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Comparison of modified Matyash method to conventional solvent systems for polar metabolite and lipid extractions

Jelena Sostare, Riccardo Di Guida, Jennifer Kirwan, Karnpreet Chalal, Elliott Palmer, Warwick B. Dunn, Mark R. Viant*

School of Biosciences, University of Birmingham, Birmingham, B15 2TT, UK

HIGHLIGHTS

- Bligh and Dyer, Matyash and a new 'modified Matyash' solvent systems were compared.
- Applied to three sample types and analysed by mass spectrometry-based metabolomics.
- Modified Matyash showed comparable or higher extraction yield than other methods.
- Reproducibility of modified Matyash method was also comparable or higher.

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ABSTRACT

In the last decade, metabolomics has experienced significant advances in the throughput and robustness of analytical methodologies. Yet the preparation of biofluids and low-mass tissue samples remains a laborious and potentially inconsistent manual process, and a significant bottleneck for high-throughput metabolomics. To address this, we have compared three different sample extraction solvent systems in three diverse sample types with the purpose of selecting an optimum protocol for subsequent automation of sample preparation. We have investigated and re-optimised the solvent ratios in the recently introduced methyl tert-butyl ether (MTBE)/methanol/water solvent system (here termed modified Matyash; 2.6/2.0/2.4, v/v/v) and compared it to the original Matyash method (10/3/2.5, v/v/v) and the conventional chloroform/methanol/water (stepwise Bligh and Dyer, 2.0/2.0/1.8, v/v/v) using two biofluids (human serum and urine) and one tissue (whole *Daphnia magna*). This is the first report of the use of the Matyash method for extracting metabolites from the US National Institutes of Health (NIH) model organism *D. magna*. Extracted samples were analysed by non-targeted direct infusion mass spectrometry metabolomics or LC-MS metabolomics. Overall, the modified Matyash method yielded a higher number of peaks and putatively annotated metabolites compared to the original Matyash method (1–29% more peaks and 1–30% more metabolites) and the Bligh and Dyer method (4–20% more peaks and 1–41% more metabolites). Additionally the modified Matyash method was superior when considering metabolite intensities. The reproducibility of the modified Matyash method was higher than other methods (in 10 out of 12 datasets, compared to the original Matyash method; and in 8 out of 12 datasets, compared to the Bligh and Dyer method), based upon the observation of a lower mRSD of peak intensities. In conclusion, the modified Matyash method tended to provide a higher yield and reproducibility for most sample types in this study compared to two widely used methods.

1. Introduction

Metabolomics has now matured into a routinely used technology for measuring the metabolic phenotypes of a wide array of sample types – including biofluids, cells and tissues – derived from plants, animals and microbes. One recent and important trend has been towards large-scale studies, in particular within biomedical and toxicological metabolic profiling [1-3]. While the necessary automation of data generation to support such large-scale studies is occurring [4, 5], and the automation of data

* Corresponding author.
E-mail address: m.viant@bham.ac.uk (M.R. Viant).

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processing workflows is increasingly being established [6, 7], the extraction of metabolites from biological samples remains a largely manual bottleneck in the metabolomics pipeline [8]; it is both a challenge for large-scale biofluid studies and an unsolved problem for studies of tissues, particularly the low-mass. High analytical reproducibility and throughput of the sample preparation step are crucial factors when measuring the metabolite compositions of hundreds to thousands of samples; however this is difficult to achieve using laborious manual extraction protocols. With advances in robotic technologies, automated sample handlers have the potential to replace manual sample processing in metabolomics [9], promising to open new horizons for large-scale studies.

The appropriate selection of extraction solvents has been a focus of the metabolomics community for several years [10-14]. Multiple factors should be considered: from maximising the chemical space of metabolites that are extracted through to maximising its operational simplicity, efficiency, reproducibility, speed and safety. The method first proposed by [15], which was originally intended to extract lipids, has proven so successful that it has been adopted by multiple laboratories worldwide [8, 16, 17]. This method utilises a chloroform/methanol/water (2/2/1.8, v/v/v) biphasic solvent system to extract both polar (methanol/water phase) and non-polar (chloroform phase) compounds separately. The extraction efficiency of chloroform stems from its ability to associate with water molecules through weak hydrogen bonds [18]. However, this solvent system has drawbacks, not least that chloroform is a carcinogen. Furthermore, the biphasic extraction results in a layer of protein and cellular debris between the upper polar and lower non-polar phases (called the interphase), which hinders the clean aspiration of the lower phase. While this is a known difficulty for manual liquid:liquid extractions, it represents a particular challenge for automated extractions using a liquid handling robot.

Significant efforts have been devoted to find an alternative to the Bligh and Dyer method such as hexane/isopropanol – 3/2, v/v [19], dichloromethane/methanol – 2/1, v/v [20], and hexane/ethanol – 5/2, v/v [21]. None of them, however, were reported to surpass the Bligh and Dyer method in terms of extraction efficiency. More recently, the Matyash method [22] was reported, which claimed to be at least as efficient as the chloroform/methanol/water method, and benefited from replacing chloroform by methyl-tert-butyl ether (MTBE), which is non-carcinogenic. The Matyash method (MTBE/methanol/water) utilises a non-polar phase (largely MTBE) that has a lower density than the methanol/water phase and hence it partitions on the top of the biphasic solvent system; this allows easier recovery of the lipid layer but correspondingly more difficult removal of the polar layer. More importantly – in terms of automation compatibility – the protein and cell debris layer is forced to the bottom of the sample tube following centrifugation, simplifying the removal of both solvent phases during the extraction. The Matyash method has been evaluated in animal [23-25] and plant samples [26, 27], proving its efficiency. The original method, however, is primarily focused on lipid extraction and unlike the Bligh and Dyer method has not been optimised for the recovery of both polar and non-polar metabolites from low-mass samples [8].

Here we have studied the extraction of two biofluids (human plasma and urine) and one tissue type (whole water flea *Daphnia magna*) in order to select a metabolite extraction protocol that offers superior metabolite yield and reproducibility and provides the highest benefit for automation (in terms of method duration, use of resources, simplicity to automate). We compare the gold-standard Bligh and Dyer extraction method (chloroform/methanol/water, stepwise) to two variations of the Matyash method (MTBE/methanol/water) – the original published protocol (MTBE/methanol/water, 10/3/2.5, v/v/v) and a modified method (MTBE/methanol/water, 2.6/2.0/2.4, v/v/v) – the latter employs solvent ratios that match those used by the Bligh and Dyer method and thereby increase the volume of the polar phase for easier handling. Specifically, we compare the extraction yields, derived from measurements of the number of peaks and putatively annotated metabolites detected in ultra performance liquid chromatography-mass spectrometry (UHPLC-MS) and direct infusion mass spectrometry (DIMS), and extraction reproducibilities, calculated as the median relative standard deviation – mRSD [28] of all detectable metabolites.

2. Materials and methods

2.1. Biological samples

Three well studied yet diverse sample types were selected to ensure our results are widely applicable: two human biofluids, plasma and urine, and a toxicological and US National Institutes of Health model organism (*D. magna*). Biofluids (100 μl aliquots from pooled frozen samples) were acquired from Sera Laboratories International Ltd (West Sussex, UK). *D. magna* was cultured in OECD media, fed on *Chlorella sp.*, and <24 h neonates (30 animals per sample, flash frozen in liquid nitrogen and stored at −80 °C) were used for experiments [29].

2.2. Metabolite extraction methods

Three extraction protocols were compared – Bligh and Dyer method (chloroform/methanol/water, 2/2/1.8, v/v/v) as optimised for metabolomics studies of tissues [8], the original Matyash method (MTBE/methanol/water, 10/3/2.5, v/v/v; [22]) and our modification of the Matyash method (MTBE/methanol/water, 2.6/2.0/2.4, v/v/v) to use solvent ratios and volumes that were equivalent to the successful Bligh and Dyer method and that were compatible with the automated extraction of both the polar and non-polar phases. Biofluids were extracted using the same protocols, however, without homogenization. Each method is described in more detail in the following sections. For each sample type-extraction method combination, 10 replicates were used.

2.3. Bligh and Dyer (stepwise) method

As described by Wu et al. [8], with some minor changes, first 75% ice cold methanol (32 $\mu l\ mg^{-1}$ methanol and 10.6 $\mu l\ mg^{-1}$ or 0.9 $\mu l\ mg^{-1}$ HPLC water for tissues and biofluids, respectively) was added to samples and they were homogenised (tissue only) in a Precellys-24 bead-based homogeniser (Bertin technologies) for 2 × 10s bursts at 6400 rpm. Homogenates were each transferred into 1.8 ml glass vials and 16 $\mu l\ mg^{-1}$ (or 2 $\mu l\ mg^{-1}$ for biofluids) of chloroform was added. Samples were mixed using a Bioshake platform (2000 rpm, 3 min; Bioshake 3000 elm (Edge Locking Mechanism), Quantifoil Instruments GmbH) and then centrifuged (2415 × g, 10 min, 18 °C; refrigerated centrifuge 6-16KR, Sigma) to pellet the protein and tissue debris. Each monophasic supernatant (~500 μl) was transferred to a clean 1.8 ml glass vial and phase separation was induced by adding 16 $\mu l\ mg^{-1}$ (or 2 $\mu l\ mg^{-1}$ for biofluids) of chloroform and 18.2 $\mu l\ mg^{-1}$ (or 2.27 $\mu l\ mg^{-1}$ for biofluids) of HPLC water. Samples were then mixed again on the Bioshake (2000 rpm, 1 min), incubated at 18 °C for 10 min to allow the partitioning of the solvent system and then centrifuged (2415 × g, 10 min, 18 °C). Polar and non-polar fractions were aliquoted into clean Eppendorf tubes or glass vials, respectively, and then dried down using a SpeedVac concentrator (SPD111V, Thermo Savant; for polar samples only) or nitrogen blow-down evaporator (TECHNE sample concentrator with Peak Scientific Genius nitrogen

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