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## Identifying Decomposition Products in Extracts of Cellular Metabolites

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### Abstract

Most methods of analyzing intracellular metabolites require extraction of metabolites from the cells. A concern in these methods is underestimation of metabolite levels due to incomplete extraction. In comparing extraction methods, it would accordingly seem that the best method for extracting a particular metabolite is the one giving the largest yield. In extracting *Escherichia coli* with different methanol:water mixtures, we observed that  $\geq 50\%$  water gave increased yield of nucleosides and bases compared to  $\leq 20\%$  water, as determined by liquid chromatography-tandem mass spectrometry analysis of the resulting extracts. Spiking of the extracts with isotope-labeled nucleotides revealed, however, that the high yield of nucleosides and bases occurred due to decomposition of nucleotides in the water-rich condition, not good extraction. Spiking combined with isotope labeling provides a general approach to detecting decomposition products in extracts of cellular metabolites. For extraction of *E. coli* with methanol:water, cold temperature and a high methanol fraction minimizes artifacts due to metabolite decomposition.

### Keywords

Metabolomics; metabolism; extraction; bacteria; sampling; stability; LC-MS/MS; triple quadrupole; small molecule

## INTRODUCTION

The cellular metabolic network plays a fundamental role in biology, converting incoming nutrients into energy, subunits of biopolymers, and signaling molecules. Due to the importance of these processes, there is great interest in obtaining as complete an understanding as possible of the metabolic activity of cells.[1-6] To this end, the past five years have seen a marked acceleration of research that attempts to study many components of the cellular metabolic network in parallel [7-9], frequently with a focus on quantifying changes in metabolite concentrations that occur upon perturbation of the cellular environment by alteration of nutrient conditions[10-13].

A long-standing challenge in metabolite analysis is extraction of compounds of interest from the biological sample[14]. While avoiding the need for extraction by measuring the analytes of interest directly within the living environment is an appealing possibility (using, for example, NMR [9,15-18], the vast majority of metabolite analysis continues to be conducted in extracts. Use of extracts has the important advantages of enabling sample concentration and separation.

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In addition, it facilitates analysis by mass spectrometry (MS), a uniquely powerful technique for identifying and quantifying low abundance components in complex mixtures[19-21]

As part of the effort to render cell-based metabolomics more quantitative and systematic, there has been a growing effort to identify extraction conditions that are appropriate to broad spectra of metabolites, and also to understand the limitations of these extraction procedures[13, 22-26] For example, Maharjan and Ferenci examined the efficiency of extracting *E. coli* with boiling ethanol:water, cold versus hot methanol:water, and perchloric acid versus potassium hydroxide in cold water, using thin layer chromatography to analyze radioactively labeled samples [23] They found cold methanol:water (which they tested in a 50:50 mixture) to be the best approach, based on its providing the highest yield of the greatest number of metabolites, while also having the intuitive appeal of avoiding hot temperatures or extremes of pH. Subsequently, Villas-Boas et al. conducted related experiments in the yeast *Saccharomyces cerevisiae*. [24] Instead of focusing on extraction of cellular material, they measured recovery of spiked metabolites using gas chromatograph-MS as their detection modality, finding that cold pure methanol, a condition not explored by Maharjan and Ferenci, provided the minimum loss of metabolites.

Based on these results and our own studies, we have previously used cold 80:20 methanol:water mixtures to extract the bacterium *Salmonella enterica*, using three rounds of serial extraction to improve total metabolite yield. We justified the efficacy of this procedure by showing that, at the end of these three rounds of extraction, subsequent extraction using other solvent mixtures did not yield substantial quantities of most metabolites [27].

While making valuable strides towards identification of appropriate extraction procedures for cell-based metabolomics, these prior studies leave open the fundamental issue of how to judge an extraction method. Is the critical feature obtaining measurable amounts of as many endogenous metabolites as possible? [23] Or maximizing yield of specific key metabolites? [22,26,28] Or minimizing loss of spiked metabolites? [24] Or extracting until further extraction yields no additional metabolites? [27]

In an effort to optimize extraction of endogenous metabolites yet further, we began a program of extracting *E. coli* under varying conditions, with some of the first conditions explored cold methanol:water mixtures differing in their fractional water content. We were surprised to find that changing from 80% to 50% methanol markedly altered the metabolite profile, with nucleotides more abundant in 80% methanol and nucleosides and bases more abundant in 50% methanol. This raised the question—is 80:20 or 50:50 methanol:water the superior extraction solvent? Taking advantage of the power of isotope-labeling[29,30], we provide a clear answer: the higher methanol content is preferred, because the apparent “good” extraction of nucleosides and bases in the water-rich condition is actually due to nucleotide decomposition. This case study highlights the importance of finding extraction methods which produce samples reflective of the true intracellular environment, not just ones yielding copious metabolites.

## MATERIALS AND METHODS

### Chemicals

HPLC-grade solvents (OmniSolv, EMD Chemical, Gibbstown, NJ) were obtained from VWR International (West Chester, PA); ammonium acetate (99.4%) from Mallinckrodt Chemicals, Phillipsburg, NJ; and ammonium hydroxide solution (29.73%) from Fisher Scientific, Pittsburg, PA. All purified metabolite standards and media components were obtained through Sigma-Aldrich (St. Louis, MO) and are  $\geq 98\%$  pure according to the manufacturer. U- $^{13}\text{C}$ -glucose (99%) was obtained from Cambridge Isotope Laboratories (Andover, MA).

## Cell growth and extraction

*Escherichia coli* K-12 strain NCM3722 [31] was used for all experiments. The cells were grown in shaker flasks at 37°C in a minimal salts media [32] with 10 mM ammonium chloride as the nitrogen source and 0.4% glucose (either unlabeled or uniformly <sup>13</sup>C-labeled) as the carbon source. Exponential-phase cultures were quenched and extracted when optical density at 650 nm (A<sub>650</sub>) reached ~ 0.35. Metabolites were serially extracted using variants of the basic protocol presented originally in Lu et al. [27] with the specific methanol:water ratios and extraction temperatures varied as described in the **Results**. Bacteria were pelleted by centrifugation for 4 min at 5000 g, the supernatant was immediately aspirated, and 300 µL of methanol:water was added to the pellet and mixed to quench metabolism and initiate the extraction process. After 15 min, the sample was spun in a micro-centrifuge at 13,200 rpm for 5 min at 4°C and the soluble extract was removed. The pellet was then re-extracted as above except using 200 µL methanol:water, and the extracts combined. The pellet was then extracted for a third time, again using 200 µL methanol:water, and this final extract combined with the first two to give a total of 700 µL of ready-to-analyze extract.

## Spiking with <sup>12</sup>C standards

<sup>13</sup>C-labeled cells were spiked with unlabeled (<sup>12</sup>C) standards at the start of the extraction process, for the purpose of assessing potential loss of the spiked standards by their absorption, sequestration, or decomposition during the extraction process, as well as for assessing any production of decomposition products from the spiked standards. *E. coli* were grown as described above in U-[<sup>13</sup>C]-glucose for a minimum of 20 generations. After centrifugation of exponentially growing <sup>13</sup>C cells into a pellet, 300 µL of extraction solvent containing a mixture of commercially available unlabeled (<sup>12</sup>C) compounds was added to quench metabolism and initiate the extraction process. The extraction process then proceeded through the full series of steps described above, without further addition of compound standards, to yield a final 700 µL extraction volume. The metabolite concentrations in the spiked extraction solvent were chosen to result, upon spiking, in a <sup>12</sup>C-standard signal roughly comparable to the endogenous <sup>13</sup>C-metabolite signal. The spiked compounds and final concentrations in the 700 µL total extraction volume were 5-methyl-tetrahydrofolate (5-MTHF, 0.015 µg/mL), acetyl-CoA (5 µg/mL), alanine (0.15 µg/mL), cytidine-5'-triphosphate (CTP, 1 µg/mL), fructose-1,6-bisphosphate (FBP, 1 µg/mL), glutamine (0.03 µg/mL), guanosine-5'-triphosphate (GTP, 1 µg/mL), inosine-5'-monophosphate (IMP, 0.1 µg/mL), S-adenosyl-methionine (SAM, 0.3 µg/mL), tryptophan (0.15 µg/mL), thymidine-5'-triphosphate (TTP, 2 µg/mL), uridine-5'-monophosphate (UMP, 1 µg/mL), valine (0.15 µg/mL), and xanthosine (1 µg/mL).

## Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Samples were analyzed using a minor variant of the procedure described in Bajad et al. [33], which involves detection of a pre-defined set of metabolites by LC-MS/MS using a triple-quadrupole instrument operated in selected reaction monitoring (SRM) mode. SRM involves detection of a particular product ion formed from a given parent ion. Each compound is identified by a combination of its chromatographic retention time, parent ion mass, and product ion mass. Different SRM scan events are employed to independently measure <sup>12</sup>C versus <sup>13</sup>C forms of a given metabolite, and data on many (e.g. 20 - 100) compounds can be obtained in a single chromatography run by quickly scanning through different SRMs.

Analytical details are as follows: the liquid chromatography (LC) approach was hydrophilic interaction chromatography using an aminopropyl column (Luna 5 µm particle size, 50 x 2 mm column dimensions from Phenomenex, Torrance, CA) at basic pH. Chromatography was accomplished on a LC-10A HPLC system (Shimadzu, Columbia, MD) with autosampler temperature 4°C, column temperature 15°C, and injection volume 10 µL. Solvent A is 20 mM ammonium acetate + 20 mM ammonium hydroxide in 95:5 water:acetonitrile at pH 9.3. Solvent

B is acetonitrile. Each sample was run twice, once in positive and once in negative ion mode. In positive mode, the gradient is  $t = 0$ , 85% B;  $t = 3$  min, 45% B;  $t = 10$  min, 0% B;  $t = 22$  min, 0% B;  $t = 23$  min, 85% B;  $t = 29$  min, 85% B; and the solvent flow rate is  $t = 0$  min, 100  $\mu\text{L}/\text{min}$ ;  $t = 18$  min, 100  $\mu\text{L}/\text{min}$ ;  $t = 20$  min, 200  $\mu\text{L}/\text{min}$ ;  $t = 27$  min, 200  $\mu\text{L}/\text{min}$ ;  $t = 27.1$  min, 100  $\mu\text{L}/\text{min}$ ;  $t = 29$  min, 100  $\mu\text{L}/\text{min}$ . In negative mode, the gradient is  $t = 0$ , 85% B;  $t = 3$  min, 45% B;  $t = 10$  min, 0% B;  $t = 20$  min, 0% B;  $t = 21$  min, 85% B;  $t = 27$  min, 85% B and the solvent flow rate is  $t = 0$  min, 100  $\mu\text{L}/\text{min}$ ;  $t = 16$  min, 100  $\mu\text{L}/\text{min}$ ;  $t = 18$  min, 200  $\mu\text{L}/\text{min}$ ;  $t = 25$  min, 200  $\mu\text{L}/\text{min}$ ;  $t = 25.1$  min, 100  $\mu\text{L}/\text{min}$ ;  $t = 27$  min, 100  $\mu\text{L}/\text{min}$ .

Mass spectrometric analyses were performed on a Finnigan TSQ Quantum Ultra triplequadrupole mass spectrometer (Thermo Electron Corporation, San Jose, CA). Electrospray ionization spray voltage was 3200 V in positive mode and 3000 V in negative mode. Nitrogen was used as sheath gas at 30 psi and as the auxiliary gas at 10 psi, and argon as the collision gas at 1.5 mTorr, with the capillary temperature 325°C. Scan time for each SRM transition was 0.1 s with a scan width of 1 m/z. Specific SRM parameters for each of the compounds reported on here are provided in Bajad et al.[33].

## RESULTS

### Effect of methanol:water ratio on endogenous metabolite yield

Figure 1 compares the yield of some  $\sim 100$  different metabolites from *E. coli* as a function of the methanol:water ratio used for extraction. The X-axis of each panel indicates the observed compound yield using 80:20 methanol:water for extraction, the methanol:water ratio we have employed in previous studies [27,33]. The Y-axis shows the yield using 100:0, 50:50, or 20:80 methanol:water. Equivalent yield, irrespective of the solvent mixture, would result in compounds falling on the line of identity. Points below and to the right of the line indicate compounds that are more abundant in 80:20 methanol:water than the comparator condition, and vice versa; compounds present only in one condition are shown on the axes. Metabolites falling far from the line of identity (i.e., that are particularly sensitive to the extraction solvent) are labeled. For each solvent mixture, the first extraction step was conducted at the coldest temperature conveniently obtained without causing the solvent to freeze ( $-75^\circ\text{C}$  for 100:0 and 80:20 methanol:water;  $-20^\circ\text{C}$  for 50:50 methanol:water;  $4^\circ\text{C}$  for 20:80 methanol:water) and the subsequent two steps were conducted at  $4^\circ\text{C}$ . The selection to conduct the first step as cold as feasible reflects the desire to quench metabolism as quickly as possible.

A first key observation in Figure 1 is that 20:80 and 50:50 methanol:water yield markedly different extracts from 80:20 and 100:0 methanol:water, which are quite similar to one another. A second key observation is the nature of some of the most profound differences between the low versus high water-content extracts: a substantial fraction of the most enriched compounds in the high water condition are nucleosides (adenosine, guanosine, cytidine, uridine, thymidine) and bases (adenine, guanine, cytosine), whereas a substantial fraction of the most depleted compounds are nucleotides (CTP, CMP, ITP, IMP, UTP, UMP, ATP, AMP, FAD), especially in the 50:50 methanol:water condition. For 20:80 methanol:water, acetyl CoA is also noticeably depleted.

### Effect of extraction temperature on endogenous metabolite yield

The above observations suggested the possibility that higher water content is associated with degradation of labile compounds to lower energy species. An alternative explanation, however, is that the key variable is not higher water content, but the associated temperature of the first extraction step. To investigate this possibility, 80:20 methanol:water was used to extract samples at various temperatures, ranging from  $-70^\circ\text{C}$  to  $4^\circ\text{C}$ . The observed metabolite yield

was similar across this temperature range (Fig. 2). Thus, metabolite yield from *E. coli* is more sensitive to the methanol:water ratio of the extraction mixture than to extraction temperature.

### Effect of methanol:water ratio on recovery of spiked metabolites

To test whether the observed pattern of high nucleosides and bases, but low nucleotides, in the higher water conditions results from nucleotide decomposition, we grew *E. coli* in uniformly  $^{13}\text{C}$ -glucose for > 20 generations, which we have shown previously results in complete replacement of  $^{12}\text{C}$ -with  $^{13}\text{C}$ -metabolites.[27,33] We then extracted these *E. coli* using methanol:water spiked with various test compounds, including some labile compounds of particular interest—nucleotides, 5-methyl-tetrahydrofolate (5-MTHF), acetyl-CoA, fructose-1,6-bisphosphate (FBP), and S-adenosylmethionine (SAM). The spiked compounds were subject to the same conditions and experiences of extraction as the cellular metabolites. In control experiments, the purified compound mixture was shown to be stable in all of the tested solvent mixes for the duration of the experiment and subsequent analysis. Nevertheless, dramatic losses of many of the test compounds occurred upon their mixing with the  $^{13}\text{C}$ -*E. coli* (Fig. 3A; note that the Y-axis is in units of  $\log_{10}$ , i.e., a value of “-1” indicates a 10-fold compound loss). Although the pattern of metabolite loss was complex and compound dependent, overall there was a clear trend towards greater compound loss in the higher water conditions. Loss of nucleotides was especially severe in the 50:50 methanol:water condition, in agreement with the observation that 50:50 methanol:water yielded the least extraction of endogenous nucleotides (Fig. 1). Loss of acetyl-CoA was most profound in the 20:80 methanol:water condition, again in agreement with the endogenous metabolite extraction results shown in Figure 1. GTP loss was greater in 100:0 than 80:20 methanol:water, perhaps indicating the value of some water for solvating certain compounds. The observed differences in spiked compound recovery as a function of solvent composition (Fig. 3A) were not due to differences in extraction temperature, which played a minimal role over the tested range (Fig. 3B).

### Formation of metabolites by degradation of other metabolites

The observation that spiked nucleotides are lost in water-rich conditions (Fig. 3) is consistent with the hypothesis that the low yield of endogenous nucleotides in these conditions is due to nucleotide loss. Such loss could occur due either to absorption or decomposition. If nucleotide decomposition were occurring selectively in the water-rich conditions, this could explain the associated large yield of endogenous nucleosides and bases. To explore this possibility, we examined  $^{13}\text{C}$ -*E. coli* extracts spiked with  $^{12}\text{C}$ -nucleotides including CTP, GTP, TTP, IMP, UMP for the presence of  $^{12}\text{C}$ -nucleosides and bases, which could have come only through the decomposition of the spiked material (controls showed insubstantial level of these compounds in  $^{13}\text{C}$ -*E. coli* in the absence of the  $^{12}\text{C}$ -compound spiking, or in the  $^{12}\text{C}$ -standard prior to its use for *E. coli* extraction). Substantial quantities of  $^{12}\text{C}$ -nucleosides and bases were found in the  $^{12}\text{C}$ -nucleotide-spiked but otherwise  $^{13}\text{C}$ -*E. coli* extract—thus, nucleosides and bases were indeed formed via nucleotide decomposition (Fig. 4). Formation of nucleosides and bases from nucleotides occurred under all extraction conditions, but by far less in the 100:0 methanol:water mixture than in the mixtures containing water (Fig. 4A). Examining the pattern of CTP and GTP decomposition in detail shows that 80:20 methanol:water yielded substantial amounts of mono- and di-phosphates and nucleosides, but relatively little decomposition all of the way to the level of bases; in contrast, 20:80 and especially 50:50 methanol:water yielded mainly nucleosides and bases from the nucleotide starting material. The differences in decomposition product formation, although showing some sensitivity to extraction temperature (Fig. 4B), were primarily driven by solvent composition.

## DISCUSSION

The ability to quantify a large number of intracellular metabolites in parallel has the potential to substantially advance basic understanding of cellular metabolic processes, as well as to contribute to applied fields including bioengineering and molecular medicine. In each of these arenas, the value of quantitative metabolic data will depend on its accurately reflecting the true cellular composition. This requires either the ability to directly measure metabolites within living cells, which is a promising approach for specific compounds but may prove challenging for larger panels of compounds [9,15,17,18,34,35], or the capacity to quench metabolism and extract metabolites without unduly perturbing the metabolome.

Here we focus on the challenge of extracting metabolites, using *E. coli* as a model system and considering only a narrow range of extraction methods involving cold methanol:water, as previous studies using both *E. coli* and yeast point to extraction with cold methanol or cold methanol:water yielding good results. A relatively broad view of the water-soluble metabolite content of the extracts is provided by LC-MS/MS analysis. With the methanol:water ratio fixed at 80:20, different “cold” extraction temperatures (from -70°C to 4°C) yield similar data (Fig. 2). In contrast, different methanol:water ratios yield substantially different metabolic extracts, highlighting the sensitivity of the extract content to the extraction solvent composition (Fig. 1).

An advantage of using LC-MS/MS to analyze the extracts is the ability to immediately determine the specific compounds that are sensitive to the extraction solvent composition. Most striking is the observation that extracts produced using  $\geq 50\%$  water contain substantially *lower amounts* of nucleotide triphosphates (and other “high energy” compounds) and *higher amounts* of nucleosides and bases than the extracts produced using  $\leq 20\%$  water (Fig. 1). This observation suggests the possibility that extraction with  $\geq 50\%$  water leads to decomposition of high energy compounds, perhaps due to incomplete quenching of enzyme activity, and that the observed high levels of nucleosides and bases are due to formation of these compounds via nucleotide decomposition.

A straightforward way to test this possibility was to spike nucleotides (and other high energy compounds) into the solvent used to initiate the quenching and extraction process, and to test both for the *loss* of the spiked compounds and for the *generation* of byproducts from them. Use of mass spectrometry as the detection technique enabled us to follow the fate of the spiked compounds via isotope labeling. To avoid the need to purchase or synthesize a broad spectrum of isotope-labeled compounds, we instead used U-[<sup>13</sup>C]-glucose to produce fully isotope-labeled *E. coli*, which we spiked with unlabeled (essentially <sup>12</sup>C) compounds.

The resulting data clearly show substantial loss of high energy compounds in all of the extraction solvents, but especially in the high water conditions (Fig. 3). They also reveal direct evidence for the nucleotides being converted into nucleosides and bases (Fig. 4). Critically, while there are many potential explanations for loss of spiked compounds (sequestration, absorption, decomposition, etc.), *only decomposition explains the associated formation of byproducts of the spiked compounds* (e.g., nucleosides from nucleotide triphosphates). Impressively, the precise extraction condition resulting in maximum loss of particular spiked compounds (e.g., 80% water for acetyl CoA; 50% water for CTP) generally matches the precise condition producing the lowest yield of the endogenous form of that compound from cells.

The present data clearly indicate that 80:20 methanol:water is a superior extraction solvent to 50:50 or 20:80 methanol:water, in terms of providing a better yield of high energy compounds and a lower yield of decomposition products. The choice between 80:20 and 100:0 methanol:water is less clear, as 80:20 methanol:water yields a larger quantity of nucleotides, especially triphosphates (Fig. 1C), which may be poorly soluble in pure methanol (see e.g.,

GTP in Figure 3A), at the expense of also yielding more decomposition products (Fig. 4A). Interestingly, similar results for 80:20 methanol:water were obtained across the temperature range of -70°C to 4°C, suggesting that sample handling on wet ice may be adequate to avoid substantial metabolite decomposition.

Two key caveats should be provided regarding our findings. One is that only methanol:water mixtures were explored. Thus, other solvent compositions [22,26,36,37], pHs [22,28,38,39], or preservatives [37,40,41] may produce substantially superior extracts, with stronger organic solvents potentially having advantages for more completely disrupting membrane structures. The other is that, to gather informative data regarding extraction without introducing complex cell handling procedures or risking metabolite loss via cell leakage, we rely here on the disadvantageously slow approach of harvesting cells by centrifugation. More sophisticated cell handling will eventually be required to address biological questions.

In conclusion, the present report focuses narrowly on improving understanding of the extraction step of metabolomic protocols. Within the realm of extraction *per se*, it makes three contributions. First, we show that very similar procedures, falling under the general rubric of “cold methanol,” yield quite different metabolite extracts depending on the fractional water composition of the methanol:water mixture used. Secondly, we clearly relate the observed metabolome differences to decomposition of high energy compounds to form low energy byproducts, which occurs preferentially under more water-rich conditions. Finally, we provide a general approach, involving spiking with isotope tracers, for identifying decomposition products in metabolite extracts. We believe that further application of this spiking procedure to a diversity of extraction conditions and cell systems should advance overall understanding of the best means of extracting cellular metabolites.

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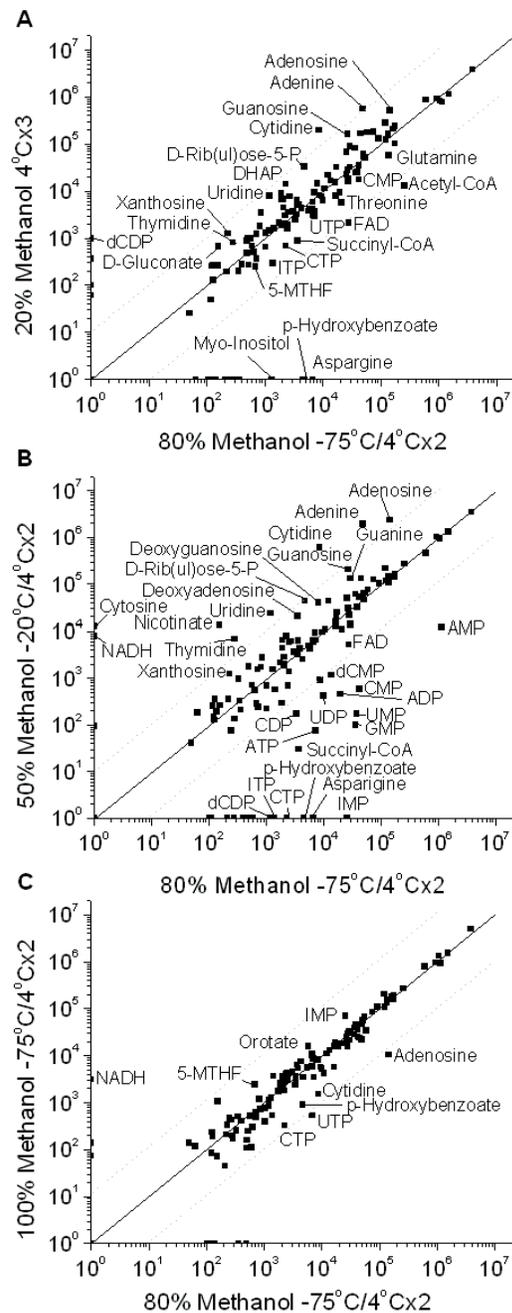
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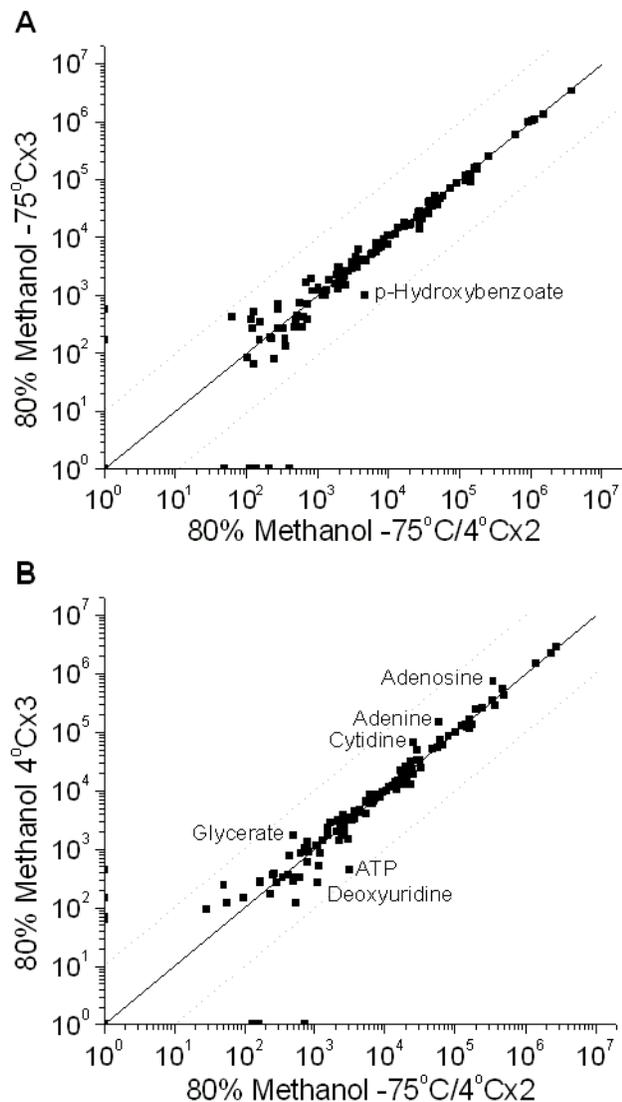
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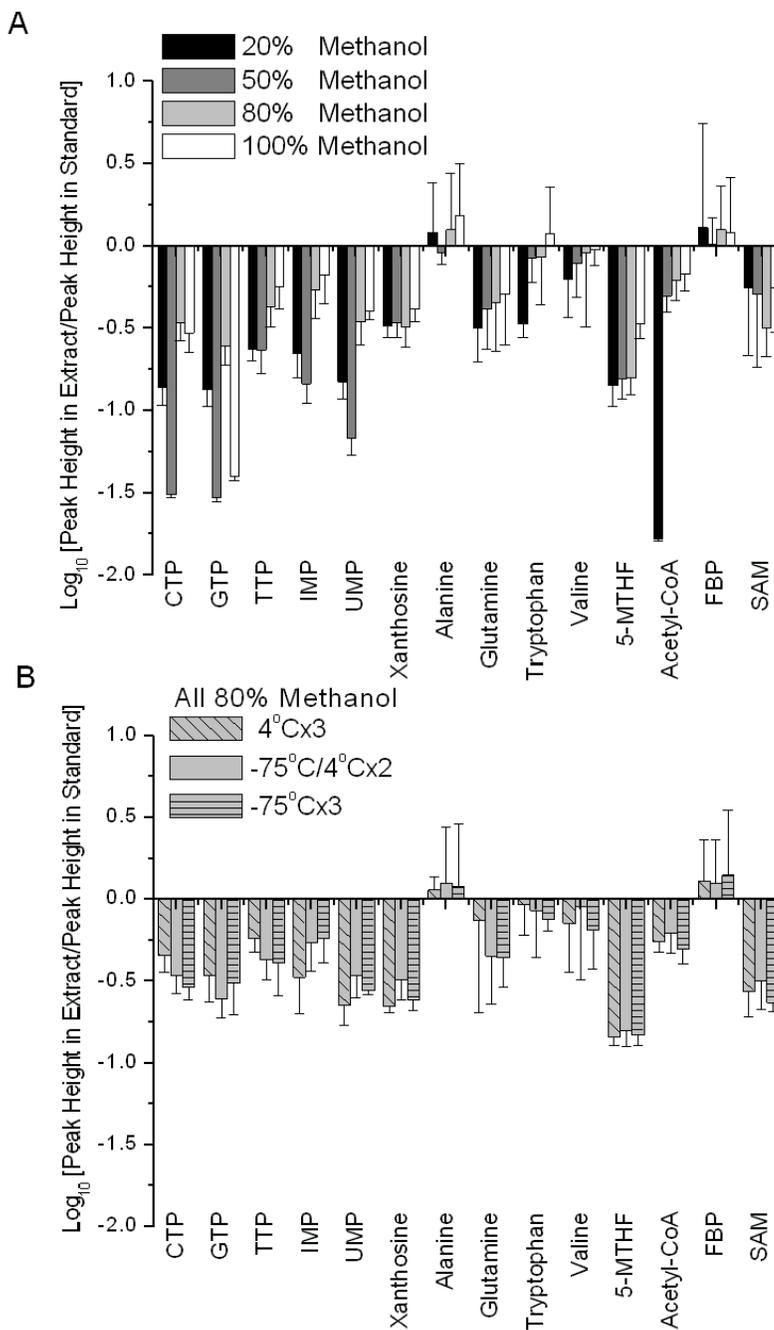
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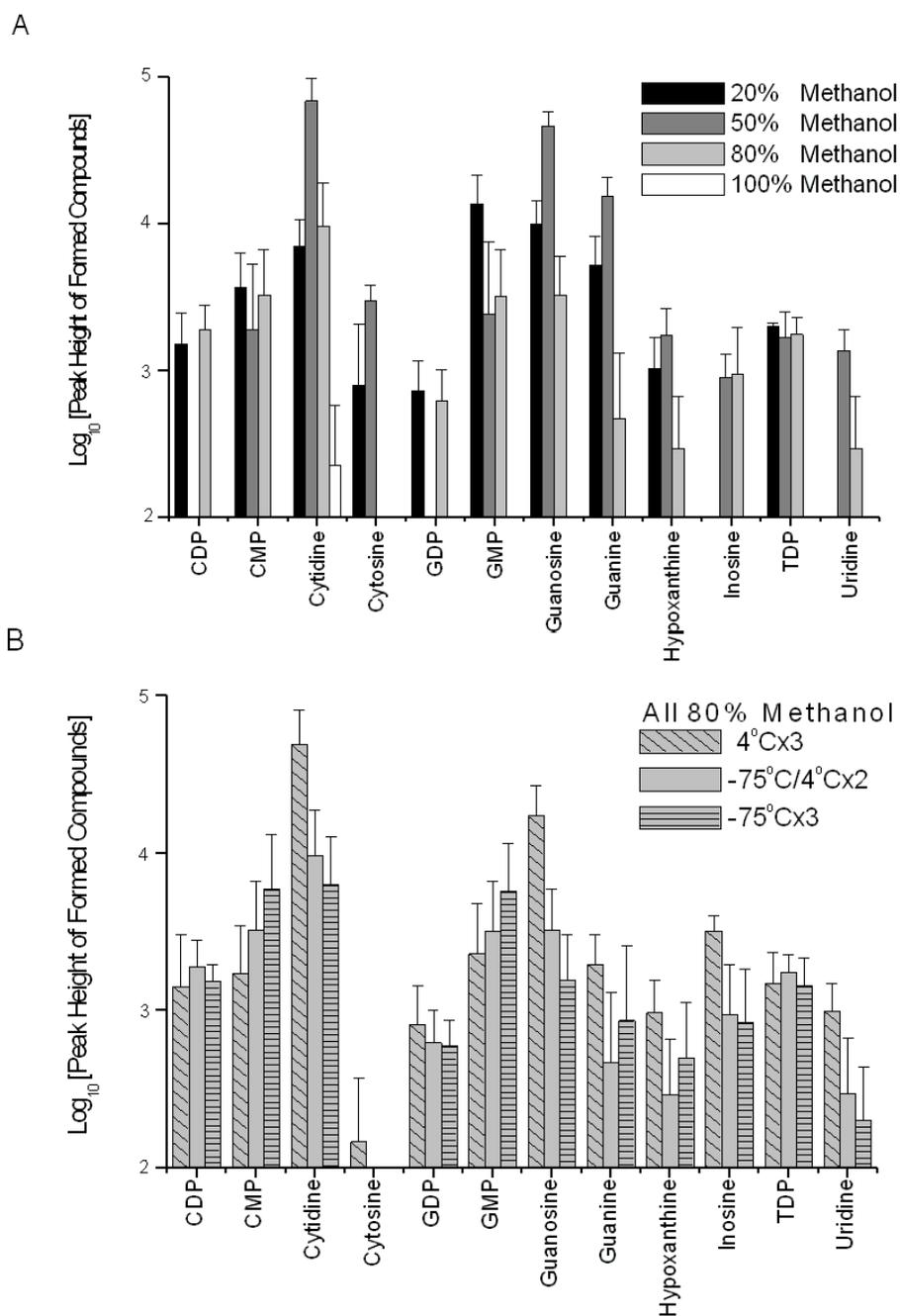
**Fig 1.** Effect of extraction solvent composition (methanol:water ratio) on endogenous metabolite yield. Each point represents a single metabolite, with the measured LC-MS/MS signal for that metabolite in 80:20 methanol:water extract plotted on the X-axis, and the signal for the same metabolite in the indicated alternative solvent composition extract plotted on the Y-axis. Each plotted value represents the mean of duplicate measurements. Compounds insensitive to extraction solvent composition fall on the line of unity (solid). Dashed lines show the boundaries of 10-fold metabolite signal changes. Metabolites not detected in a particular condition are shown on the axes. Compounds that are particularly sensitive to the extraction conditions are labeled.



**Fig 2.** Effect of temperature on endogenous metabolite yield. Each point represents a single metabolite measured from two different 80:20 methanol:water extracts. The X-axis extract was produced by first extracting once at -75°C, and then twice at 4°C. The Y-axis extract was produced by extracting three times, each at -75°C (A) or 4°C (B). Each plotted value represents the mean of duplicate measurements from independent extracts. Compounds insensitive to extraction temperature fall on the line of unity (solid). Dashed lines show the boundaries of 10-fold metabolite signal changes. Metabolites not detected in a particular condition are shown on the axes. Compounds that are particularly sensitive to the extraction conditions are labeled.



**Fig 3.** Recovery of spiked metabolites. Fully  $^{13}\text{C}$ -labeled *E. coli* were quenched and extracted using solvent spiked with  $^{12}\text{C}$  metabolites. The signal of the spiked  $^{12}\text{C}$  metabolites obtained in the resulting extract is compared to the signal anticipated assuming no metabolite loss, with downward facing bars indicating metabolite loss and upward facing bars metabolite gain. (A) Effect of extraction solvent methanol:water ratio. (B) Effect of extraction temperature. Plotted data are the mean  $\pm$  standard deviation of quadruplicate measurements.



**Fig 4.** Formation of nucleotide decomposition products. Fully  $^{13}\text{C}$ -labeled *E. coli* were quenched and extracted using solvent spiked with  $^{12}\text{C}$  nucleotides. The presence of  $^{12}\text{C}$  decomposition products of these nucleotides in the resulting extract was then measured. (A) Effect of extraction solvent methanol:water ratio. (B) Effect of extraction temperature. Plotted data are the mean  $\pm$  standard deviation of quadruplicate measurements. Background signal arising from  $^{12}\text{C}$  nucleotide decomposition products present in the nucleotide standard mix have been subtracted from the reported values.