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ORIGINAL PAPER

Towards an unbiased metabolic profiling of protozoan parasites: optimisation of a *Leishmania* sampling protocol for HILIC-orbitrap analysis

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Abstract Comparative metabolomics of *Leishmania* species requires the simultaneous identification and quantification of a large number of intracellular metabolites. Here, we describe the optimisation of a comprehensive metabolite extraction protocol for *Leishmania* parasites and the subsequent optimisation of the analytical approach, consisting of hydrophilic interaction liquid chromatography coupled to LTQ-orbitrap mass spectrometry. The final optimised protocol starts with a rapid quenching of parasite cells to 0 °C, followed by a triplicate washing step in phosphate-buffered saline. The intracellular metabolome of 4×10^7 parasites is then extracted in cold chloroform/methanol/water 20/60/20 (*v*/*v*/*v*) for 1 h at 4 °C, resulting in both cell disruption and comprehensive metabolite dissolution. Our developed metabolomics platform can detect approximately 20% of the predicted *Leishmania* metabolome in a single experiment in positive and negative ionisation mode.

Keywords Metabolomics · *Leishmania* · Liquid chromatography–mass spectrometry (LC–MS) · HILIC · Systems biology

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Introduction

Leishmania is a group of kinetoplastid protozoans that infect mammals following transmission by sand flies (*Psychodidae*: *Phlebotominae*). The life cycle of this parasite consists of two distinct morphological forms: (a) promastigotes, adapted to life in the sand fly intestinal tract and easily cultured axenically in vitro, and (b) amastigotes, living intracellularly within mammalian macrophages. *Leishmania* parasites cause a wide spectrum of poverty-related neglected diseases called leishmaniasis, a disease characterised by diversity and heterogeneity endemic in large areas of the tropics, subtropics and the Mediterranean basin. The heterogeneity of *Leishmania* species is reflected in the variety of clinical manifestations they cause, ranging from self-healing skin lesions to disfiguring mucocutaneous lesions up to lethal visceral disease. Even infection with one particular *Leishmania* species can result in diverse clinical profiles in terms of disease severity and treatment response. This clinical polymorphism challenges health professionals to manage leishmaniasis patients effectively [1]. Unfortunately, little is known about the intra- and inter-species molecular differences underlying this clinical polymorphism [2, 3]. Therefore, characterising the diversity of parasite populations

is considered as a key step towards a better control of this disease. Metabolomics is an emerging field that allows in-depth characterisation of the metabolome, the closest correlate to the phenotype on molecular level [3, 4]. This technology has great potential for resolving the metabolic variation between different *Leishmania* species and among multiple parasite isolates of a single species (reviewed in [3]).

Protocols for monitoring the metabolome of protozoan species have not been fully optimised yet, in contrast to other unicellular organisms [4–6]. This study aimed to optimise a sample preparation protocol for subsequent liquid chromatography–mass spectrometry-based metabolomics analysis of *Leishmania donovani* promastigotes.

Generally, a sampling protocol needs to start with immediate arrest of the intracellular metabolism. Several methods are described in the literature for unicellular organisms, ranging from immersing the cells in cold organic solvent followed by centrifugation of the quenched cells [4, 7] to applying rapid filtration of cells followed by freezing them with liquid nitrogen [6, 8]. It is essential that, during this initial step, rupture of the cell envelope causing leakage of intracellular metabolites is prevented. Furthermore, removal of the extracellular environment is a challenging task, especially since *L. donovani* is generally cultured in rich medium with 20% (*v*/*v*) foetal calf serum. De Souza et al. [9, 10] have described rapid (seconds) quenching of *Leishmania* cultures to 0 °C using a dry ice–ethanol bath. They reported that this method quenches metabolism of *Leishmania* reliably and reproducibly.

In the second step, the quenched and washed cells need to be disrupted. Chemical and physical dissimilarities between cell envelopes hamper the design of a universal cell disruption method for global metabolomics analysis of single-cell organisms. Some species simply burst by permeabilisation in organic solvents (e.g. *Lactobacillus plantarum* [4], *Escherichia coli* [11] or cultured mammalian cells [9]), while others demand mechanical interference to disrupt the rigid cell envelope (e.g. *Chlamydomonas reinhardtii* [10, 12] or *Mycobacterium bovis* [13]). *Leishmania* promastigotes are protected by a specific membrane enclosure, i.e. a thick glycoconjugate surface coat also referred to as the glycocalyx [14]. Since adequate cell lysis is essential to release the bulk of intracellular metabolites, we evaluated six different cell disruption procedures based on heating, milling or mixing of promastigotes.

Finally, the released metabolites need to be characterised. Hydrophilic interaction liquid chromatography (HILIC) coupled to LTQ-orbitrap mass spectrometry (liquid chromatography–mass spectrometry, LC–MS) has already been identified as an excellent tool for metabolic profiling of trypanosome parasites, a distant relative of *Leishmania* [15]. Phospholipids tend to be retained with reversed-phase chromatography (C18), causing ion suppression effects over long periods of chromatography by co-eluting into the ion source with other analytes of interest [16, 17]. However, with HILIC chromatography, these lipid compounds elute in the void volume, allowing more reliable quantification of compounds eluting later. These polar metabolites, which encompass the majority of the intracellular metabolome, include signature metabolites of the trypanosomatid species such as trypanothione. Moreover, LC–MS covers a wide mass range which allows analysis of many compound classes not detectable by the alternative gas chromatography coupled to MS [18].

Here, we report the optimisation of a complete protocol for metabolome analysis of *Leishmania*, from the quenching of promastigotes, cell disruption and metabolite extraction, up to the HILIC-orbitrap analysis. The full protocol was validated by applying it to three *L. donovani* isolates.

Experimental

Chemicals and materials

Formic acid (ULC grade), acetonitrile (ULC grade), water (ULC grade), methanol (ULC grade) and chloroform (high-performance liquid chromatography (HPLC)-S grade) were purchased from Biosolve (Valkenswaard, the Netherlands). The ZIC®-HILIC PEEK Fitting Guard columns and ZIC®-HILIC PEEK HPLC columns were obtained from HiChrom (Reading, UK). Phosphate-buffered saline (PBS) was obtained from Invitrogen (Merelbeke, Belgium).

Regarding the culturing of *Leishmania* promastigotes, modified Eagle's medium (designated HOMEM medium, Invitrogen) [19] has been supplemented with 20% (*v*/*v*) heat-inactivated foetal calf serum (PAA Laboratories GmbH, Linz, Austria) pH 7.5 at 26 °C. Disposable count chambers (Uriglass) were obtained from Menarini Diagnostics (Reading, UK). The *L. donovani* strains MHOM/NP/02/BPK282/0 and MHOM/NP/03/BPK275/0 were isolated from bone marrow aspirates from confirmed visceral leishmaniasis patients recruited at the B.P. Koirala Institute of Health Sciences, Dharan, Nepal, as described by Rijal et al. [20]. The clinical parasite isolates were identified as *L. donovani* based on a CPB PCR–RFLP assay [21]. The *L. donovani* strain 1S has its origin in Sudan (MHOM/SD/00/1S-2D). Extraction/cell disruption devices included Thermomixer (Eppendorf AG, Hamburg, Germany), Retsch mill (Retsch GmbH & Co. KG, Haan, Germany), Ultra Turrax mixer (IKA Werke GmbH & Co KG, Staufen, Germany) and Dispomix (Xiril AG, Hombrechtikon, Switzerland).

High-performance liquid chromatography equipment consisted of a Surveyor HPLC pump (ThermoElectron, Hemel Hempstead, UK) and two different ZIC-HILIC column setups differing mainly in the column diameter: either (a) a ZIC-HILIC guard column (20 mm×2.1 mm;

5 μm) and analytical column (150 mm×4.6 mm; 3.5 μm) or (b) a ZIC-HILIC guard column (15 mm×1.0 mm; 5 μm) and analytical column (150 mm×2.1 mm; 3.5 μm). High-resolution mass measurements were obtained with a Finnigan LTQ-orbitrap mass spectrometer (Thermo Fisher Scientific Inc., Hemel Hempstead, UK). The LC–MS system was controlled by Xcalibur version 2.0 (Thermo Fisher Scientific Inc., Hemel Hempstead, UK).

Parasite growth conditions

Leishmania promastigotes were grown in modified Eagle's medium [19] supplemented with 20% (*v*/*v*) heat-inactivated foetal calf serum pH 7.5 at 26 °C. The cultures were initiated by inoculating parasites from a culture at days 3–4 of stationary phase of growth into 5-ml culture medium to a final concentration of 5×10^5 parasites per millilitre. Independently growing cultures of the parasite were treated as biological replicates. After 7 days of growth, when parasites had been in stationary phase for 4 days, parasite density was determined using disposable count chambers, and subsequently an aliquot corresponding to 1×10^8 parasites (for optimisation cell disruption/metabolite extraction experiments) or 4×10^7 parasites (for all other experiments) was taken from each culture. Experiments are described following their order in the final sample preparation protocol. However, the quenching method was evaluated after optimisation of the cell disruption/metabolite extraction step and the LC–MS approach. All optimisation experiments were done with BPK282/0 in late-stationary promastigote stage, except for the cell leakage part, where both BPK282/0 and 1S were used.

Metabolite extraction

Optimisation experiments for cell disruption and metabolite extraction were performed in triplicate (biological replicates), using one-phase chloroform/methanol/water 20:60:20 (*v*/*v*/*v*) at 0 °C as a comprehensive extraction solvent. Following harvest of 1×10^8 promastigotes (BPK282/0 isolate) and three wash steps in PBS, cell disruption procedures were applied. Tested procedures include heating block (HB; 70 °C; 60 min), Thermomixer (TM; 60 min; 4 °C; 1,400 rpm), vortex with glass beads (V; 0.5 mm i.d.; 3×1 min; cool in between in ice bath), Retsch mill with glass beads (RM; 0.5 mm i.d.; 3×1 min; cool in between in ice bath), Ultra Turrax mixer (UT; 3×1 min; in ice bath) and Dispomix (DM; 3×1 min; 0 °C). After cell disruption and metabolite extraction, all samples were centrifuged for 10 min at 16,100×*g* (4 °C). The resulting supernatants were separated from cell debris and analysed immediately on the HILIC-orbitrap platform. The raw data files of all different extraction procedures are processed and aligned together.

The cell leakage experiment was done with two *L. donovani* strains, BPK282/0 and 1S. Three biological replicates were grown for each strain and harvested as described above. Their spent culture medium (M) and the successive washing solutions (W1-3) were aliquoted during the sample preparation and stored at −70 °C until further analysis within the next 48 h. Before LC–MS analysis, 75 μl of each fraction was taken and deproteinised with 300 μl 20:60 chloroform/methanol (*v*/*v*) to obtain identical solubility of compounds as in the parasite extracts. Following centrifugation (16,100×*g*; 4 °C; 10 min), deproteinised supernatant was separated and used for analysis.

The final, optimised metabolite extraction protocol consists of (a) quenching (<20 s) of *L. donovani* promastigotes in their culture flasks to 0 °C in a bath containing a mixture of dry ice/ethanol, (b) isolating the necessary volume for harvesting 4×10^7 parasites, (c) triplicate washing of parasite cells in 1 ml of cold (0 °C) PBS (pH 7.4) by centrifugation (20,800×*g*, 0 °C, 3 min) and re-suspending cells using a vortex, (d) cell disruption and metabolite extraction of the washed cell pellet in 200 μl chloroform/methanol/water 20:60:20 (*v*/*v*/*v*) during 1 h in a Thermomixer (1,400 rpm, 4 °C), (e) isolating the metabolite extract from cell debris by centrifugation (20,800×*g*, 0 °C, 3 min) and (f) deoxygenating the extracts with a gentle stream of nitrogen gas for 1 min prior to tube/vial closure (Fig. 6).

LC–MS analysis

Gradient elution was performed on two different ZIC-HILIC column setups differing mainly in the column diameter: either (a) 150 mm×4.6 mm or (b) 150 mm×2.1 mm. Elution of the ZIC-HILIC columns was carried out with a gradient of (A) 0.1% formic acid in acetonitrile and (B) 0.1% formic acid in water. The flow rate was 300 and 100 μl/min, with an injection volume of 10 and 5 μl, respectively. Gradient elution chromatography was always performed starting with 80% solvent A. Within a 6-min time interval, solvent B was increased to 40% and maintained for 12 min, followed by an increase to 90% within 4 min. This composition was maintained for 2 min, after which the system returned to the initial solvent composition in 2 min. The whole system was allowed to re-equilibrate under these conditions for 14 min.

The LTQ-orbitrap mass spectrometer was operated in both positive and negative ion electrospray mode. Optimal instrument parameters were based on previous results [15, 16, 22]. Briefly, ESI source voltage was optimised to 4.0 kV, and capillary voltage was set to 30 V. The source temperature was 250 °C, and the sheath and auxiliary gas flow rates were 30 and 10, respectively, in machine-specific units. Full-scan spectra were acquired over an *m*/*z* range of 50–1,000 Da, with the mass resolution set to 30,000 full

width at half maximum (FWHM). The target for mass accuracy was <1 ppm. By using a resolution of 30,000 FWHM, this was routinely achievable and allowed rapid spectrum acquisition compatible with the peak widths obtained by the chromatographic system, leading to at least 20 full scans across the width of a peak. All spectra were collected in continuous single MS mode.

Data processing

Raw data files acquired from analysed samples were converted to the mzXML format by the readw.exe utility (a tool of the Trans-Proteomic Pipeline software collection, downloaded from http://tools.proteomecenter.org/wiki/index.php?title=Software:ReAdW). Further processing was handled by a flexible data-processing pipeline mzMatch [23] (http://mzmatch.sourceforge.net/). mzMatch is a modular, open-source and platform-independent data-processing pipeline for metabolomics LC-MS data written in the Java language. It was designed to provide small tools for the common processing tasks for LC-MS data. The mzMatch environment was based entirely on the PeakML file format and core library, which provides a common framework for all the tools. mzMatch comprises integrated chemistry (e.g. molecular formulae, mass conversion and periodic table), math (e.g. statistics, wavelet transform, function fitting and loess and Savitzky-Golay) and visualisation (JFreeChart and SWT for user interface applications) routines. Starting from the PeakML data, signal detection [24], retention time alignment [25, 26], blank removal, noise removal [26] and signal matching were performed. Masses whose abundance was not reproducible for all biological and technical replicates, as indicated by a relative standard deviation (RSD) larger than 35%, were discarded, as quantification is expected to be at least 20% accurate over multiple runs [27]. Derivative signals (isotopes, adducts, dimers and fragments) were automatically annotated by correlation analysis on both signal shape and intensity pattern [28]. The derivative signals were removed before further statistical tests, as they would give excessive weight to abundant analytes with many derivatives. The selected mass chromatograms were putatively identified by matching the masses (mass accuracy <1 ppm) progressively to those in metabolite-specific databases. In the first round of identification, LeishCyc [29], LipidMAPS [30] and a contaminant database were used [31]. The latter allows removal of typical impurities and buffer components often detected in metabolomics experiments. Only the remaining unidentified peak groups went through a second round of matching with KEGG [32] and a peptide database; and finally a third round was done with the Human Metabolome Database for any remaining unidentified analytes [33]. This iterative process was used in order to restrict the number of potential matches to the most likely [34]. A selection of standards was run routinely to ensure that the instrument was returning true masses, thereby enabling the use of retention time in the identification process [22].

Statistical analysis and graphical routines were handled in R (R Development Core Team, R: A Language and Environment for Statistical Computing, Vienna, Austria: 2010; http://www.R-project.org). Principal component analysis (PCA) is an unsupervised multivariate analysis technique frequently used in metabolomics [35]. It implements a data dimensionality reduction of complex data matrices, so that clustering tendencies, trends and outliers can be visualised among samples. Unit variance scaling was used in PCA calculations, and no additional normalisation was performed on the datasets. The R code consisting of reading and writing routines of data from/to PeakML file format (XML representation of processed data produced by the mzMatch pipeline) is available from the authors upon request. Total metabolite yield is calculated as the sum of all metabolite intensities.

Results and discussion

Quenching and washing of *Leishmania* promastigotes

A quenching methodology based on De Souza et al. [36] was assessed here for *L. donovani* promastigotes. The method involves quenching parasites in their culture medium, which avoids leakage of metabolites during quenching. This is in contrast with commonly used quenching methods based on suspension of cells in cold organic solvent, which increases the risk of cell lysis and subsequent loss of intracellular metabolites [6, 12, 37]. However, in order to separate and extract only the intracellular *Leishmania* metabolome, it is crucial that the growth medium is effectively removed after the quenching step by washing the parasites thoroughly. These washing steps involve a risk of cell lysis and subsequent leakage of intracellular metabolites [6]. PBS (pH 7.4), a common washing buffer for *Leishmania* promastigotes, was chosen here for the washing steps to remove culture medium after quenching. Possible leakage of cells was monitored by analysing the supernatants (SN) removed after each washing step using our LC–MS setup. Figure 1 shows the effective removal of selected medium compounds and extracellular metabolites during the successive washing steps. The data are representative for all detected metabolites. Phospholipids were the most difficult to remove; very small percentages (<2.35%) of the culture medium were still detected in the final wash solution. No intracellular metabolites (e.g. glutamate, glutathione, nicotinamide adenine dinucleotide (NAD) and trypanothione) could be detected in

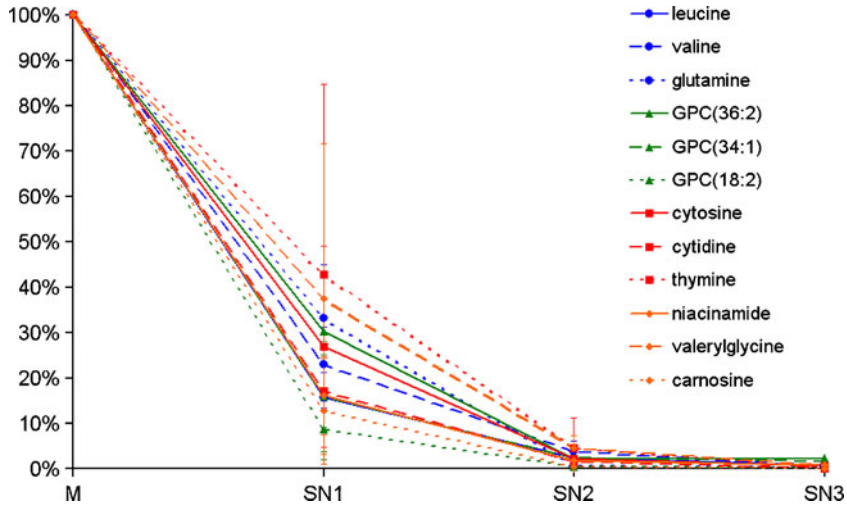

Fig. 1 Washing efficiency—metabolite leakage during the successive washing steps for selected metabolites. Signal intensities in the SN of three sequential washing steps are compared to those of spent culture medium (M). The data clearly show that at least three washing steps are required to obtain clean parasite pellets without medium contamination (*GPC* glycerophosphocholine)

the supernatants of the different washing steps, confirming the absence of leakage of intracellular metabolites. Overall, three successive washing steps are needed to remove the vast majority of culture medium compounds and extracellular metabolites.

Cell disruption and metabolite extraction

Comprehensive metabolite extraction is a crucial step in untargeted metabolomics. As no guideline currently exists on comprehensive metabolite extraction of *Leishmania* parasites, different cell lysis approaches were tested in order to disrupt the *Leishmania*-specific glycocalyx. All procedures were compared using chloroform/methanol/water 20:60:20 (*v*/*v*/*v*) as extraction solvent, which has been shown to provide comprehensive metabolite dissolution after cell disruption [38]. Procedures were based on heating (HB) [15, 36], mixing (Thermomixer, TM; Ultra Turrax, UT; Dispomix, DM), vortexing (vortex, V) or milling (Retsch mill, RM) [13]. For each cell disruption procedure, three biological replicates were analysed. PCA clearly shows a denser clustering of samples prepared by TM or DM, compared to extracts prepared with the other approaches (Fig. 2), indicating a superior reproducibility with both methods. Calculation of the average RSD of the intensities of all detected preliminary annotated metabolites (a number of 63) confirms this (Table 1). The total metabolite yield of the detected metabolites was very similar for the different extraction procedures; only the RM method showed significantly lower signals.

Some labile metabolites in the higher mass range, which are among the targeted compounds, showed some significant differences between the different methods. NAD was detected in all cold approaches, while it was absent in the heated one (HB). On the other hand, nicotinamide could only be detected when heating was used, clearly showing up as a degradation product of NAD at elevated temperatures, as described before [39]. Trypanothione, a thiol metabolite typical for these parasitic protozoa [40], could not be detected in the heated method, while the level of its oxidised form, trypanothione disulfide, clearly exceeded the amount detected in the cold approaches. Ultra Turrax mixing of the parasites also led to a higher oxidation level of trypanothione. In conclusion, applying heat during metabolite extraction causes chemical degradation of some targeted metabolites.

Finally, the cold TM method was selected as the most adequate extraction approach for untargeted *Leishmania*

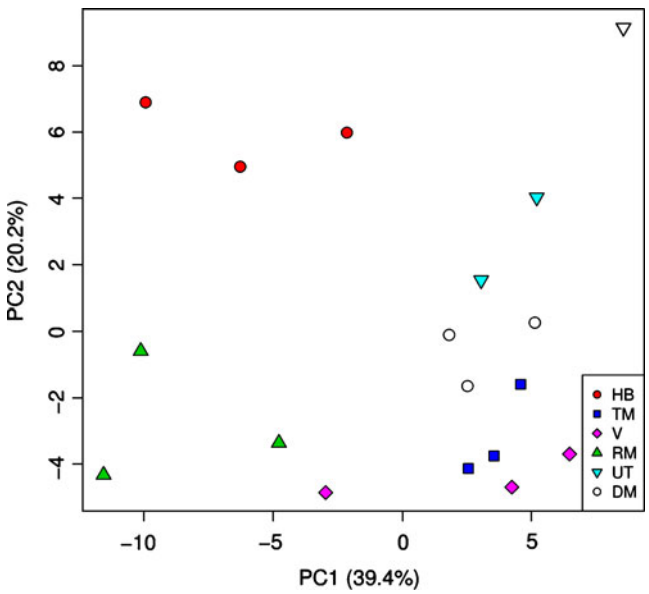

Fig. 2 Principal component analysis of samples obtained by six different cell disruption/extraction procedures. The score plot representing the first two principal components explains 59.6% of the variation present in the LC–MS dataset. Thermomixer (*TM*) and Dispomix (*DM*) show the tightest clustering and thus the best reproducibility, compared with the other cell disruption methods (*HB* heating block, *V* vortex, *RM* Retsch mill, *UT* Ultra Turrax)

Table 1 Average relative standard deviation (RSD%) of the signal intensities and total metabolite yield of all annotated metabolites obtained with six different extraction procedures

Method	Average RSD% ± SD	Total metabolite yield
HB	15.84%±6.71	1.73×10^8
TM	9.81%±7.34	1.75×10^8
V	15.91%±8.01	1.67×10^8
RM	18.93%±7.17	1.35×10^8
UT	17.97%±18.65	1.87×10^8
DM	9.77%±7.94	1.78×10^8

RSD% was calculated for each metabolite detected (n=3), and an average RSD% ± SD was calculated for all metabolites. Total metabolite yield was calculated as the sum of all signal intensities

HB heating block, *TM* Thermomixer, *V* vortex, *RM* Retsch mill, *UT* Ultra Turrax, *DM* Dispomix

metabolomics. This method was found to provide excellent reproducibility and good yield, and—in contrast to DM—it is an easy and fast method which is a relevant criterion for large-scale metabolomics studies.

Other extraction solvent compositions (aqueous methanol, aqueous ethanol, aqueous acetonitrile, aqueous isopropanol and 50:50 methanol/chloroform (*v*/*v*)) were tested alongside the chloroform/methanol/water 20:60:20 (*v*/*v*/*v*) one-phase composition. However, with the exception of 50:50 (*v*/*v*) methanol/chloroform, none of the alternative extraction solvents resulted in visual cell disruption when working in cold conditions (<4 °C). The presence of chloroform in the extraction solvent seems to be a key element for disrupting the *Leishmania* promastigote glycocalyx when using a cold-sample methodology. Other cell disruption methods might be advantageous with different solvents, but at the expense of reproducibility or degradation of labile metabolites.

Finally, the duration of the cell disruption process and extraction was also optimised. We compared extraction times of 15, 30 and 60 min for the selected Thermomixer method for three technical replicates. The average relative standard deviation was 13.16%, 8.23% and 4.96%, showing that longer extraction times, somewhat surprisingly, led to more reproducible results. More specific experimental analysis should be done to identify possible reasons for this phenomenon.

Optimisation of cell number and analytical sensitivity

Initial experiments were done with 1×10^8 promastigotes in 1 ml of extraction solvent. In vitro cultures of *L. donovani* strains typically reach a density of $2.5–5\times10^7$ cells per millilitre in late-stationary phase. This means that a relatively large volume of cell culture (2–4 ml) is required to obtain 1×10^8 parasites, and this is unpractical for high-throughput experiments. Hence, we decreased the number of parasites to 4×10^7 cells, while at the same time decreasing the amount of extraction solvent to 200 µl, thereby increasing the actual cells/extraction solvent ratio by a factor of 2. This allowed handling of the parasite cultures in 1.5-ml tubes which further facilitated the extraction approach. The resulting metabolite profiles of three biological replicates showed an increased reproducibility (average RSD of 10.05%±7.40 for the 4×10^7 cells approach versus 13.09%±7.11 for the 1×10^8 cells approach) and a higher signal intensity for polar metabolites representing different metabolite classes (total signal intensity of 40 representative compounds is 1.9-fold higher with the 4×10^7 cells approach compared to the 1×10^8 cells approach).

HILIC was chosen as the optimal separation step, following the conclusions of earlier studies [15]. However, we further compared the performance of columns with different inner diameters, 4.6 mm (conventional HPLC as used in the previous studies) and 2.1 mm (micro-bore HPLC), to optimise analytical sensitivity (Fig. 3). Down-scaling the column diameter generally offers better detection sensitivity of metabolites [41]. This is confirmed by our results; a total of 43,580 signals were detected in positive ESI mode on the 2.1-mm column, compared with 28,060 signals on the conventional column. If we subtract signals originating from eluent and extraction solvent [26] and filter for reproducibility (35% RSD cutoff; [27]) and good peak shape (Coda-DW value >0.8; [26]), the number of "quality peaks" in both analyses is 3,650 and 2,264, respectively, for the 2.1- and 4.6-mm column. A tentative identification based on database matching at a mass accuracy <1 ppm results in 390 versus 220 putative identifications for the 2.1- and 4.6-mm column. Thus, using a micro-bore column instead of a conventional HPLC column significantly increases the number of "quality peaks", resulting in an almost twofold increase in the number of putative identifications.

Length of analytical block of LC–MS analysis of *Leishmania* extracts

The length of the analytical block can influence the quality of the analytical metabolomics data acquired with LC–MS platforms [42]. Setting up the instrumentation is rather time-consuming, so everyone strives to analyse as many samples as possible in a single analysis batch. However, instability of samples during autosampler storage can affect the metabolomics data negatively, showing decreasing trends of metabolites due to oxidation and/or degradation. Apart from this, the performance of the LC–MS analysis itself can decrease over time, leading to a decrease in sensitivity of the mass spectrometer, loss of mass accuracy and/or shifting of retention times. To determine the optimal analysis time, we analysed two identical extracts (designated A and B, aliquots taken from a pool of several technical

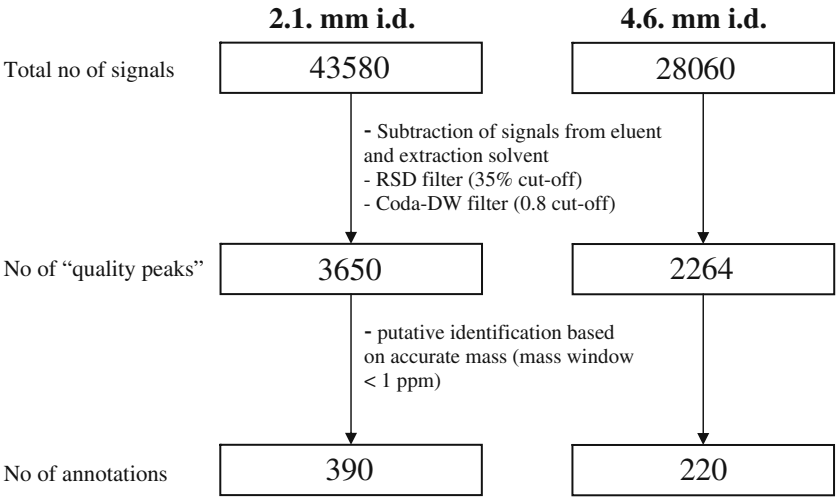

Fig. 3 Comparison of the performance of columns with inner diameter 4.6 and 2.1 mm. The process of signal detection and signal filtering is described in the "Experimental" section

replicates) repeatedly during 72 h to identify changes during autosampler storage of extracts. Extract B was placed in the autosampler 12 h after the analysis of extract A was started, allowing us to discriminate between a decrease in LC–MS performance and actual sample instability in the autosampler. A decrease in signal of both extracts (A and B) at the same time would suggest a drop in LC–MS performance, while sample instability would show as an independent decrease in signal of extracts A and B, after comparable time intervals following the start of the analysis of each extract. Principal component analysis of the dataset shows that the LC–MS performance continuously changes, with a clear shift after 26 and 48 h (Fig. 4 plots samples from extract A; extract B showed an identical pattern). Overall, the majority of detectable metabolites did not significantly vary over time, with only slight increases in the average relative standard variation over time (11.18%, 12.77% and 14.37% after 24, 48 and 72 h, respectively; see Electronic Supplementary Material Figure S1 for trends of individual metabolites in extract A). However, a subset of 50 out of 235 metabolites was the source of the variability seen in the PCA score plot. These compounds showed either a signal intensity close to the minimum detection level and were thus not detected continuously or they were sensitive to degradation during storage in the autosampler. The latter can be identified as a consistently decreasing trend across time. Figure 5 clearly shows the oxidation of glutathione and formation of its oxidised form, glutathione disulfide, during storage in the autosampler, for both extracts A and extract B (note the 12-h time shift as expected). Trypanothione and its oxidised product, trypanothione disulfide, show an even faster oxidation rate, making quantification of this marker metabolite impossible without special precautions. Other oxidation-sensitive products like cysteine, glutamylcysteine and glutathionylspermidine also show oxidation during autosampler storage. These results clearly demonstrate the necessity for oxidation prevention. Nitrogen and argon gas were shown to prevent extracts from oxidation and degradation caused by atmospheric air [43, 44]. A deoxygenating step using nitrogen gas was consequently included in the sample preparation protocol. Deoxygenated samples show stable profiles of oxidation-sensitive metabolites (see Electronic Supplementary Material Figure S2).

Coverage of HILIC-orbitrap analysis

To illustrate the final performance of our optimised protocol (Fig. 6), we compared the global metabolome of three

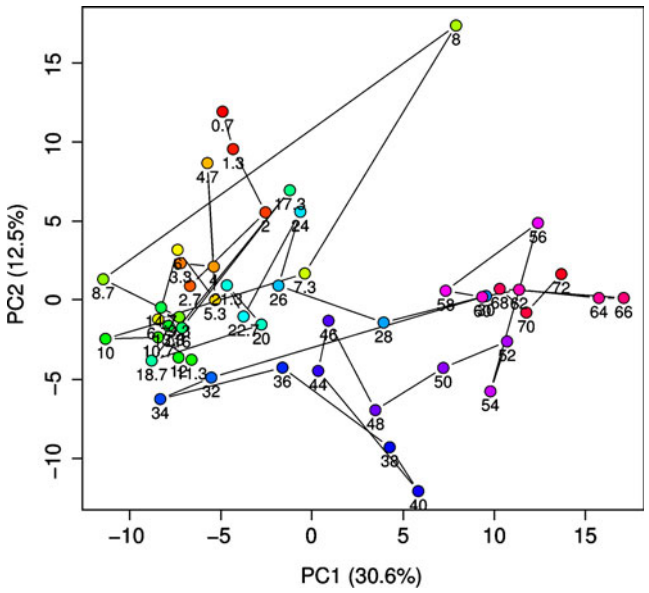

Fig. 4 Principal component analysis showing the LC–MS time trend during analysis of extract A. The hour of analysis is indicated underneath each point in the plot, and points are connected in order of analysis. A constant drift in performance is visible in the first principal component that corresponds to the highest variation in the dataset, with major shifts after 26 and 48 h

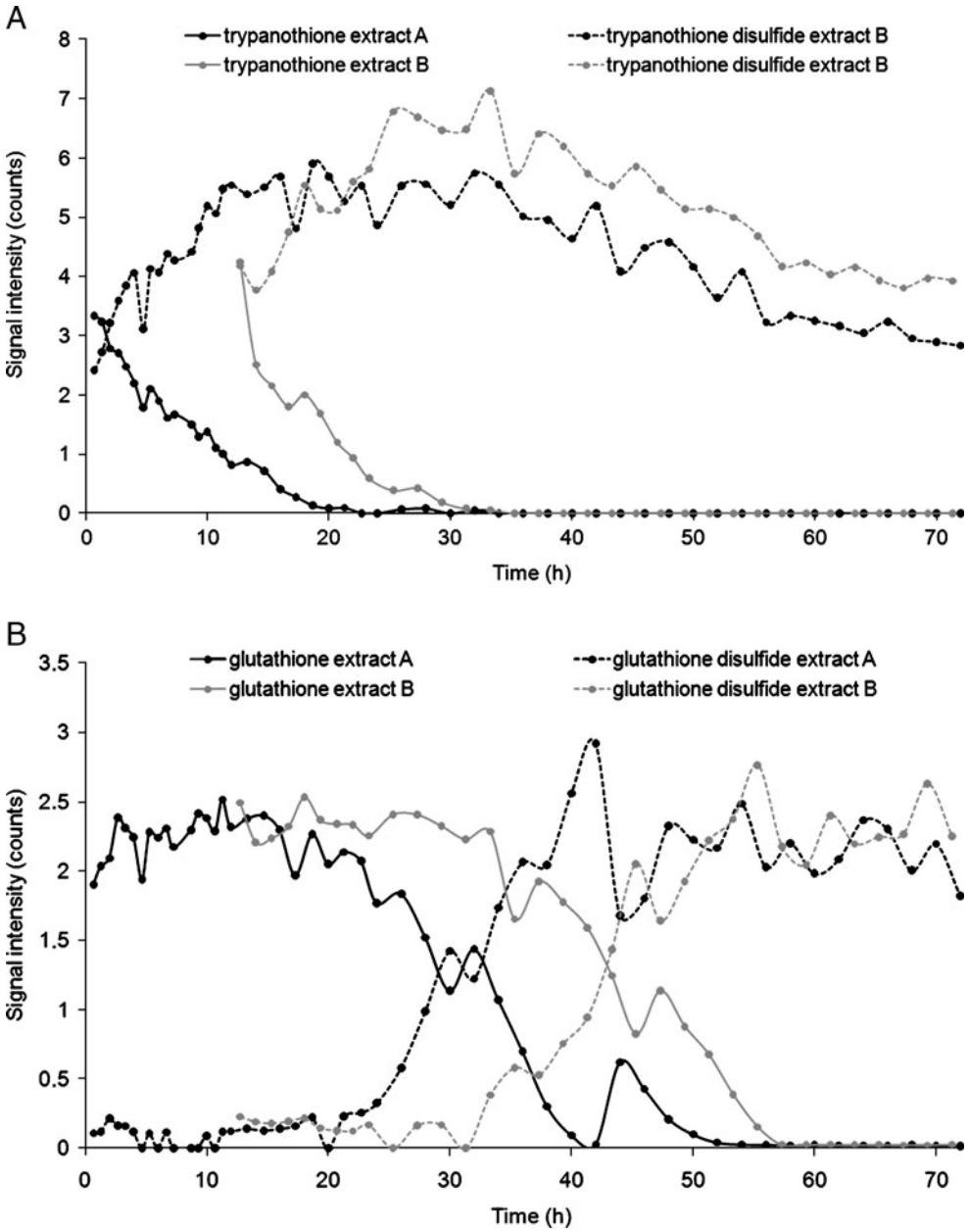

Fig. 5 Levels of metabolites sensitive to oxidation during storage in the autosampler (4 °C), for extract A and extract B (measurement started 12 h after extract A). Intensity values (in counts) are scaled to unit variance. The signal of trypanothione immediately drops due to formation of its oxidation product, trypanothione disulfide, showing the necessity of oxygen deprivation during autosampler storage

different *L. donovani* strains. HILIC-orbitrap analysis was performed in both negative and positive ionisation mode to maximise the coverage. All cultures were growth-synchronised to ensure harvest at the same growth stage. Principal component analysis shows huge variation between the different strains on metabolome level, clearly demonstrating the potential of metabolomics in protozoa-related research, as described before [3] (see Electronic Supplementary Material Figure S3). About 20–50% of the detected metabolome variation can be linked to differences between strains.

To assess the metabolite coverage obtained by our protocol, we generated a conservative list of detected metabolites using the following inclusion criteria: (a) reproducible signal intensity (RSD%<0.35 across the biological replicates), (b) consistent detection (detected in all samples; $n=11$), (c) peak shape quality (Coda-DW value>0.8), (d) retention time matching in positive and negative ESI mode, and (e) high mass accuracy (<1 ppm). We matched the resulting metabolite list to the LeishCyc database [29], a biochemical pathway database for the closely related *Leishmania major*, and estimated the approximate coverage of the *L. donovani* metabolome achieved with our approach. The LeishCyc database is still being updated and contains 566 metabolites so far; the entire *Leishmania* metabolome is predicted to contain about 1,101 metabolites [45]. However, a cell-based metabolomics analysis encompasses the different compartments within a cell. If one corrects for the metabolites occurring in multiple cellular compartments, there are about

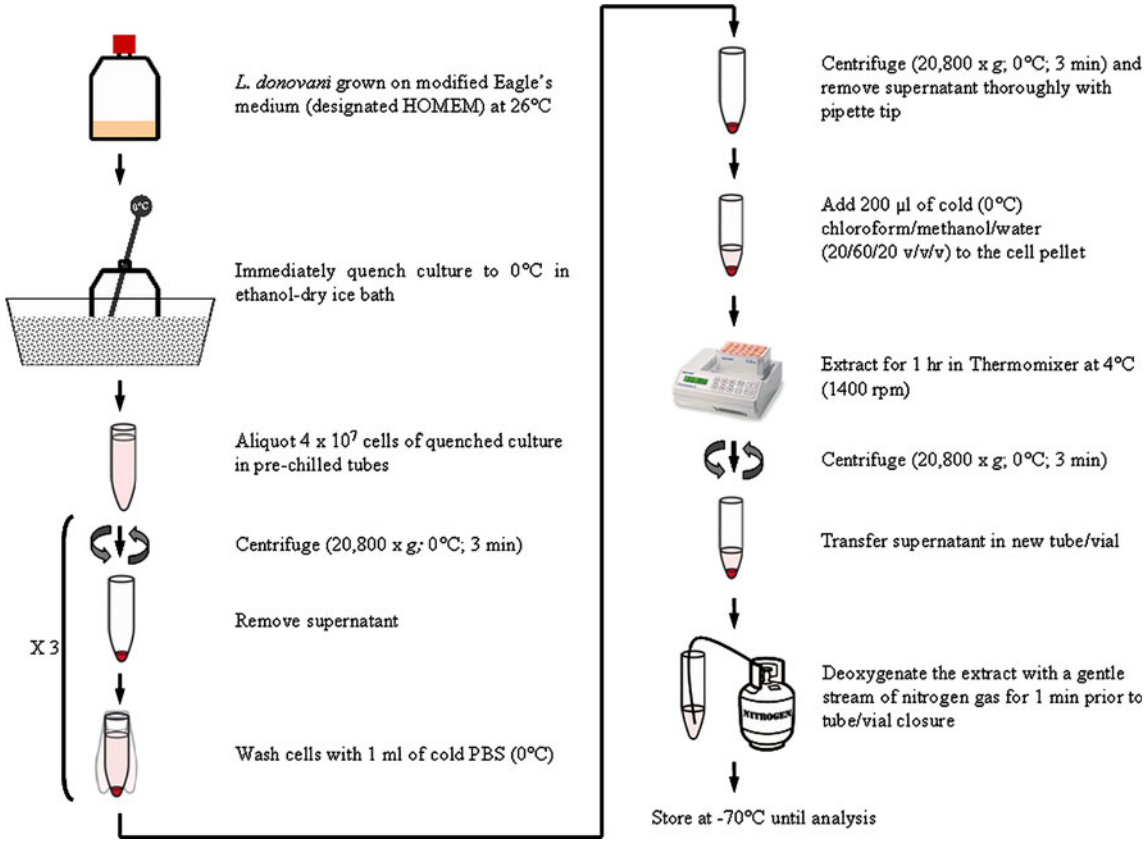

Fig. 6 Final optimised protocol for the study of the intracellular metabolome of *Leishmania* parasites

600 unique metabolites [3]. A total of 118 metabolites of the LeishCyc database were present in our list of putatively identified metabolites detected with our developed LC–MS approach, corresponding to 20% coverage of the predicted *Leishmania* metabolome. All identified LeishCyc metabolites are listed in the Electronic Supplementary Material Figure S4. As the LeishCyc database is not complete (e.g., phospholipids are not included), we also matched our list to

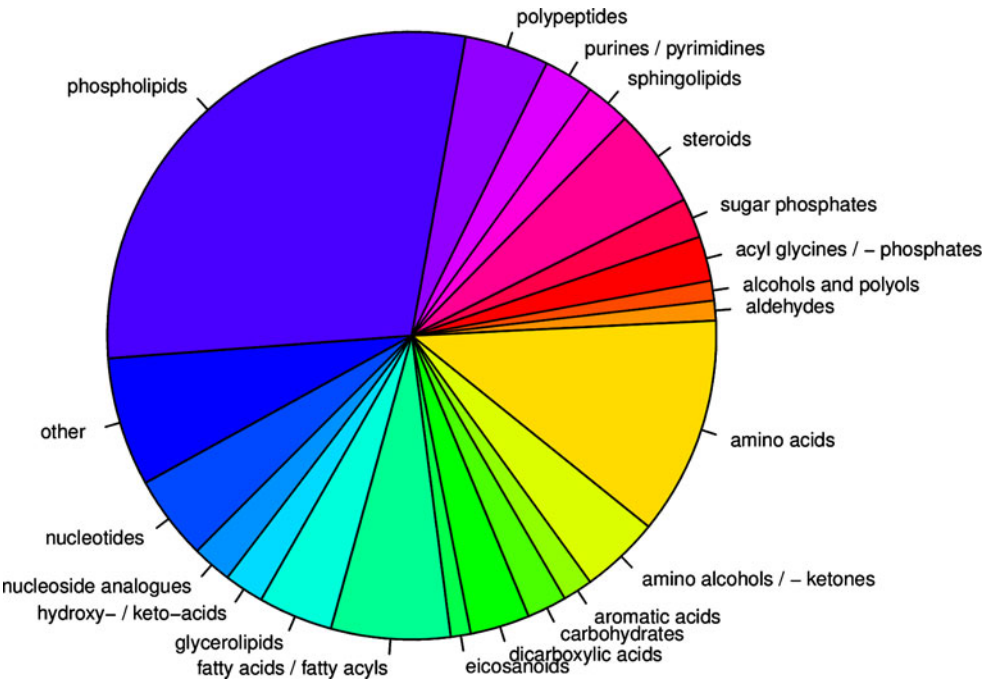

Fig. 7 Pie chart representing coverage of metabolite classes using our optimised analytical protocol. Metabolite classes are gathered from the Human Metabolome Database [34]. The class defined as *other* contains all metabolite classes that have only one or two representative compounds (i.e. pyridoxals and derivatives, quaternary amines, pterins, prenol lipids, indoles and derivatives) or metabolites that are not classified (e.g. ovothiol and trypanothione). A total of 379 metabolites were detected and putatively identified

more extensive databases like KEGG [32], LipidMAPS [30], Human Metabolome Database [33] and a peptide database. Overall, a total of 379 mass signals (combining positive and negative ESI) are annotated using all databases combined. An overview of the metabolite classes represented in our data is shown in Fig. 7. The largest groups of annotated metabolites consisted of phospholipids (29%) and amino acids (12%). Also, a high number of fatty acids/fatty acyls (6%), steroids and steroid derivatives (5%), polypeptides (5%) and nucleotides (5%) could be annotated.

Conclusions

In this study, we aimed to develop a specific method for the analysis of the metabolome of *Leishmania* species. We have chosen to work with the promastigote life stage of *Leishmania* as this form is relatively easy to culture in vitro and has an extracellular lifestyle. Biologically speaking, it would also be interesting to study the clinical relevant life stage, amastigotes, but their intracellular lifestyle in mammalian macrophages hampers a reliable and accurate parasite-specific metabolite extraction and subsequent detection. Our final sampling protocol starts with a rapid quenching of promastigotes to 0 °C in their culture medium. A triplicate washing step with phosphate-buffered saline is required to remove the rich culture medium. We could demonstrate that cell leakage is absent during this extensive washing procedure. Cell disruption and comprehensive metabolite extraction were found to be optimal using 4×10^7 parasites in 200 μl cold chloroform/methanol/water 20:60:20 (*v*/*v*/*v*) for 1 h at 4 °C. We also demonstrated that selected key metabolites show rapid oxidation during storage in the autosampler, which we try to minimise by including a deoxygenation step of the extract.

The obtained coverage of 20% of the predicted metabolome, involving metabolites from many different pathways, is a good starting point for the untargeted detection of metabolome changes in our large collection of *L. donovani* clinical isolates. However, a coverage of 20% compares unfavourably with what can be achieved by proteomic or transcriptomic analyses [3, 46, 47]. But even if not all metabolites can be detected (due to low abundance, instability or incompatibility with the separation conditions), the broad coverage of the metabolome ensures that "marker metabolites" of most metabolic processes are included in our dataset. This will provide a unique insight into the cellular function of genetically and phenotypically diverse strains of this complex pathogen. The improvement of metabolite coverage using multiple analytical platforms is a major focus of our ongoing research efforts.

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ERRATUM

Erratum to: Towards an unbiased metabolic profiling of protozoan parasites: optimisation of a *Leishmania* sampling protocol for HILIC-orbitrap analysis

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Unfortunately, the references in the following sentence in the introduction were incorrect.

"De Souza et al. [9, 10] have described rapid (seconds) quenching of *Leishmania* cultures to 0 °C using a dry ice–ethanol bath. They reported that this method quenches metabolism of *Leishmania* reliably and reproducibly."

The citations [9, 10] should be replaced by the following 2 references:

De Souza DP, Saunders EC, McConville MJ, and Likic VA (2006) Bioinformatics 22:1391–1396. (reference [36] of the manuscript)
Saunders EC, De Souza DP, Naderer T, Sernee MF, Ralton JE, Doyle MA, MacRae JI, Chambers JL, Heng J, Nahid A, Likic VA, and McConville MJ (2010) Parasitology 137:1303–13 (reference not listed in the manuscript)

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