball, 2007[19]	*E. coli*					+	+	+++	LC-MS, semiquantitative for efficacies (peak heights for nearly 100 metabolites) and recoveries, by spiking (peak heights for 12 metabolites). Tested 2 other methods (and some variations).
Faijes et al., 2007[43]	*Lactobacillus plantarum*	+++			+++	++	+++		Quantitative, enzymatic analysis, 3 metabolites, recoveries by spiking (only FTM). Tested 1 other method (chloroform−water).
Winder et al., 2008[42]	*E. coli*	+	+		+++	+++	+++		GC/MS, qualitative (number of peaks detected) and semiquantitative (relative abundance of 21 metabolites) for efficacies, no recoveries.

[a] Legend: +, poor/bad; ++, fair; +++, good; underlined, best among the methods tested. Some variations in extraction time, buffers, solvent concentrations, and temperatures are considered within the same method.

the techniques in Table 1 only acid (PCA) and base (KOH) extractions were not included, because their salt-rich extracts present limitations for our analytical platform (e.g., ion suppression). These methods have also seen relatively little use in recent years, due to reported near-total losses of specific coenzymes.[7,24,25] We have based our evaluation only on strictly quantitative data of metabolite concentrations in cell extracts. Samples were obtained under well-defined, reproducible culture conditions using optimized sampling procedures. Two different growth states were investigated (glucose limitation and excess), to check for growth condition dependency. Three analytical methods (GC/MS and two LC-MS/MS) were employed, to exclude analytical bias, covering a total of 44 metabolites of different classes and molecular properties (phosphorylated intermediates, organic acids, amino acids, nucleotides). For the determination of metabolite recoveries, we introduced and applied a novel approach based on differential addition of ^{13}C-labeled internal standards at the beginning and end of the sample treatment.

We are confident that the outcomes of this work will serve researchers in the microbial metabolomics community by providing an improved assessment of the extraction techniques at their disposal. The experimental strategy employed, including the new ^{13}C-based approach for determining recoveries, should also be useful as a framework for systematic validation of these, and newer methods, in other microorganisms.

EXPERIMENTAL PROCEDURES

Solvents and Chemicals. HPLC-grade solvents were from J.T. Baker. Analytical grade standards were from Sigma-Aldrich.

Strain and Medium. The strain used was the prototrophic haploid *Saccharomyces cerevisiae* CEN.PK 113-7D (MATa).[26] Strain stocks were kept in 20% glycerol at −80 °C. A new low-salt minimal medium was designed, for sustaining a biomass concentration of 1.5−4.5 g_{DW}/L. Ensuring low residual concentrations of salts minimizes interferences in the analysis (e.g., peak-shifting, ion suppression) by ions carried over in samples, in case of limited washing.[1] No change in morphology or physiology has been observed in the cells compared to growth on standard salt-rich medium.[27,28] The composition of the optimized medium was, per liter: 0.3 g of $(NH_4)_2SO_4$, 3 g of $NH_4H_2PO_4$, 0.3 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 1 g of vitamin solution,[29] and 1 g of trace element solution.[28] As carbon source, the medium contained 7.5 g/L glucose, and as antifoam, it contained 0.05 g/L silicone antifoaming agent (BDH, U.K.). The final pH was adjusted to 5 with KOH.

Cultivation Conditions. Cultivations were performed aerobically in a 7 L fermentor (Applikon, The Netherlands) equipped with a DCU3 controller and continuous data acquisition via MFCS (B. Braun Biotech, Germany). A working volume of 4 L was maintained by weight control. The cultivations were carried out at 30 °C, pH 5.0 (via addition of 2 M KOH), with 0.3 bar overpressure, an aeration rate of 0.5 vvm (120 L/h), and a stirrer speed of 500 rpm. Base utilization and effluent accumulation were monitored by weight. Oxygen and CO_2 fractions in dry exhaust gas and air were measured using a combined paramagnetic/infrared analyzer (NGA 2000, Rosemount, U.S.) via a MUX multiplexer unit (B. Braun Biotech, Germany). Precultures were carried out in unbaffled 500 mL conical flasks on 100 mL of minimal medium as described above, adjusted to pH 6, and inoculated with 2 mL strain stocks. These were grown overnight in a rotary shaker, at 30 °C, 200 rpm, and then used to inoculate the fermentor. Two growth conditions were investigated: chemostat and batch. The glucose-limited chemostat culture was carried out at a dilution rate of 0.1 h^{-1}. Sampling took place after 6 residence times (60 h) of steady-state growth with constant dissolved oxygen and off-gas readings, at a biomass concentration of 3.7 g_{DW}/L. For the batch culture, the medium was supplemented before inoculation with a sterile concentrated glucose solution, for an initial concentration of 30 g/L. Sampling took place in exponential glucose-consumption phase, 11 h after inoculation, at a biomass concentration of 3.2 g_{DW}/L. Biomass dry weight concentration was determined gravimetrically on membrane disk filters (PES, 0.45 μm, Pall, U.S.) dried for 48 h at 70 °C. Preweighed filters were loaded with 5−10 g of broth and washed with 3 volumes of demineralized water.

Samples for Intracellular Metabolites. For an overview of the experimental design, see Figure 1. Samples were taken using a custom-made low dead-volume rapid sampling setup,[23] and leakage-free quenching was performed according to Canelas et al.[1] Briefly, 1.2 g (±0.06) of broth was withdrawn (<1 s) into 5 mL of pure methanol at −40 °C, the mixture was quickly vortexed (≈1 s) and placed back in the cryostat (−40 °C) (Lauda, Germany). Extracellular medium was separated by centrifugation (5000*g*, 5 min, −20 °C, rotor precooled to −40 °C). No additional washing step was performed. After decanting, 200 μL of U-^{13}C-labeled cell extract was added to half of the samples as internal standard (IS).[8,30] Each of the 5 different extractions was performed in quadruplicate: two samples with ^{13}C-labeled IS (named AQ: after quenching) and two samples without (BA, see below) (Figure 1).

Metabolite Extraction Methods. The procedures for each extraction method were adapted, in some cases with minor simplifications, from the most relevant literature sources. For HW, BE, CM, and FTM, these were the protocols most commonly used for *S. cerevisiae*. In the case of AANM, for which there is yet no source in the literature referring to its application to yeast cells, we employed the protocol that gave optimal results in *E. coli*. The details of each procedure are described individually below.

Hot Water (HW) Extraction. This procedure was adapted from refs 4 and 31−34. Tubes containing 2 mL of demineralized water were preheated in a water bath at 95−100 °C for 6 min. Then, each sample was taken from the cryostat; the hot water was quickly poured over the cell pellet; the mixture was immediately vortexed, and the sample was placed in the water bath. After 15 min, each tube was placed on ice, and the extracts were stored at −80 °C until further use.

Boiling Ethanol (BE) Extraction. This procedure was adapted from refs 7, 23, 31, and 35. Tubes containing 5 mL of 75% ethanol were preheated in a water bath at 95 °C for 5 min. Then, each sample was taken from the cryostat; the boiling ethanol was quickly poured over the cell pellet; the mixture was immediately vortexed, and the sample was placed in the water bath. After 3 min, each tube was transferred back to the cryostat (−40 °C), and the extracts were stored at −80 °C until further use.

Chloroform−Methanol (CM) Extraction. This procedure was adapted from refs 6, 24, 36, and 37. Throughout the procedure,

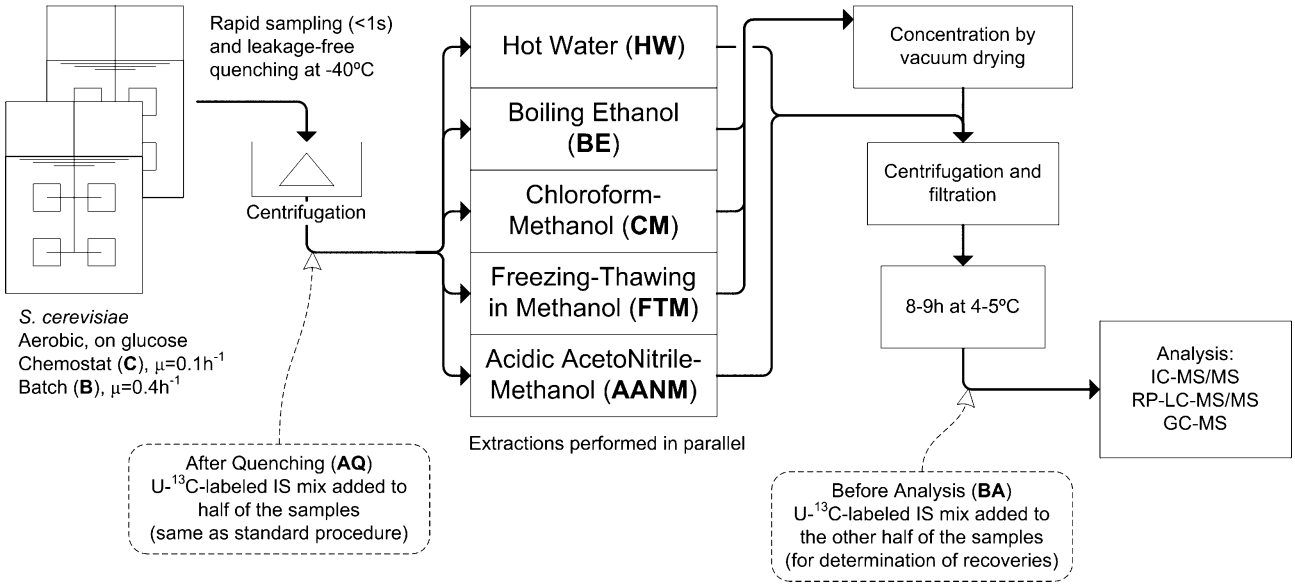

Figure 1. Schematic representation of the experimental design.

the temperature of samples and solutions was maintained as close as possible to −40 °C. Each sample was resuspended in 2.5 mL of precooled 50% (v/v) aqueous methanol, after which 2.5 mL of precooled chloroform was added. All samples were then vigorously shaken for 45 min with an orbital shaker, using a custom-made tube adaptor, inside a −40 °C freezer (temperature never above −32 °C). The samples were then centrifuged (5000*g*, 5 min, −20 °C, rotor precooled to −40 °C); the upper water/methanol phases were collected separately, and the lower layers were reextracted with 2.5 mL precooled 50% (v/v) methanol by vortexing for 30 s. After centrifugation, the upper phases were pooled with the first extracts, and the combined extracts were stored at −80 °C until further use.

Freezing-Thawing in Methanol (FTM) Extraction. This procedure was adapted from refs 18, 24, and 38. Each sample was resuspended in 2.5 mL of 50% (v/v) aqueous methanol precooled to −40 °C; then, the resulting solution was frozen in liquid nitrogen for 3−5 min and thawed on ice for 3−5 min. After three freeze−thaw cycles, the samples were centrifuged (5000*g*, 5 min, −20 °C); the supernatants were collected separately, and the pellets were reextracted with 2.5 mL precooled 50% (v/v) methanol by vortexing for 30 s. After centrifugation, the supernatants were pooled with the first extracts, and the combined extracts were stored at −80 °C until further use.

Acidic Acetonitrile−Methanol (AANM) Extraction. This procedure was adapted from ref 19. Each sample was resuspended in 1 mL of precooled (−20 °C) acidic acetonitrile/methanol/water (40:40:20 v/v, containing 0.1 M formic acid) solution and placed in a cryostat at −20 °C for 15 min. The samples were then centrifuged (5000*g*, 5 min, 4 °C); the supernatants were collected separately, and the pellets were reextracted twice with 200 μL of ice-cold AANM solution, each time by vortexing for 20 s. Finally, the pooled extracts (1.4 mL) were neutralized with 100 μL of 1.4 M NH_4OH and stored at −80 °C until further use.

Sample Concentration and Conditioning. After thawing, BE, CM, and FTM extracts were evaporated under vacuum (120 min, 30 °C, <10 mbar) using a RapidVac (Labconco, U.S.). Dried residues of samples with ^{13}C-labeled IS (AQ) and without (BA) were resuspended in 600 μL or 400 μL of Milli-Q water, respectively. All samples, including the HW and AANM extracts, were then centrifuged (15 000*g*, 5 min, 4 °C) to remove cell debris. The supernatants were transferred to filter caps (Ultrafree, PVDF, 0.22 μm, Millipore) and centrifuged again, and the filtrates were collected and stored at −80 °C. Finally, to simulate the effect of typical waiting times during an analysis run, all samples were thawed and left to stand in the refrigerator (4−5 °C) for 8−9 h. At the end of this period, 200 μL of ^{13}C-labeled IS was added to the half of the samples which had not received it yet (named BA: before analysis), and all samples were placed back at −80 °C until analysis.

Metabolite Analysis. The concentrations of glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), glucose-1-phosphate (G1P), mannose-6-phosphate (M6P), fructose-1,6-bisphosphate (FBP), trehalose-6-phosphate (T6P), 6-phospho-gluconate (6PG), 3-phosphoglycerate + 2-phosphoglycerate (3PG), phosphoenolpyruvate (PEP), glycerol-3-phosphate (G3P), citrate + isocitrate (Citrate), oxoglutarate, succinate, fumarate, malate, sedoheptulose-7-phosphate (S7P), and UDP-glucose were determined by anion-exchange LC-MS/MS.[39] The concentrations of AMP, ADP, ATP, UMP, UDP, UTP, GDP, and CDP were determined by ion-pair reverse-phase LC-MS/MS.[40]

The concentrations of 19 amino acids were determined by GC/MS using the EZ:Faast Free Amino Acid Kit (Phenomenex, U.S.). Extraction and derivatization of the amino acids were carried out according to the instructions of the manufacturer. Briefly, after a solid-phase extraction (SPE) step, the amino acids are simultaneously derivatized in an aqueous medium on the amino- and carboxyl group with *n*-propyl chloroformate in the presence of *n*-propanol and 3-picoline, resulting in *n*-propoxycarbonyl *n*-propylesters. Owing to the SPE step and the high specificity of the derivatization reagent propyl chloroformate, sample matrix effects such as high salt and buffer concentrations are limited and other components as sugars and organic acids do not interfere with the measurement. The derivatized extracts were analyzed with a Trace GC Ultra equipped with a programmed temperature vaporizer (PTV) injector and autosampler AI 3000 which was

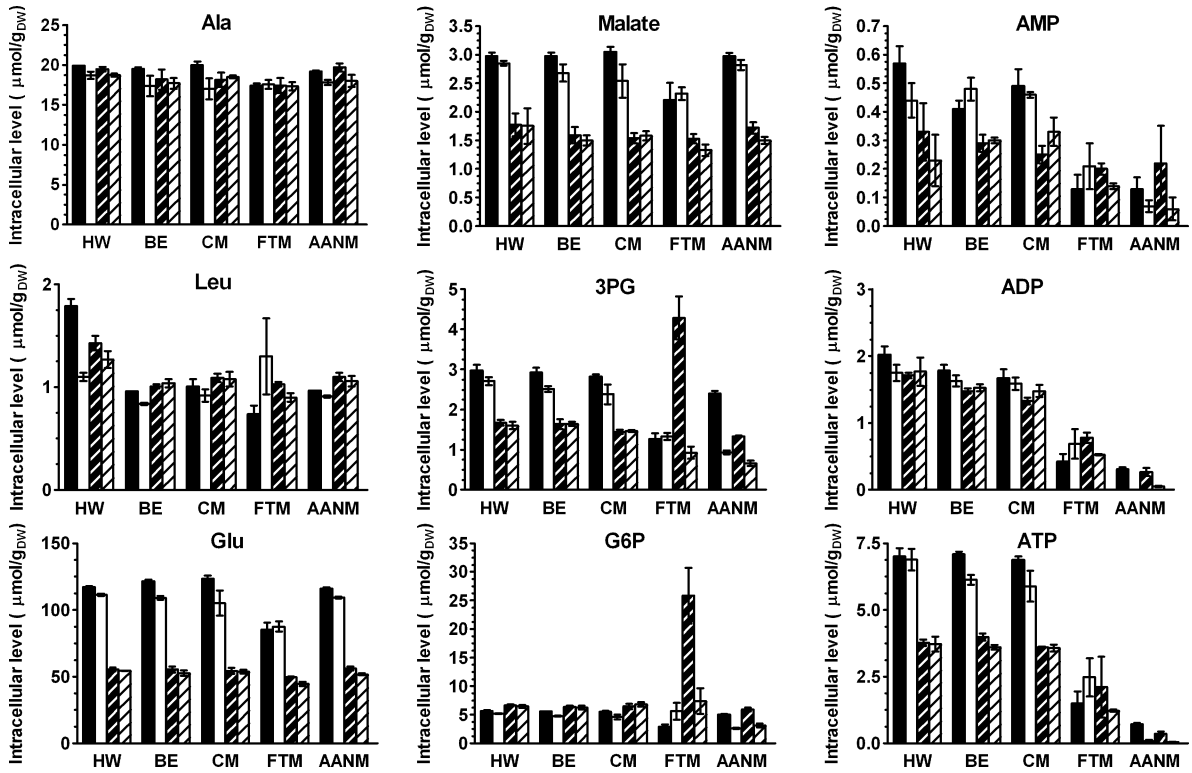

Figure 2. Intracellular levels of representative metabolites, obtained by extraction of cells from two growth conditions using five extraction methods (see Figure 1 for the experimental design). Data are averages and standard deviations of duplicate samples each analyzed twice. Legend: dark, AQ samples; light, BA samples; solid, chemostat; striped, batch. Comparison of dark and light bars indicates the effect of the sample treatment procedure, including the extraction, on the metabolite levels. Comparison of the solid and striped bars shows the differences in metabolite levels related to the growth condition.

directly coupled to a Trace DSQ single quadrupole mass spectrometer with an electron ionization source (Thermo Finnigan, U.S.). One microliter of derivatized sample was injected on a ZB-AAA column (medium polarity fused silica capillary column 10 m $\times$ 0.25 mm ID with a film thickness of 0.25 μm). The PTV injector, equipped with a glass liner with, carbofrit, was set in PTV-splitless mode with an initial temperature of 45 °C and a splitless time of 1 min. The temperature of the PTV was raised at 14.5 °C/s to 280 °C, held for 5 min, and subsequently raised at 14.5 °C/s to 300 °C. The GC temperature was initially set at 45 °C and then raised at 30 °C/min to 320 °C and held for 1 min. Helium was used as carrier gas with a gas flow of 1.5 mL/min. The transfer line to the MS was set to 250 °C and the ion source to 240 °C. The electron ionization was operated with 70 eV. For quantitative measurements, the MS was used in selected ion monitoring (SIM) mode.

All analyses were performed in duplicate. U-^{13}C-labeled cell extract was added as internal standard to both samples and calibration standards for IDMS-based quantification.[8,30]

RESULTS AND DISCUSSION

Whether for Quantitative or for Qualitative Purposes, the Choice of Extraction Method can Affect the Results. An objective evaluation of intracellular metabolite extraction methods must be based primarily on the fulfillment of three essential criteria: completeness of extraction, prevention of interconversion (e.g., due to enzymatic activity), and absence of extensive degradation (e.g., chemical, thermal). Here, we have evaluated these criteria using a strictly quantitative approach, that is, based on the absolute determination of metabolite levels in cell extracts obtained using different extraction techniques.

In these experiments, we have employed a novel approach for determination of metabolite recoveries, based on the addition of ^{13}C-labeled IS at the beginning and end of the sample treatment (see Figure 1). For samples named AQ, ^{13}C-labeled IS was added right after quenching (as is standard procedure), while for samples named BA, it was added just before analysis. Thus, in BA samples, the ^{13}C-labeled IS will only correct for analytical artifacts (e.g., sample matrix effects), while in AQ samples, it will also compensate for losses during sample treatment (e.g., volume losses, partial degradation). Levels in AQ samples provide a measure of the ability of each extraction method to release metabolites from the cells, independent of their stability during treatment, that is, its efficacy. By comparing levels in BA and AQ samples, we can determine the change in concentration of each metabolite over the entire sample treatment, that is, its recovery.

In this respect, note that we are determining an overall process recovery, including extraction, concentration, and a conditioning period at 4−5 °C which was meant to simulate the effect of the time samples spend waiting for analysis (see Figure 1). The reason for doing so is that the extraction method applied determines the extent of changes in metabolites not only during the extraction itself but also potentially further down the sample treatment until the actual point when the sample is injected in the LC or GC (for example, if enzymatic activity is not fully inactivated). By determining overall process recoveries, all such changes are taken into account.

In Figure 2, we present the measured metabolite levels in samples extracted with each of the 5 methods tested, under the two growth conditions investigated (chemostat and batch), for a set of 9 representative metabolites (of the 44 analyzed) chosen to cover a wide range of molecular weight (from <100 to >500 Da), abundance (from <1 to >100 μmol/g_{DW}), functional class (amino acids, organic acids, phosphorylated intermediates, nucleotides) and analytical method used (3 each). From these data, it is clear that different extraction techniques can indeed result in substantial differences in the measured metabolite levels. Overall, the CM, BE, and HW methods seem to yield comparable metabolite levels, while AANM stands out with somewhat lower levels for the phosphorylated compounds, and FTM can result in both higher and lower levels, depending on the metabolite and growth condition. Furthermore, even for experiments where the absolute metabolite levels are not required but only a qualitative impression of their changes, the choice of extraction method can still drastically influence the results. This can be seen for Leu, 3PG, G6P, and the nucleotides in Figure 2, for which even the direction of change observed between growth conditions (glucose excess vs glucose limitation) depends on the extraction technique used.

Comparison of Extraction Efficacy. As a measure of a method's ability to release metabolites from the cells, we have defined extraction efficacy, versus the maximum among the five techniques, for metabolite x under extraction method i, as

$$\text{efficacy(vs max)}_{x,i} = \frac{[x]_i^{\text{AQ}}}{\max[x]^{\text{AQ}}} \quad (1)$$

However, it became clear that in certain situations an extraction method might yield higher metabolite concentrations than the others; if, for example, remaining substrate is converted to intermediates or macromolecules are broken down, in which case, the efficacy of all other methods would appear, by comparison, misleadingly low. If so, an alternative measure would be to calculate an efficacy versus the median, rather than the maximum, which we defined as

$$\text{efficacy(vs median)}_{x,i} = \frac{[x]_i^{\text{AQ}}}{\text{median}[x]^{\text{AQ}}} \quad (2)$$

Note that these efficacy factors are comparative measures of effectiveness, so the results do depend on the set of techniques investigated. However, by comparing a sufficiently varied set of methods, based on different extraction principles and separately optimized for optimal release of metabolites, it seems safe to assume that at least one of them, if not more, will completely release any given metabolite. In this case, efficacies may be treated as near-absolute measures.

The extraction efficacies of all 44 metabolites analyzed, for both experiments, are plotted, for each extraction method, in Figure 3. The efficacy profiles for HW, BE, and CM are all very similar, with efficacies near 1 for most of the metabolites. The profile for AANM is similar to the former for a wide range of metabolites but shows a clear discrimination of the larger, more polar metabolites (e.g., di- and triphosphate compounds), for which efficacies drop significantly. Finally, the profile for FTM is the most scattered, with efficacies below 1 for a large number of metabolites, as well as exceptionally high efficacies for a small subset of compounds in the batch experiment. A clear bias is also observed, as with AANM, toward lower extractability of the larger, more polar compounds.

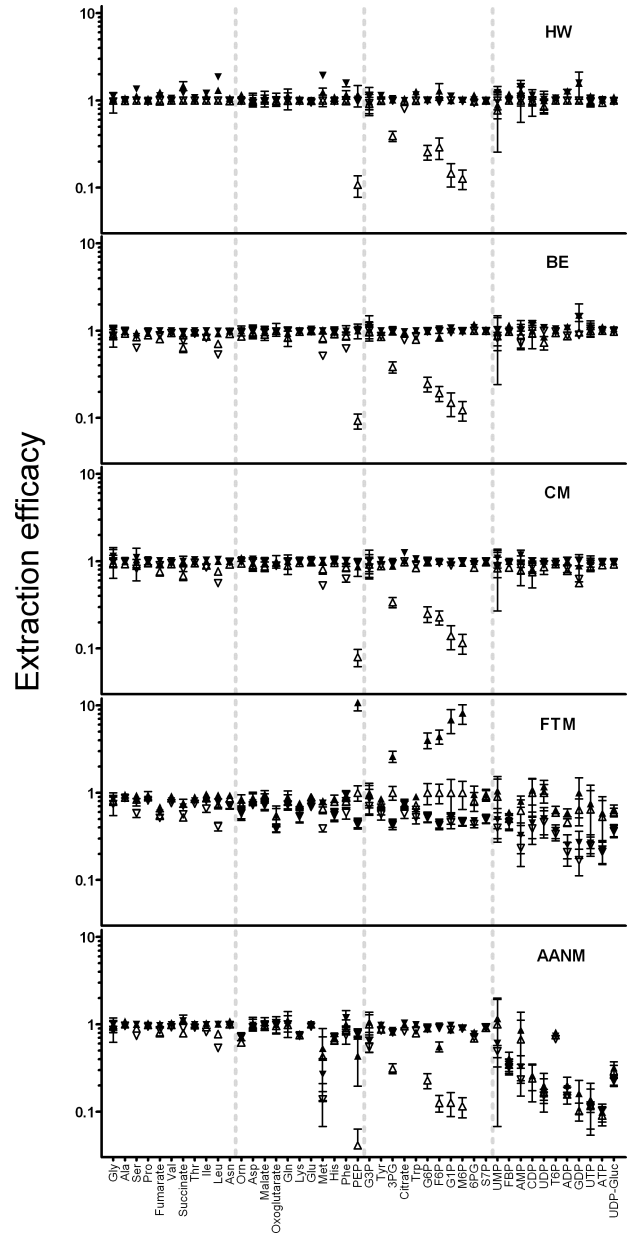

Figure 3. Extraction efficacies for all 44 metabolites analyzed, in order of increasing molecular weight, for each of the extraction methods, under both growth conditions (see Results and Discussion for definitions of efficacy vs max and efficacy vs median). Data are averages and standard deviations of duplicate samples each analyzed twice. Legend: ▽ and △, efficacy vs max in chemostat and batch, respectively; ▼ and ▲, efficacy vs median in chemostat and batch, respectively. Gray dashed vertical lines are for guidance only.

The uniformity of the results with HW, BE, and CM strongly indicates that these three methods achieve near-complete extraction of all metabolites analyzed. The only remarkable exceptions are six metabolites (PEP, 3PG, G6P, F6P, G1P, and M6P) which were found at much higher levels in FTM extracts in the batch experiment. Strikingly, the very same metabolites are found at comparatively lower levels in the chemostat experiment. Further-

more, in both growth conditions, the FTM method achieved overall poor efficacies, so it seems implausible that the difference lies in metabolite extractability. Rather, the fact that all six metabolites are fast-turnover glycolytic intermediates and their levels are comparatively higher in the condition of glucose-excess, but not glucose-limitation, points at the possibility of metabolite formation from the substrate (the ^{13}C-labeled IS would not correct for that). Furthermore, the metabolites involved imply that at least the whole glycolysis is active. This hypothesis is strengthened by the evidence of more widespread enzyme activity in FTM extracts provided by the recoveries, which we shall discuss below.

Among the similarity between the results of HW, BE, and CM, a few differences stand out upon closer inspection. The profiles for BE and CM are particularly close, while HW yielded somewhat higher levels of certain metabolites, especially Leu, Met, GDP, succinate, Phe, and Ile (>2 standard deviations), followed by Trp, fumarate, ADP, Ser, and Tyr (>1 standard deviation). In principle, higher efficacies mean that HW has a better ability to extract these metabolites. However, it is unclear why a few amino acids and organic acids, but not others, should be better released by HW than, say, BE. The occurrence of several amino acids among the largest differences suggested an alternative possibility: that the higher levels could originate from decomposition of macromolecules such as proteins. If protein hydrolysis occurred, the largest relative contribution would be for those amino acids which are most abundant in whole-protein compared to their free pool. If we calculate, for each amino acid, the quotient between its percentage in protein[41] and its percentage in the free pool, the highest are for Leu (31), Phe (22), Met (16), and Ile (13), followed by Gly, Trp, Tyr, and Pro (5−10). The striking correspondence with the amino acids which are higher in HW samples strongly indicates that partial protein hydrolysis could indeed be the source. Protein is a major component of biomass ($\approx$0.4 g/g_{DW}). We estimate that the hydrolysis of as little as 0.2% of the cell protein would be sufficient to explain the measured differences in free pools. This also complicates experimental verification of amino acid release from purified protein, as it is possible that only a small number of labile proteins are degraded. It is conceivable that the other metabolites elevated in HW extracts also originate from macromolecular degradation, although the exact biomass components that would generate succinate, fumarate, GDP, and ADP upon thermal hydrolysis remain to be elucidated.

Comparison of Metabolite Recoveries. As a measure of the stability of each metabolite throughout sample treatment with each extraction technique, we have defined the overall process recovery for metabolite x under extraction method i as

$$\text{recovery}_{x,i} = \frac{[x]_i^{\text{BA}}}{[x]_i^{\text{AQ}}} \quad (3)$$

Since we are determining an overall process recovery, nonspecific losses are to be expected simply by volume loss during centrifugation, filtration, etc. In our samples, estimated losses of 30−60 μL would represent 5−10% (recoveries 90−95%). Thus, even allowing for measurement error, losses of more than 15−20% indicate metabolite degradation, especially if they are metabolite specific. Conversely, mixed recoveries above as well as below 100% would mean that metabolite conversion, either enzymatic or chemical, is taking place during the sample treatment. Metabolite conversion will distort the metabolic "snapshot" that each sample represents. An extraction technique which does not effectively prevent it (e.g., by inactivating enzymatic activity) cannot be considered suitable for metabolomics research.

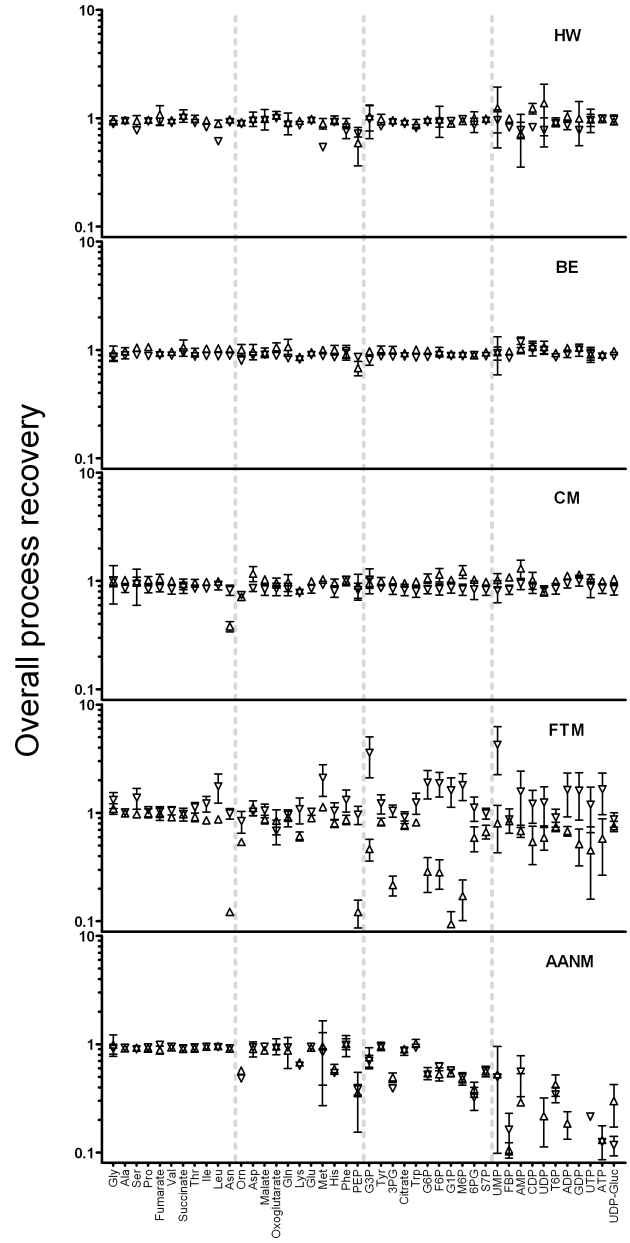

Figure 4. Overall process recoveries for all 44 metabolites analyzed, in order of increasing molecular weight, for each of the extraction methods, under both growth conditions (see Results and Discussion for the definition of recovery). Data are averages and standard deviations of duplicate samples each analyzed twice. Legend: ▽, chemostat; △, batch. Gray dashed vertical lines are for guidance only.

The overall process recoveries of all analyzed metabolites, for both experiments, are plotted, per extraction method, in Figure 4. The recovery profiles for HW, BE, and CM are quite comparable, with recoveries near 1 across all metabolites. The profile for BE is noticeably the "flattest", indicating the near absence of metabolite-specific losses and excellent reproducibility. With CM and HW only a few metabolite-specific effects were observed: in

the case of CM, the recovery was low for Asn (0.38, batch) and Orn (0.71−0.74), while in the case of HW, it was low for Leu (0.61, batch) and Met (0.54, chemostat).

Extraction with AANM led to much more pronounced losses. While the recoveries for smaller, less polar compounds were very reproducible and near 1, a few mid-MW metabolites had low recoveries: Orn (0.48−0.52), Lys (0.63−0.68), His (0.54−0.59), PEP (0.35−0.38), and 3PG (0.39−0.49). For the larger phosphorylated intermediates, the recoveries were systematically below 60% and some nucleotides (e.g., GDP, CDP, UTP, ADP, UDP) must have been so affected that they could not be detected in some of the samples. What these metabolites have in common is that they correspond exactly to the positively charged side chain-containing amino acids (the remaining, Arg, was not analyzed) and the phosphorylated compounds. Why they are lost, however, is unclear. Exposure to low pH is not very long (45−60 min total) and is at low temperatures. Besides, both compound classes are thought to be reasonably stable under acidic conditions. The AANM and ammonia solutions were titrated against each other before use (as a check), so prolonged exposure to low pH due to inadequate neutralization can be excluded. An alternative explanation could be that the most polar, charged metabolites are not entirely soluble in the cold solvent-rich mixture and, if partially precipitated (e.g., with cell debris, trace elements), would be lost during centrifugation and filtration.

The profile of recoveries for FTM extraction clearly shows that metabolite stability is most problematic with this method. Not only are there several observations of very low recoveries but also there is a large number of recoveries above 100%. This confirms the suspicions, discussed above, of remaining enzymatic activity during the procedure. In addition, rather than being reproducibly high or low in both experiments, for many metabolites, the recovery depended on the origin of the samples. For example, Asn, Orn, Lys, PEP, 3PG, 6PG, S7P, T6P, and UDP-glucose had low recoveries in samples from the batch experiment but not from the chemostat, while Gly, Ser, Leu, Met, and UMP had recoveries above 100% in samples from the chemostat but not from the Batch. Furthermore, for a series of fast-turnover metabolites: G3P, G6P, F6P, G1P, M6P, AMP, CDP, UDP, ADP, GDP, UTP, and ATP, recoveries were above 100% in chemostat samples and well below 100% in Batch samples. Consider for instance the hexose-phosphates: in the batch their levels in AQ samples were far higher than with the other extraction methods, which we have attributed to assimilation of glucose (see above). However, in BA samples, their levels had decreased again, indicating conversion of the elevated pools into other intermediates later on in the sample treatment. This would have been partly compensated by the ^{13}C-labeled IS in the AQ samples, giving the very low recoveries but not in BA samples. Conversely, in the chemostat, the hexose-phosphate levels in AQ samples were comparatively low, indicating poor extractability, but in BA samples, the levels were higher, again indicating interconversion between metabolite pools after extraction. This could, in principle, occur at any stage after extraction, implying that enzymes were extracted from the cells but not actually inactivated. Additionally, it cannot be ruled out that conversion occurs also during extraction, since the samples spend several minutes thawing at 0 °C, and methanol and low temperatures do not prevent enzymatic activity but merely slow it down.[21,22] While the latter could perhaps be prevented by thawing instead at −40 °C, the former cannot. Note that the use of ^{13}C-labeled IS is not meant to deal quantitatively with metabolite conversion (that would require the substrate/product ratios in the IS to be exactly the same as in the sample) but rather with degradation and analytical artifacts (e.g., matrix effects). In conclusion, the changes observed in glycolytic intermediates are consistent with the presence of enzymatic activity, and the extent of other metabolites exhibiting condition-dependency effects suggests this activity is widespread. Since inactivation of enzymatic activity is an essential requirement of an extraction method, these results mean that FTM cannot be considered a suitable technique for metabolomics research, at least in *S. cerevisiae*.

Finally, we would like to draw attention to the advantages of the approach used here for the determination of recoveries in comparison to the "traditional" approaches of spiking and standard additions. In principle, these involve additions (at one or multiple concentrations) of each metabolite to different samples. Furthermore, if the influence of sample treatment and analysis are to be distinguished, separate additions must be performed at different stages. Doing this separately for every metabolite requires so many samples that in practice mixes of metabolites are used instead. Not only does the addition of multiple metabolites make interconversion difficult to notice but also the concentrations added are sometimes so high that they may no longer reflect what takes place in a real sample. This implies that the outcomes are dependent on the actual mix of metabolites chosen and concentrations applied. These limitations are circumvented by the ^{13}C-labeled IS approach designed here. Since the added (labeled) metabolites do not interfere with the (unlabeled) metabolites already present, recoveries reflect changes as they actually occur in physiological samples. Since the IS mix is a cell extract itself, thus containing all metabolites, distinguishing metabolome-wide losses over the entire sample processing can be achieved with a modest experimental workload. Furthermore, if the recoveries at individual stages of sample treatment need to be distinguished, it is possible to extend the approach by applying the ^{13}C-labeled IS to additional replicate samples before and after each processing step. Although that will multiply the experimental effort several-fold, it should allow losses to be tracked down to specific operations.

Our Verdict: Best Performance with BE and CM. From the quantitative data presented above, the five extraction methods investigated can be objectively evaluated in terms of completeness of extraction, prevention of metabolite conversion, and metabolite stability. We conclude that the best extraction performance is obtained with the BE and CM methods. Both techniques achieved quantitative extraction of all metabolites analyzed with near-100% recoveries and good reproducibility. In the investigation of biological phenomena (RSD > 15%), the metabolite profiles obtained with these two methods should be nearly indistinguishable, which is quite remarkable considering they are based on rather different extraction principles. The data from CM and BE samples have been combined, and the resulting intracellular metabolite levels for the two growth conditions investigated are presented in Table 2.

Table 2. Comparison of Intracellular Metabolite Levels under the Two Growth Conditions Investigated

	intracellular level (μmol/g_{DW})[a]	
metabolite	chemostat (μ = 0, 1 h^{-1})	batch (μ = 0, 4 h^{-1})
Glycolysis		
G6P	5.53 ± 0.08	6.4 ± 0.4
F6P	1.38 ± 0.04	1.0 ± 0.1
FBP	0.47 ± 0.02	18 ± 1
3PG	2.9 ± 0.1	1.5 ± 0.1
PEP	2.1 ± 0.1	0.18 ± 0.03
Carbohydrate Synthesis		
G1P	1.07 ± 0.05	0.40 ± 0.02
UDP-Glucose	3.03 ± 0.09	0.60 ± 0.03
T6P	0.37 ± 0.02	0.080 ± 0.004
M6P	1.75 ± 0.03	0.72 ± 0.03
PPP and Glycerol Synthesis		
6PG	0.69 ± 0.03	1.0 ± 0.1
S7P	2.97 ± 0.06	1.03 ± 0.03
G3P	0.09 ± 0.02	0.66 ± 0.05
TCA Cycle		
Citrate	7 ± 1	2.4 ± 0.1
Oxoglutarate	1.9 ± 0.1	2.8 ± 0.1
Succinate	1.09 ± 0.05	1.8 ± 0.2
Fumarate	0.655 ± 0.008	0.45 ± 0.03
Malate	3.01 ± 0.09	1.6 ± 0.1
Nucleotides		
ATP	7.0 ± 0.2	3.8 ± 0.2
ADP	1.7 ± 0.1	1.40 ± 0.09
AMP	0.45 ± 0.06	0.27 ± 0.03
UTP	1.8 ± 0.2	1.2 ± 0.1
UDP	0.78 ± 0.05	0.38 ± 0.04
UMP	0.10 ± 0.02	0.06 ± 0.01
GDP	0.21 ± 0.04	0.13 ± 0.04
CDP	0.17 ± 0.02	0.10 ± 0.01
Amino Acids (Aromatic and His)		
Phe	0.47 ± 0.02	1.5 ± 0.1
Tyr	1.41 ± 0.05	1.01 ± 0.06
Trp	0.40 ± 0.02	0.190 ± 0.008
His	5.3 ± 0.2	7.7 ± 0.5
Amino Acids (from 3PG)		
Ser	4.0 ± 0.9	12.4 ± 0.8
Gly	2.3 ± 0.4	12 ± 1
Amino Acids (from Pyruvate)		
Ala	19.8 ± 0.4	18 ± 1
Leu	0.98 ± 0.05	1.05 ± 0.05
Ile	1.72 ± 0.04	1.99 ± 0.09
Val	8.1 ± 0.1	8.9 ± 0.3
Amino Acids (from Oxoglutarate)		
Glu	122 ± 2	55 ± 2
Gln	45.1 ± 0.6	43 ± 5
Pro	3.9 ± 0.1	2.7 ± 0.2
Orn	3.9 ± 0.1	15 ± 1
Amino Acids (from Oxaloacetate)		
Asp	15.8 ± 0.3	12 ± 2
Asn	4.52 ± 0.04	2.7 ± 0.1
Lys	4.4 ± 0.1	20.2 ± 0.8
Met	0.218 ± 0.007	0.42 ± 0.02
Thr	4.1 ± 0.2	12.3 ± 0.7

[a] Data are averages and standard deviations of four samples, two extracted with BE and two with CM, each analyzed twice.

Extraction with HW gave results very similar to BE and CM for most metabolites but raised some concerns regarding possible hydrolysis of macromolecules, which is undesirable. Although an incubation of 15 min is until now standard for extraction of yeast cells, it seems very likely that a shorter extraction time would lead to a performance comparable to that of BE. Extraction with AANM gave poor results, with limited efficacy and low recoveries for specific types of metabolites. Nevertheless, if the reason behind the problems encountered with the more polar, charged metabolites is found and solved, it could eventually provide a viable alternative to CM. Finally, according to our results, FTM cannot effectively prevent metabolite conversion throughout the samples treatment, most likely by failing to inactivate enzymatic activity, and thus, cannot be considered an adequate extraction method.

Considering the inconsistency in the literature concerning the evaluation of extraction methods illustrated in Table 1, it is not surprising that our conclusions are partly in agreement with some previous works and in disagreement with others. In the interest of clarifying possible causes for the differences in outcome, it is worth comparing our conclusions with those of previous studies. At first sight, our most striking conclusion would appear to be that FTM is not adequate as an extraction method, since much of the recent literature, including some of the most influential papers on this topic, consider it the best technique.[18,24,42−44] However, three of these studies, including the original proponents of the method, did not determine metabolite recoveries,[18,42,44] thereby leaving two of the essential requirements untested. In the study by Villas-Boas et al., metabolite recoveries were determined for 27 metabolites (14 of which also measured here) in extracts spiked with a mix of the same compounds.[24] Recoveries differed substantially between extraction methods and were generally quite low, including for BE and CM extractions. Some metabolites, such as sugar phosphates, were not recovered acceptably (>30%) with any of the procedures, for which no explanation could be found. CM and FTM had, overall, the least unsatisfactory recoveries so the authors concluded FTM was the best choice, based on higher extraction efficacy (judged from the number of GC peaks detected) and lower solvent toxicity. A more recent study actually found evidence of conversion of ATP to ADP and AMP in ATP-spiked FTM extracts.[43] Despite this, the authors concluded that presence of enzyme activity was only a minor drawback of the method and, based on experimental simplicity and metabolite yields comparable to BE and PCA (in *L. plantarum*, for 3 metabolites), selected it as their preferred extraction method. From these considerations, we conclude that, in the evaluation of the FTM method, the main sources of discrepancy between this work and the literature lie in the definition of evaluation criteria and the type of data used for their verification.

Our results also differ from the literature regarding the evaluation of the AANM extraction. The proponents of the method compared it to FTM and CM (and a few other variations) for the extraction of metabolites from *E. coli*.[19] Contrary to our results, they found that CM gave lower metabolite levels than AANM or FTM. However, the authors had adapted the procedure for CM extraction[6] by applying no mixing of the two-phase system. Interestingly, another study also in *E. coli* where only short periods of mixing (3 × 30 s) were applied for CM extraction also concluded it had limited efficacy.[18] Insufficient interfacial stress could well explain why CM failed to perform better than FTM and AANM, although it does not explain why it performed worse. This discrepancy may well be related to organism-specific differences. In the comparison between FTM and AANM, the authors preferred AANM because they found less metabolite conversion (attributed to chemical degradation) in nucleotide-spiked samples than with the FTM method. This is partially in agreement with our

findings, since presence of enzymatic activity in FTM extracts (see above) would explain the conversions between metabolites which they observed. Finally, the authors also concluded that AANM gave the best performance for the extraction of nucleotides, which is opposite to our findings. If our hypothesis of metabolite precipitation were true, it is unclear why the *E. coli* samples would be less affected. On the other hand, it also cannot be excluded that losses were also taking place in their study since metabolite recoveries were not determined.

To our knowledge, BE, CM, and HW extractions had not been compared together before, although several works compared at least two of them. Among those studies, half concluded they gave similar results,[31,42,45,46] while the other half found significant differences.[18,24,43,47] As discussed above, in some instances, the poor performance found with CM might be related to insufficient mixing of the two-phase system.[18,43] Villas-Boas et al.,[24] also using *S. cerevisiae*, found particularly poor recoveries with BE extraction for several metabolites, among them hexose-phosphates (100% loss), Lys, Phe, Trp, citrate, and oxoglutarate, which were completely stable in our experiments. The cause of this discrepancy is unknown. However, it should be noted that their finding of 0% recovery for hexose-phosphates is not in agreement with the fact that these metabolites can be routinely assayed in BE extracts at several laboratories.[48−52] In the study of Hiller et al.,[47] HW extraction was considered superior to BE in terms of reproducibility and linearity of standard additions (from which detection limits were determined). These aspects cannot be easily compared between studies since reproducibility can be affected by so many different factors. In our study, the reproducibility with HW and BE was comparable. In fact, the average RSD between metabolite levels ($n = 88$) in duplicate biological samples was below 5% with BE, HW, or CM.

Finally, let us consider how our findings relate with the idea that comprehensive coverage of the metabolome can only be achieved using a combination of parallel extraction methods. This notion can be traced back to the works of Maharjan and Ferenci and Villas-Boas et al.[18,24] Having observed a large disparity in results with different extraction methods and in the absence of one method that was better for all metabolites, Maharjan and Ferenci concluded it was unlikely that a single extraction method could cover all metabolites, given their variety in chemical and physical properties. Their conclusion was based on relative measurements of 13 metabolites, most with poor reproducibility, and no data on metabolite recoveries. This frame of mind may have led Villas-Boas et al. to express their results in terms of metabolite classes rather than individual compounds. Having observed a large disparity in the recoveries of 27 metabolites, they concluded that every method discriminated particular metabolite classes and concluded that developing a method that could extract the large variety of compounds in the cell was an impossible task. Since then, these notions have been supported by at least two reviews on the topic (one of which was by our group)[53,54] and by some of the most recent literature.[42] An additional contributing factor may have been the inconsistency between published method comparisons, illustrated in Table 1. However, the results we have presented here are not consistent with that view. Admittedly, we have analyzed only a subset of the metabolome, from which is it dangerous to extrapolate. It cannot be excluded that extraction performances differ significantly for functional classes not analyzed here, such as lipids, sugars, CoA derivatives, etc. Nevertheless, the fact that all metabolites analyzed could be extracted with high efficacies and near-100% recoveries by at least two very different extraction techniques deserves being considered before assuming that metabolome-wide extraction is an impossible feat, in yeast as well as other microorganisms.

CONCLUDING REMARKS AND RECOMMENDATIONS

For accurate determination of intracellular metabolite levels in *S. cerevisiae*, samples should be extracted using boiling ethanol or chloroform−methanol. Both methods gave high efficacies and excellent recoveries for the whole range of metabolites analyzed here. Hot water also performs generally well but raises the risk of overestimating some metabolite pools, presumably due to macromolecule hydrolysis, although this can probably be minimized with a shorter incubation time. The performance of acidic acetonitrile−methanol is inferior, for reasons not yet elucidated. Freezing-thawing in methanol is inadequate as an extraction method, as it does not ensure inactivation of enzymatic activity.

Some aspects of metabolite extraction are species dependent, so the performance of the different techniques available should be adequately validated with each microorganism. Furthermore, extraction performance may, in some cases, depend on the history of the sample, so method comparisons can benefit if investigations are performed under more than one growth condition (e.g., chemostat and batch).

The approach outlined here, of applying ^{13}C-labeled internal standards at different stages of the sample treatment process, is particularly useful for the determination of metabolite recoveries.

The choice of methods for the earliest steps in sample treatment can affect the outcome of a metabolomics-based experiment to the extent that even the direction of changes in metabolite levels may be distorted. Insufficient validation of the experimental procedures used, thus, compromises the results of subsequent research and conclusions derived thereof.

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