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Evaluation of metabolite extraction strategies from tissue samples using NMR metabolomics

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Metabolomic analysis of tissue samples can be applied across multiple fields including medicine, toxicology, and environmental sciences. A thorough evaluation of several metabolite extraction procedures from tissues is therefore warranted. This has been achieved at two research laboratories using muscle and liver tissues from fish. Multiple replicates of homogenous tissues were extracted using the following solvent systems of varying polarities: perchloric acid, acetonitrile/water, methanol/water, and methanol/chloroform/water. Extraction of metabolites from ground wet tissue, ground dry tissue, and homogenized wet tissue was also compared. The hydrophilic metabolites were analyzed using 1-dimensional (1D) ^{1}H nuclear magnetic resonance (NMR) spectroscopy and projections of 2-dimensional J-resolved (p-JRES) NMR, and the spectra evaluated using principal components analysis. Yield, reproducibility, ease, and speed were the criteria for assessing the quality of an extraction protocol for metabolomics. Both laboratories observed that the yields of low molecular weight metabolites were similar among the solvent extractions; however, acetonitrile-based extractions provided poorer fractionation and extracted lipids and macromolecules into the polar solvent. Extraction using perchloric acid produced the greatest variation between replicates due to peak shifts in the spectra, while acetonitrile-based extraction produced highest reproducibility. Spectra from extraction of ground wet tissues generated more macromolecules and lower reproducibility compared with other tissue disruption methods. The p-JRES NMR approach reduced peak congestion and yielded flatter baselines, and subsequently separated the metabolic fingerprints of different samples more clearly than by 1D NMR. Overall, single organic solvent extractions are quick and easy and produce reasonable results. However, considering both yield and reproducibility of the hydrophilic metabolites as well as recovery of the hydrophobic metabolites, we conclude that the methanol/chloroform/water extraction is the preferred method.

KEY WORDS: metabolomics; NMR; J-resolved; metabolite extraction; sample preparation; tissue.

Abbreviations: UCD: University of California, Davis; UB: University of Birmingham; NMR: nuclear magnetic resonance; PCA: principal components analysis; p-JRES: projected J-resolved; 1D: 1-dimensional; 2D: 2-dimensional; TMSP: sodium 3-trimethylsilyl-2,2,3,3-d_4-propionate; AW: acetonitrile/water; MW: methanol/water; MC: methanol/chloroform/water

1. Introduction

Metabolites, as the end products of metabolism, represent the functional responses of a cell. Their characterization can provide insight into the underlying mechanisms of genomic or environmental actions on metabolism. In general, two types of samples – biofluids and tissues – can be utilized for metabolic analysis. While biofluids effectively integrate the metabolic changes that occur in an animal's organs (Holmes *et al*., 2000; Heijne *et al*., 2005), tissue samples can be used to determine organ-specific metabolic fingerprints. These can be of particular interest when investigating certain diseases (Pears *et al*., 2005; Stentiford *et al*., 2005) or sites of toxicity (Garrod *et al*., 2001). Although metabolomic analysis of tissues is widely applied across fields such as medicine, toxicology and the environmental sciences, there still lacks a thoroughly validated method for sample preparation.

It is well known that the rapid termination of enzyme activity is important to achieve an accurate measure of the metabolome. Typically, tissues are flash frozen in liquid N_2 to temporarily halt metabolism, then the enzymes are precipitated by treatment with acid (Viant 2003; Pears *et al*., 2005) or cold mixtures of organic solvents such as methanol, ethanol, acetone, or acetonitrile (Coen *et al*., 2003; Kim *et al*., 2004; Stentiford *et al*., 2005). After they are flash frozen, tissues should either be kept frozen below −70 °C or lyophilized to avoid recovery of enzymatic activity. Metabolite extraction using either wet frozen or freeze-dried samples has been reported (Warne *et al*., 2001; Bundy *et al*., 2004; Viant *et al*., 2005a). However, even though tissues remain cold under the vacuum of the freeze drying process, the actual tissue temperature may not always be

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low enough to stop all enzymatic activity. It is therefore important to compare these two extraction methods using replicate biological samples.

Prior to sample extraction, different methods can be used to break up tissues and cells. The most common methods include grinding in a liquid N_2-cooled mortar and pestle (Rosenblum *et al*., 2005; Viant *et al*., 2005a) or homogenization by an electric tissue homogenizer directly in the extraction solvent (Warne *et al*., 2001; Pears *et al*., 2005). Traditionally, grinding frozen tissues by mortar and pestle has been the 'gold standard,' but is very labor intensive. Great care must also be taken to avoid partially thawing the tissues while grinding. Homogenization is relatively straightforward, but this approach may not be practical for small amounts of tissue.

It is easier to establish an efficient extraction method for a specific metabolite or class of metabolites. For example, ultrasonication of berries with methanol and water produces the highest recovery of secologanin, a monoterpene glycoside, compared with other extraction methods (Kim *et al*., 2004). Due to the large variety of metabolites contained within tissues, all with highly differing physical and chemical properties, there is no one ideal way to simultaneously extract all classes of metabolites with high efficiency. For metabolic fingerprinting studies, perchloric acid is widely used to precipitate proteins and extract hydrophilic metabolites. Although advantageous for extracting amines (Bouchereau *et al*., 2000), acidic treatments are problematic for many analytical methods and may irreversibly damage the structure of metabolites. Polar organic solvents such as methanol, ethanol, acetonitrile and acetone, are typically mixed with water to extract hydrophilic metabolites (Coen *et al*., 2003; Kim *et al*., 2004; Stentiford *et al*., 2005). Chloroform can be used to extract hydrophobic metabolites (Choi *et al*., 2004; Stentiford *et al*., 2005). There are a few published studies that compare the mass spectrometry-based metabolic fingerprints of plants, microorganisms, or biofluids extracted using different systems (Castrillo *et al*., 2003; Gullberg *et al*. 2004; Want *et al*., 2006). A comparison of methanol/chloroform and perchloric acid extractions of brain tissue for high-resolution NMR analysis showed that the former method gave higher yields and reproducibility for selected metabolites (Le Belle *et al*., 2002). However, to our knowledge, a thorough comparison of several different extraction solvents for NMR-based metabolomic studies of tissues has not been published. An optimized method with good yield and reproducibility, which can be demonstrated to be consistent between research laboratories, will ultimately provide more reliable metabolic fingerprint data.

This study was undertaken jointly by two metabolomic research laboratories, one at the University of California, Davis (UCD), USA, the other at The University of Birmingham (UB), UK. Our goal was to identify a rapid, straightforward and reproducible method to extract metabolites from tissues. Moreover, two different tissues were used in two different laboratories in order to help confirm that our findings are universal and not specific to one laboratory or tissue type. Both of our laboratories have used the same sample preparation protocols but different types of tissues (muscle and liver) to evaluate several tissue preparation strategies, including (1) tissue disruption methods by grinding with pestle and mortar, and by electric homogenisation, and (2) extraction using seven solvent systems for metabolic analysis. Moreover, spectral data from 1-dimensional (1D) ^{1}H NMR and projections from 2-dimensional (2D) J-resolved (p-JRES) spectroscopy were also compared for these different solvent systems. Principal components analysis (PCA) compared the reproducibility of metabolic fingerprints derived from replicate biological samples.

2. Materials and methods

2.1. Animals

Muscle from Chinook salmon (*Onchorhyncus tshawytscha*) smolts and liver from adult chub (*Leuciscus cephalus*) were used in this study at UCD and UB, respectively. Salmon smolts were obtained from the California Department of Fish and Game's American River hatchery, while chub were collected from a UK river as part of the CITYFISH project. After fish were sacrificed, tissues were immediately dissected, flash frozen in liquid N_2, and stored at −80 °C until extraction.

2.2. Metabolite solvent extraction

Extraction methods were taken, and in some cases modified, from previous work (Viant *et al*., 2003a; Coen *et al*., 2003; Stentiford *et al*., 2005). Frozen muscle samples were pooled and then ground together in a liquid N_2-cooled mortar and lyophilized overnight. The homogenous dry tissue powder was weighed and divided into 40 Eppendorf vials, and then extracted with 30 ml/g (dry mass) of either (1) ice cold 6% perchloric acid, (2) acetonitrile/water (1/1), (3) acetonitrile/water (2/1), (4) methanol/water (1/1), (5) methanol/water (2/1), (6) methanol/chloroform/water, or (7) methanol/chloroform/water with 0.8% KCl ($n = 5$ each, except for the perchloric acid extractions with $n = 10$). The final ratio for the bi-phasic methanol/chloroform/water extraction for separating the polar and non-polar fractions was 2/2/1.8 (Bligh and Dyer, 1959). Samples were vortexed for 15 s three times and put on ice in between. Following centrifugation (10,000 g, 10 min, 4 °C), 0.46 ml of supernatant (for methanol and acetonitrile extractions) or the entire upper layer (for methanol/chloroform/water extractions) was removed and then lyophilized. The supernatant from the perchloric acid extraction was

neutralized to a pH of ca. 7.2 with 2 M K_2CO_3 (measured by dropping a few μl of supernatant onto pH paper), kept on ice for 30 min, and then centrifuged again as above. The resulting supernatant was removed in 0.46 ml aliquots and then lyophilized prior to NMR analyses (see figure 1 for summary of experimental design).

Liver tissues from two chub were ground separately under liquid nitrogen by mortar and pestle. The homogenous tissue powder from one liver was divided into 20 glass vials and extracted with either (1) acetonitrile/water (1/1), (2) acetonitrile/water (2/1), (3) methanol/water (1/1), or (4) methanol/water (2/1); each $n = 5$. The second liver was extracted using acetonitrile/water (2/1, $n = 6$), methanol/water (2/1, $n = 6$) and methanol/chloroform/water (2/2/1.8, $n = 5$) (see figure 1).

2.3. Tissue disruption methods

One piece of frozen muscle tissue from a salmon smolt was broken apart in a liquid N_2-cooled pestle and mortar. Larger pieces of the tissue were then weighed and homogenized using an electric tissue homogenizer (Tissumizer model TP 18/10S1; Tekmar, Cincinnati, OH) with 5 ml/g (wet mass) methanol/water (2/1, $n = 5$). The remaining tissue was ground to powder with a liquid N_2-cooled pestle and mortar and divided into 10 Eppendorf vials; samples remained frozen throughout. Five of the ground tissues were then lyophilized and extracted in 30 ml/g (dry mass) methanol/water (2/1, $n = 5$), while the other five samples were extracted directly using 5 ml/g (wet mass) methanol/water (2/1, $n = 5$). Tissue extracts were centrifuged (10,000 g, 10 min, 4 °C), and the supernatants were removed and lyophilized prior to NMR analyses (see figure 1).

2.4. 1D ^{1}H and 2D J-resolved NMR spectroscopy

Metabolomic analyses were performed as previously described, with slight modifications (Viant, 2003). First, all lyophilized extracts were resuspended with sodium phosphate buffer in D_2O (0.1 M, pH 7.4) containing sodium 3-trimethylsilyl-2,2,3,3-d_4-propionate (TMSP), which served as an internal chemical shift standard. All NMR spectra were measured at 500.11 MHz using Avance DRX-500 spectrometers (Bruker, Fremont, CA), one at UCD and one at UB. Acquisition parameters for the 1D NMR spectroscopy consisted of a 9-μs (60°) pulse, 6-kHz spectral width, 2.5-s relaxation delay with presaturation of the residual water resonance, and 100 transients collected into 32 k data points, requiring a 9-min total acquisition time. All data sets were zero-filled to 64 k points, exponential line-broadenings of 0.5 Hz were applied before Fourier transformation, the spectra were phase and baseline corrected and then calibrated (TMSP, 0.0 ppm) using XWINNMR software (Version 3.1; Bruker).

2D J-resolved NMR spectra were acquired using 4 transients per increment for a total of 32 increments, which were collected into 16 k data points using spectral widths of 6 kHz in F2 (chemical shift axis) and 40 Hz in F1 (spin–spin coupling constant axis). A 3.0-s relaxation delay was employed, giving a total acquisition time of 11 min. Datasets were zero-filled to 128 points in F1, and both dimensions multiplied by sine-bell window functions prior to Fourier transformation. Spectra were tilted by 45°, symmetrized about F1, calibrated (TMSP, 0.0 ppm), and the proton-decoupled skyline projections (p-JRES) obtained, all using XWINNMR. Selected peaks were assigned by comparison to tabulated chemical shifts (Fan, 1996; Viant *et al.*, 2003b, 2005b) and by the Chenomx NMR software suite (Professional Edition, Version 4.0, Chenomx, Inc.).

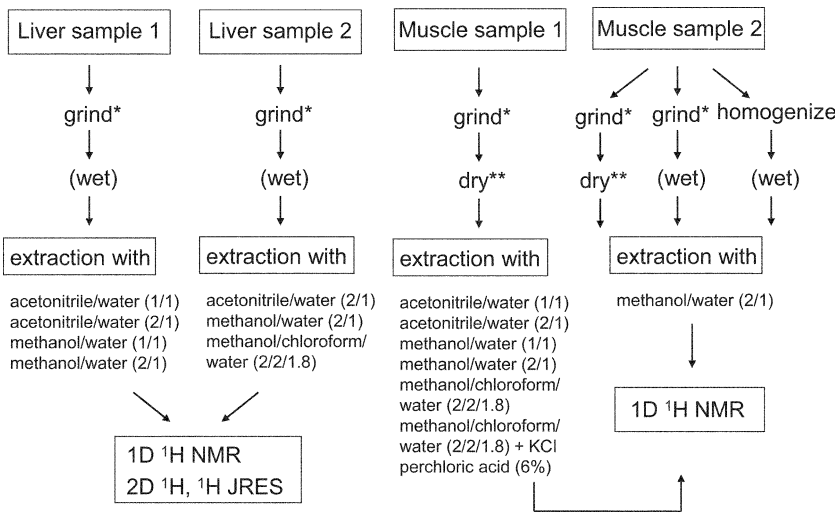

Figure 1. A flow chart summarizes the tissue preparation methods conducted at UB (liver) and UCD (muscle). * denotes grinding in a mortar and pestle under liquid nitrogen. ** denotes drying of tissue in a lyophilizer prior to extraction.

2.5. NMR spectral pre-processing and statistical analyses

Each spectrum was segmented into 1960 chemical shift bins between 0.2 and 10.0 ppm, corresponding to a bin width of 0.005 ppm (2.5 Hz), using custom-written *ProMetab* software (Version 2.1; Viant, 2003) in MATLAB (The MathWorks, Natick, MA). The area within each spectral bin was integrated to yield a 1×1960 vector containing intensity-based descriptors of the original spectrum. Bins representing the residual water peak (from 4.60 to 5.20 ppm for muscle and 4.68 to 5.20 ppm for liver) were removed. In some cases, groups of bins were compressed into a single bin in order to capture peaks with variable chemical shifts into a single bin. The total spectral area of the remaining bins was normalized to unity to facilitate comparison between the spectra. The binned data was subject to the generalized log transformation ($\lambda = 8\times10^{-9}$; Purohit *et al.*, 2004), and the columns mean-centered before multivariate analysis.

PCA of the pre-processed NMR data was conducted using the PLS_Toolbox (Version 3.5; Eigenvector Research, Manson, WA) within MATLAB. Each data set was examined to identify potential outliers as well as the degree of similarity between the metabolic fingerprints, in an unsupervised manner. In this pattern recognition technique, the algorithm calculates the highest amount of correlated variation along PC1, with subsequent PCs containing correspondingly smaller amounts of variance. For each model built, the loading vector for the PC was examined to identify which metabolites contributed to these clusters.

3. Results

3.1. Metabolite solvent extraction

Representative 1D and 2D NMR spectra showing the metabolic fingerprints of muscle and liver extracts are presented in figure 2. Following PCA, the spectra of muscle perchloric acid extracts showed the largest sample-to-sample variation, as evidenced by the lack of clustering in the PCA scores plot that was likely due to pH-induced variability in the peak chemical shifts (figure 3a). The NMR data from the perchloric acid extracts was then excluded in order to examine the metabolic fingerprints that resulted from the other solvent extractions. Extracts of methanol/chloroform/water with KCl were separated from other solvent systems along PC1 (data not shown) due to numerous peaks moving to higher chemical shift values. After this shifting was effectively removed by combining the several bins that contained a given peak, PC1 was able to partially differentiate the metabolic fingerprints from the six extraction methods (figure 3b). The loadings plot for a specific PC explains which metabolites contribute to the variation in that component, as shown for PC1

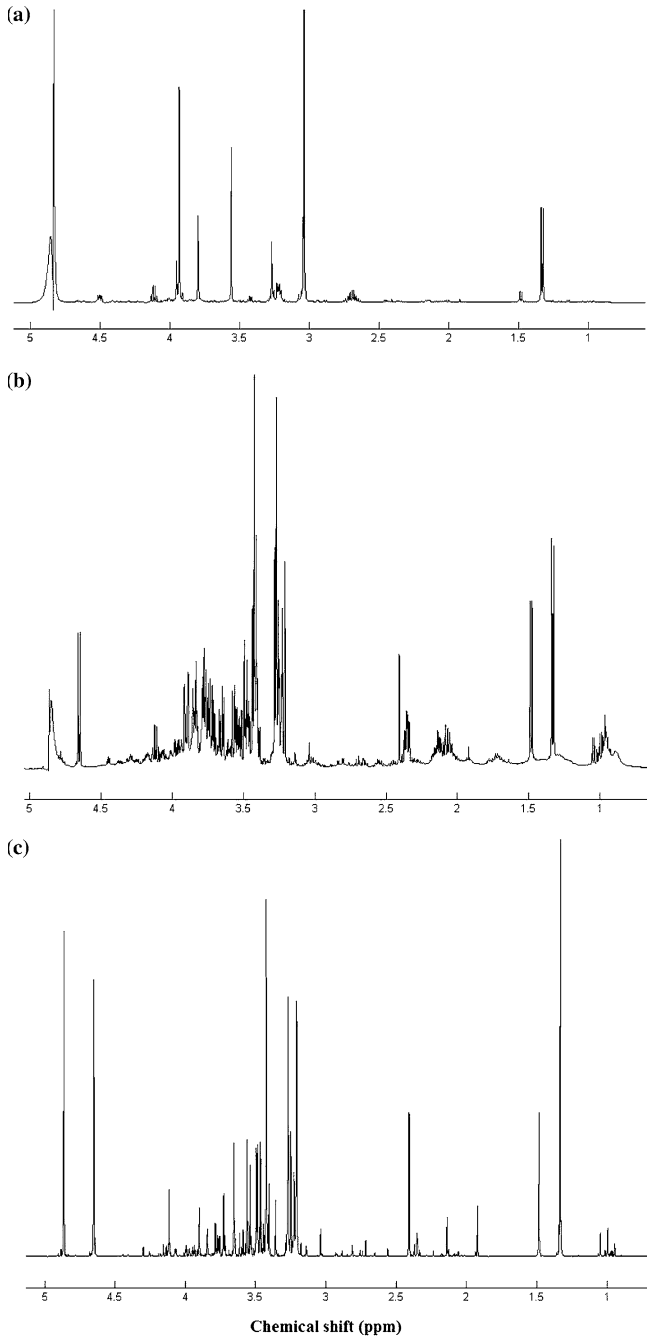

Figure 2. Representative 1D ^{1}H NMR spectra of the methanol/water (2/1) extracts from (a) ground, freeze-dried smolt muscle and (b) ground, wet chub liver, and (c) a 1D projection of a 2D J-resolved NMR spectrum of the extracts from the same chub liver.

(figure 3c). Therefore, methanol/water, methanol/chloroform, and methanol/chloroform + KCl were found to extract mostly low molecular weight metabolites that give rise to sharp peaks, whereas acetonitrile extraction tends to also recover non-specific lipids and various macromolecules (e.g., lipoproteins) which give rise to the broad negative peaks in figure 3c. Several peaks were identified from the loadings plots associated with PC1 (figure 3c), including lactate (chemical shift (δ) 1.33,

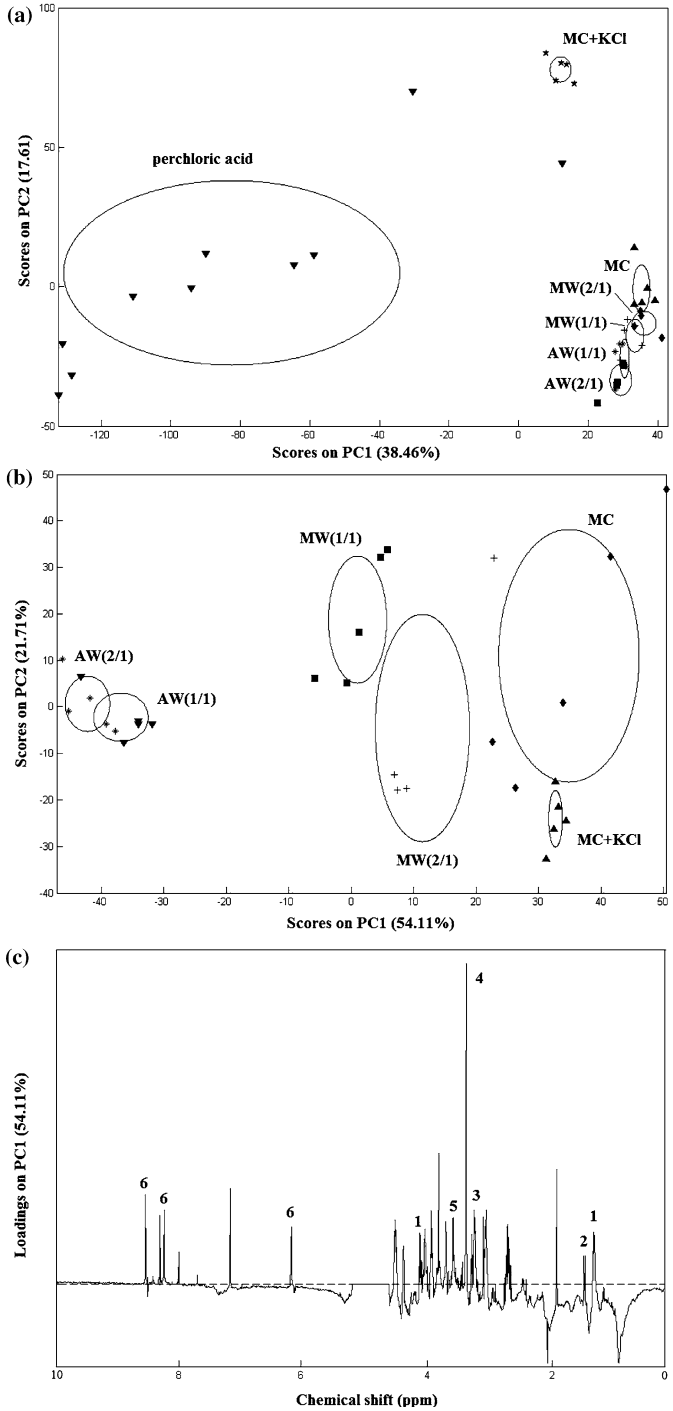

Figure 3. PCA scores plots from the NMR spectra of salmon smolt muscles extracted using (a) perchloric acid, AW (1/1), AW (2/1), MW (1/1), MW (2/1), MC and MC + KCl (n = 5 each, except n = 10 for perchloric acid), (b) the six remaining extraction solvents after removal of perchloric acid and after correction of peak shift artefacts, and (c) the PC1 loadings plot that corresponds to the scores data in (b). Ellipses represent mean ± SD for each extraction method. Legend: AW (1/1) = acetonitrile/water (1/1), AW (2/1) = acetonitrile /water (2/1), MW (1/1) = methanol/water (1/1), MW (2/1) = methanol/water (2/1), MC = methanol/chloroform/water, and MC + KCl = MC with 0.8% KCl. Metabolite assignments: 1. lactate; 2. alanine; 3. taurine; 4. glycerophosphorylcholine; 5. glycine; 6. ATP (coincident with ADP).

4.12 ppm), alanine (δ 1.49), taurine (δ 3.27, 3.42), glycerophosphorylcholine (δ 3.36), glycine (δ 3.56), and ATP (coincident with ADP; δ 6.16, 8.27, 8.55).

The homogenous frozen liver powder was extracted and analyzed using both ^{1}H and p-JRES NMR (figure 2b, c). The p-JRES NMR spectra possess flatter baselines and lower peak densities than corresponding 1D ^{1}H NMR spectra, as observed previously (Viant, 2003). For example, broad peaks (possibly from macromolecules such as lipids and lipoproteins) were

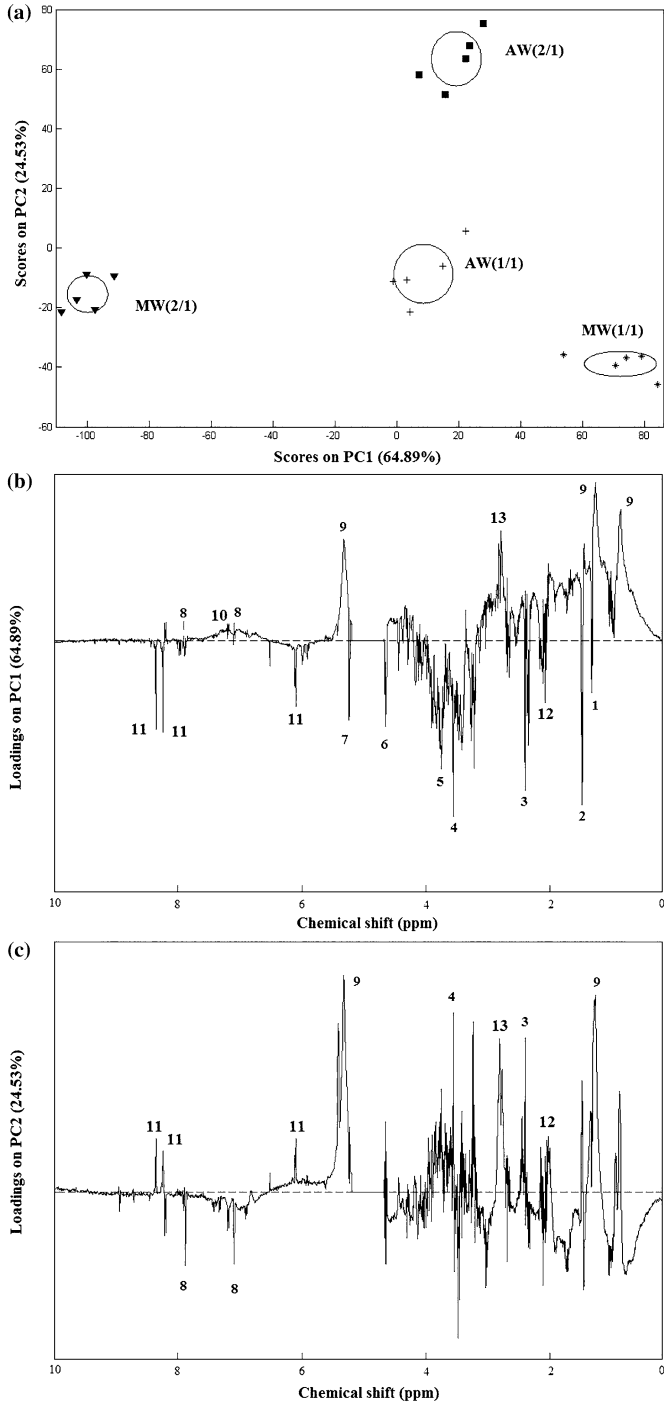

Figure 4. (a) PCA scores plot, (b) PC1 loadings plot and (c) PC2 loadings plot from the analysis of the 1D ^{1}H NMR spectra of the chub liver extracted using acetonitrile/water [AW (1/1), AW (2/1)] and methanol/water [MW (1/1) and MW (2/1)]. Ellipses represent mean ± SD for each extraction method. Metabolite assignments: 1. lactate; 2. alanine; 3. succinate; 4. glycine; 5. glutamate; 6. α-glucose; 7. β-glucose; 8. histidine; 9. lipid; 10. tyrosine; 11. inosine; 12. proline; 13. aspartate.

observed near 1 ppm in the 1D ^{1}H NMR spectra, but were absent in the p-JRES spectra. The PCA scores plots from analysis of both the 1D ^{1}H and p-JRES NMR spectra (figures 4a and 5a, respectively) indicate clear differences between the liver metabolic fingerprints from the four extraction methods. Features causing these groupings were identified from the loadings plots associated with the PC1 and PC2 axes (figures 4b, c, 5b, c). The loadings plots from the 1D NMR data showed a highly variable baseline and a significant contribution from macromolecules, while the corresponding plots from the p-JRES data were considerably simpler, with multiple sharp resonances superimposed on flat baselines. Considering PC1 from the analysis of p-JRES NMR data, higher levels of leucine, valine, lactate, aspartate, glucose and inosine were extracted by

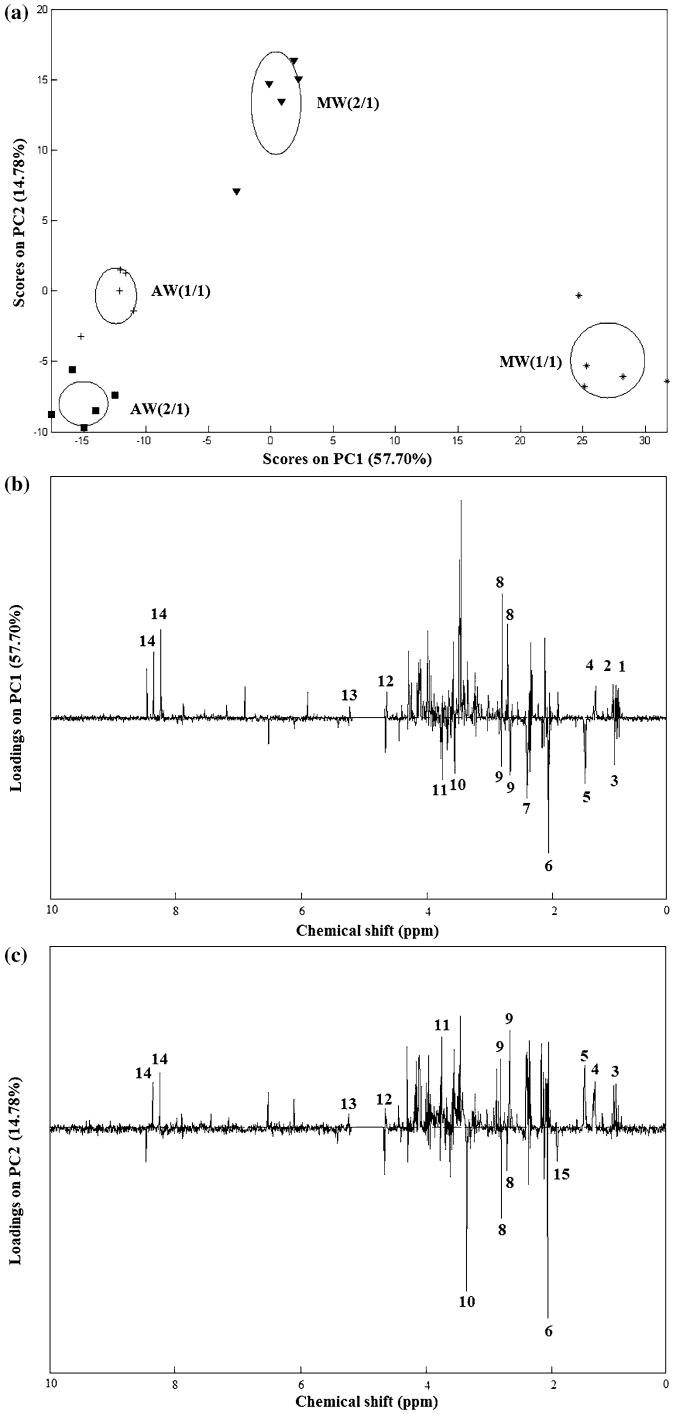

Figure 5. (a) PCA scores plot, (b) PC1 loadings plot and (c) PC2 loadings plot from the analysis of the projections of the 2D J-resolved NMR spectra of the chub liver extracted using acetonitrile/water [AW (1/1), AW (2/1)] and methanol/water [MW (1/1) and MW (2/1)]. Ellipses represent mean ± SD for each extraction method. Metabolite assignments: 1. leucine; 2. valine; 3. isoleucine; 4. lactate; 5. alanine; 6. proline; 7. succinate; 8. aspartate; 9. citrate; 10. glycine; 11. glutamate; 12. α-glucose; 13. β-glucose 14. inosine; 15. acetate.

methanol/water (1/1). Conversely, higher concentrations of isoleucine, alanine, proline, succinate, citrate, glycine and glutamate occurred in the extracts of acetonitrile/water (1/1 and 2/1). Considering PC2, methanol/water (2/1) extracted more isoleucine, lactate, alanine, citrate, glucose, glutamate and inosine.

Additional solvent extractions were conducted using a second chub liver. Both 1D ^{1}H and p-JRES NMR spectra (from both chub) were analyzed by PCA, and the resulting scores plots are shown in figure 6a and b, respectively. Again, a large distinction between the 1D and p-JRES NMR data sets is visible with the PCA

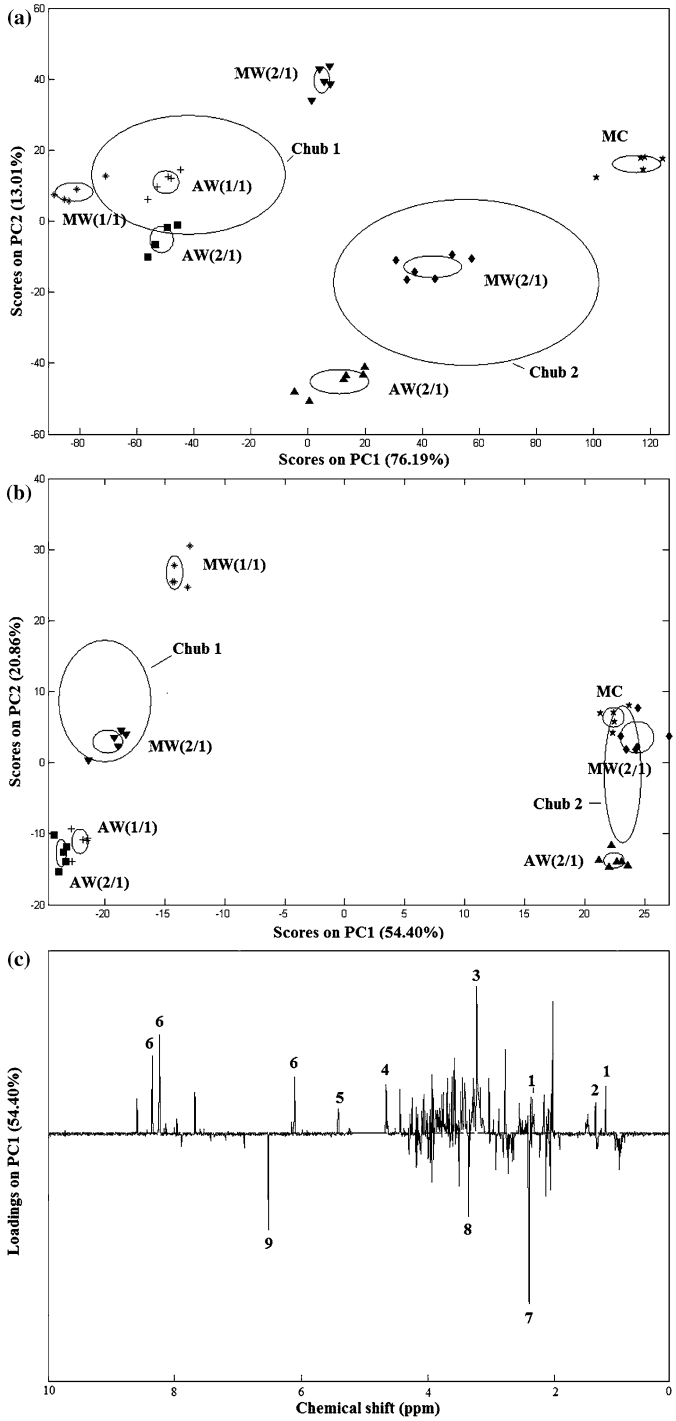

Figure 6. PCA scores plots from the analysis of (a) the 1D ^{1}H and (b) projections of the 2D J-resolved NMR spectra of the extracts from both chub livers that were extracted using acetonitrile/water [AW (1/1) and AW (2/1)], methanol/water [MW (1/1) and MW (2/1)] and methanol/chloroform/water [MC]. Ellipses represent mean ± SD for each extraction method as well as for the mean ± SD for chub 1 and 2 considering all methods together. (c) PC1 loadings plot that corresponds to the scores plot in (b). Metabolite assignments: 1. 3-hydroxyisobutyrate; 2. 2-hydroxyisobutyrate; 3. betaine; 4. α-glucose; 5. β–glucose; 6. inosine; 7. succinate; 8. glycerophosphorylcholine; 9. fumarate.

scores plot from the p-JRES analysis (figure 6b) showing a significantly more obvious discrimination between the individual fish along PC1 compared to the 1D NMR data (figure 6a); the corresponding PC1 loadings plot for the p-JRES data is shown in figure 6c.

3.2. Tissue disruption methods

The 1D NMR spectra of extracts from ground dry tissue showed much flatter baselines than those from both the ground wet and the homogenized wet tissues (data not shown). Moreover, the replicate extractions of

ground dry tissue showed the least variation in PCA space (figure 7a). Extraction of freeze-dried versus wet tissues caused a significant difference in the metabolic fingerprints of the muscle tissue along PC1. The corresponding loadings plot is shown in fig 7b, which shows the contribution of the undulating baseline as well as broad resonances in separating the treatment groups. The extracts from ground wet (frozen) tissues yielded spectra with higher baselines even when extracted with other solvent systems (data not shown).

4. Discussion

4.1. Peak shifting in the spectra

Inconsistencies in the positions of the NMR peaks resulted in the major source of variation between replicate samples, especially in the perchloric acid extraction group. Similar findings have been suggested previously (Defernez and Colquhoun, 2003). Even after buffering of the perchloric acid extracts, metabolites with pK values close to 7.2 (the pH at which many NMR measurements are conducted) are extremely sensitive to small variations in sample pH, which results in peak shifting. Other factors such as metal concentration, metabolite–protein interactions, or chemical exchanges may also alter peak position (Cloarec *et al.*, 2005). Peak shifting was also observed in the spectra of the aqueous layer following methanol/chloroform/water extraction with KCl. KCl serves to improve partitioning of polar phospholipids into the chloroform layer by increasing the polarity of the aqueous phase. Unlike the variable peak shifting in the spectra of perchloric acid extracts, a few peaks from the methanol/chloroform/water with KCl extraction consistently moved to a higher chemical shift. This is most likely caused by the salt inducing a small but consistent change in the solution (matrix effect) compared to the other extraction methods. These results highlight the problem of extracting tissues using perchloric acid (or any other acid) as the somewhat

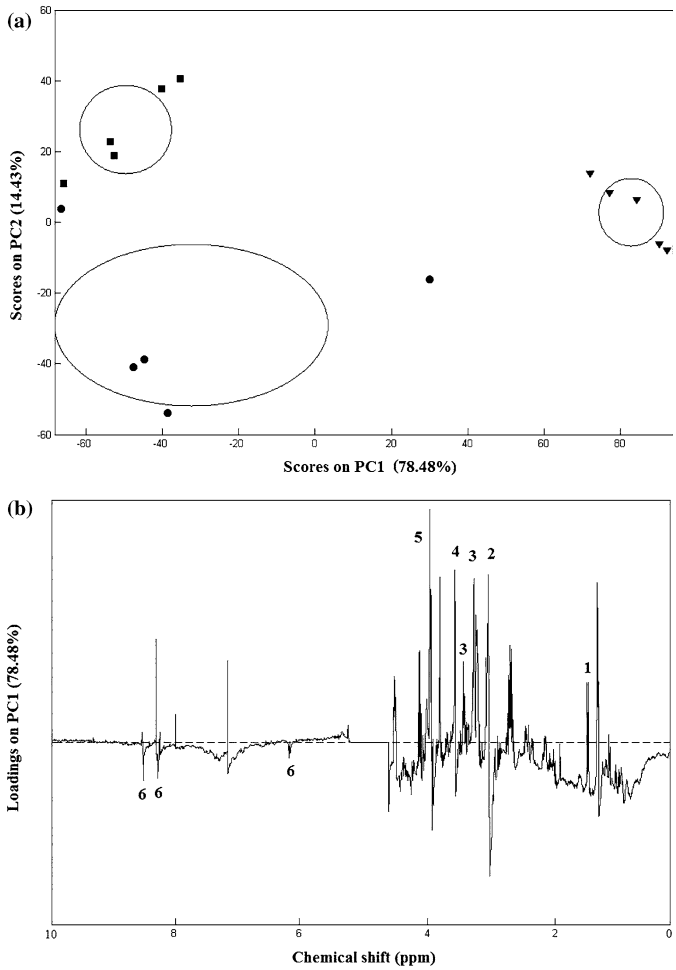

Figure 7. (a) PCA scores plot from analysis of 1D ^{1}H NMR spectra of muscle tissues that were extracted using MW (2/1) after being ground and freeze-dried (▼), ground only (●) or homogenized only (■) and (b) PC1 loadings plot that corresponds to the scores plot in (a). Ellipses represent mean ± SD for each extraction method. Metabolite assignments: 1. lactate; 2. alanine; 3. lysine; 4; taurine; 5. glycine; 6. ATP (coincident with ADP).

variable final pH will induce peak shifting in the NMR spectra. The small but constant peak shift caused by KCl in the methanol/chloroform/water extraction is not in itself detrimental. However, NMR measurements of metabolites using a cryoprobe as well as metabolite analysis using mass spectrometry would both be compromised by addition of KCl, and therefore this approach is also not recommended.

4.2. Metabolite solvent extraction

The criteria for assessing the quality of a tissue extraction protocol for metabolomics include yield, reproducibility, ease and speed, each of which is addressed below (see table 3). Since low molecular weight metabolites are often co-extracted with compounds such as lipids and lipoproteins (which produce broad resonances in 1D NMR spectra), the net yield of extracts (i.e. the total peak area of the 1D NMR spectra) can only provide an approximate tool for comparing metabolite yields from different extraction protocols. Total NMR spectral area of each extraction was normalized to TMSP signal area and to tissue mass in order to compare yields between extractions. Further, total spectral areas were normalized around acetonitrile/water (2/1) to a yield of 1 (see table 1). According to the 1D NMR data, methanol/water (2/1) and methanol/chloroform/water extractions produce smaller yields, while lipids or lipoproteins are generally recovered to greater extent by acetonitrile/water. In essence, methanol/water (2/1) and methanol/chloroform/water tend not to extract macromolecules and lipids (in the methanol layer). Methanol-based extraction has also been shown to efficiently precipitate proteins from serum (Want *et al*., 2006). The extraction yields were actually elevated in our 2D NMR data where neither lipids nor lipoproteins were observed. The slightly lower yield in the methanol/chloroform/water extraction probably arises from partitioning of some non-polar metabolites into chloroform, so fewer metabolites are in the aqueous phase. Even though methanol/water (1/1) produced the highest yields of small metabolites, the variation was large. Therefore, we conclude that methanol/water (2/1), acetonitrile/water (1/1 or 2/1), or methanol/chloroform/water all produce good consistent yields, although the acetonitrile-based extractions tend to recover more lipids and macromolecules in with the polar metabolites (artificially elevating the observed yield in the 1D NMR data).

Since the different solvent systems have somewhat different polarities, it is expected that different metabolites will be extracted with differing efficiencies. For example, this is evident when considering the extraction of amino acids with R groups of a range of polarities. The PC1 loadings plot of the p-JRES spectra (figure 5b) shows that methanol/water (1/1), containing a relatively high ratio of highly polar solvent (water), preferentially extracted more polar amino acids such as aspartate ($R = CH_2$–COOH), but acetonitrile/water (1/1 or 2/1) extracted more hydrophobic metabolites such as isoleucine ($R = (CH_3)CH(C_2H_5)$), alanine ($R = CH_3$), and proline ($R = C_3H_6$). Clearly a major advantage of the methanol/chloroform/water method is the simultaneous extraction of both hydrophilic and hydrophobic metabolites into two different compartments, which is especially important when addressing the lipid metabolome.

Reproducibility is essential for good sample preparation. We have determined the reproducibility of each extraction strategy using PCA scores plots, which provide an unbiased assessment of the similarities and

Table 1
Comparison of the yields of several metabolite extraction methods

Extraction method[a]	Tissue	Yield[b]	
		1D ^{1}H NMR	2D J-resolved NMR
AW (1/1)	Liver (fish 1)	1.088 ± 0.019	1.193 ± 0.010
	Muscle	1.263 ± 0.049	–
AW (2/1)	Liver (fish 1)	1.000 ± 0.031	1.000 ± 0.033
	Liver (fish 2)	1.000 ± 0.032	1.000 ± 0.025
	Muscle	1.000 ± 0.024	–
MW (1/1)	Liver (fish 1)	1.268 ± 0.052	2.102 ± 0.336
	Muscle	1.109 ± 0.066	–
MW (2/1)	Liver (fish 1)	0.849 ± 0.020	1.107 ± 0.052
	Liver (fish 2)	0.872 ± 0.040	1.158 ± 0.020
	Muscle	0.952 ± 0.022	–
MC	Liver (fish 2)	0.592 ± 0.035	0.902 ± 0.042
	Muscle	0.748 ± 0.074	–
MC + KCl	Muscle	0.872 ± 0.038	–
Perchloric acid	Muscle	0.845 ± 0.039	–

[a]AW: acetonitrile/water; MW: methanol/water; MC: methanol/chloroform/water.
[b]Mean and SD of the yield determined from the ratio of total spectral area/TMSP/tissue mass. Then the yields were normalized around acetonitrile/water (2/1) to a yield of 1.

differences between the metabolic fingerprints of multiple replicates of one biological sample. Specifically, we determined method reproducibility based upon the area of the one standard deviation ellipse (i.e. derived from the standard deviation of the replicate samples along PC1 multiplied by the standard deviation along PC2), using the data from figures 3b, 6a and b (see table 2). In general, both UCD and UB laboratories found that acetonitrile/water extractions yielded comparable sample-to-sample variation compared with methanol/chloroform/water extractions, and much less variation than for methanol/water extractions. Considering reproducibility of the p-JRES spectra of liver only, methanol-based extractions exhibited, on average, twice the variability of the acetonitrile-based extractions. The origin of this difference is unclear. Considering the 1D NMR data, one possibility is that if the lipids and lipoproteins known to be recovered in greater yield by acetonitrile/water are extracted reproducibly, then this would tend to increase the overall reproducibility of the NMR spectra. However, the 2D NMR data shows that this is not the case, since the lower variation in the acetonitrile based extractions is retained in the 2D data where lipids and lipoproteins are not observed. Overall, based upon reproducibility only, these results support the use of either acetonitrile-based or methanol/chloroform/water extractions.

Within the group of methanol-based extractions, the methanol/chloroform/water yielded the least variable metabolic fingerprints based upon the p-JRES spectra of liver, and methanol/chloroform/water with KCl the least variable result in muscle. There was an unexpectedly large effect on reproducibility by adding KCl. Since the extremely high variability of methanol/chloroform/water without KCl was observed only once, this was probably an aberrant result caused by the greater technical challenges associated with bi-phasic extraction. Another unexpected result was the magnitude of the metabolic difference between the 1/1 and 2/1 methanol/water extraction groups (clearly evident in figure 5a), which is large compared to the corresponding difference between the 1/1 and 2/1 acetonitrile/water extraction groups. This highlights an additional advantage of the acetonitrile-based approach since small pipetting errors that may alter the ratio of water to acetonitrile and small differences in the water content of tissue samples will each have a smaller impact on the consistency of the NMR metabolic fingerprints.

Comparing the ease and speed of the methods is somewhat subjective, but in general all the single organic solvent extractions are both easier and faster. However, methanol-based extractions produce less reproducible spectra, while acetonitrile-based extractions recover lipids and macromolecules in with the polar metabolites. Using methanol/chloroform/water is more time consuming, requiring careful transfer of the solvents from the bi-phasic solution. However, it provides both hydrophilic and hydrophobic metabolites, as well as having high yield and reproducibility (see table 3).

Table 2
Comparison of the reproducibility of several metabolite extraction methods

Extraction method[a]	Tissue	Reproducibility[b]	
		1D ^{1}H NMR	2D J-resolved NMR
AW (1/1)	Liver (fish 1)	14.470	1.152
	Muscle	22.796	–
AW (2/1)	Liver (fish 1)	15.523	0.823
	Liver (fish 2)	31.403	0.918
	Muscle	22.288	–
MW (1/1)	Liver (fish 1)	19.329	1.673
	Muscle	64.122	–
MW (2/1)	Liver (fish 1)	10.276	2.025
	Liver (fish 2)	30.906	3.058
	Muscle	185.858	–
MC	Liver (fish 2)	21.500	1.256
	Muscle	308.377	–
MC + KCl	Muscle	7.044	–

[a]AW: acetonitrile/water; MW: methanol/water; MC: methanol/chloroform/water.
[b]Determined from PCA scores (in figures 3b, 6a and 6b) by multiplying standard deviation (SD) along PC1 by SD along PC2 for the replicates within each extraction method.

4.3. NMR methods

Liver contains a large amount of lipids and macromolecules, which give rise to peaks that overlap with those from low molecular weight metabolites in 1D NMR spectra. The UB laboratory therefore applied 2D J-resolved NMR spectroscopy, which removes the contribution from macromolecules and yields sufficiently well resolved peaks to examine the effects of solvent extraction on metabolic fingerprints more accurately (Viant, 2003).

The resulting scores plot (figure 6b) shows a significantly more obvious discrimination between the individual fish along PC1 compared to the 1D NMR data (figure 6a). Furthermore, the differences between the extractions can largely be accounted for by PC2 alone (figure 6b). In fact, the different extraction strategies for

Table 3
Summary of metabolite solvent extraction methods

Extraction method[a]	Yield	Reproducibility	Ease/speed	Other advantages
AW	+[b]	+	+	
MW	+		+	
MC	+	+		Obtain hydrophobic fraction

[a]AW: acetonitrile/water; MW: methanol/water; MC: methanol/chloroform/water.
[b]Extracts significant amount of macromolecules and lipids with the polar metabolites.

the two livers were extremely consistent, with the acetonitrile/water 2/1 groups having the most negative PC2 scores (approximately-10 to-15 for both fish), followed by the methanol/water 2/1 groups (PC2 scores from 0 to 5), and the methanol/water 1/1 group having the largest positive PC2 score. Collectively this demonstrates that although the differences between individuals dominated the PCA (54% variability along PC1), the choice of solvent system also had a major effect on the observed metabolome (21% variability along PC2), highlighting the importance of selecting the optimal extraction method. This is consistent with the conclusion by Defernez and Colquhoun (2003) whom, in a study of metabolite variation, showed that individual differences are larger than the differences arising from sample preparation and NMR acquisition. We also suggest that by virtue of reducing peak congestion and removing peaks from macromolecules the p-JRES NMR approach increases the likelihood of separating metabolic fingerprints of biological samples.

4.4. Tissue disruption methods

Although manually grinding tissues in liquid N_2 is an established method for preparing tissues for extraction, several groups now use an electric tissue homogenizer since it is considerably less labor intensive. We found that homogenization of wet tissue yielded considerably less sample-to-sample variation than grinding wet tissue (figure 7a). Furthermore, although grinding lyophilized tissue improved the sample-to-sample variation still further, it was relatively minor (figure 7a). The NMR spectra of the lyophilized samples yielded flat baselines compared to the homogenized tissues, which is likely because macromolecules did not re-solubilize efficiently following lyophilization. Overall, based on metabolite reproducibility, we conclude that either grinding lyophilized tissue or homogenizing wet tissue is best. When also considering ease and speed of extraction, wet tissue homogenization is clearly preferable (see table 4).

5. Concluding remarks

Reproducible tissue extraction protocols combined with appropriate NMR techniques minimize metabolic variation from analysis and increase the likelihood of distinguishing metabolic differences between samples. The importance of selecting the appropriate methods should not be underestimated. Considering yield, reproducibility, ease and speed, we recommend homogenizing wet tissues and extracting them in methanol/chloroform/water. This method also benefits from extracting the hydrophilic and hydrophobic metabolites into different fractions. Reassuringly, our recommendation is consistent with the conclusions from an earlier metabolite extraction study by Le Belle *et al.* (2002). The methanol/chloroform/water method may in fact be mandatory for particularly lipid-rich tissues such as liver and brain. Furthermore, if the amount of tissue is not limited (at least 50 mg), we recommend the application of 2D J-resolved spectroscopy. We have previously demonstrated that this approach will produce considerably less congested spectra while still detecting the same metabolites as in 1D NMR, thereby facilitating unambiguous metabolite identification and quantification (Viant, 2003). If the tissue mass is less than 50 mg, which effectively precludes 2D J-resolved spectroscopy, a traditional 1D method can be employed.

Table 4
Summary of tissue disruption methods

Tissue disruption method	Remove macromolecules and lipids[a]	Reproducibility	Ease/ speed
Grind (dry)	+	+	
Grind (wet)			
Homogenize (wet)		+	+

[a]Based upon the appearance of the ^{1}H NMR spectra, and likely a result of the drying process rather than the tissue disruption method.

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