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Fast filtration sampling protocol for mammalian suspension cells tailored for phosphometabolome profiling by capillary ion chromatography – tandem mass spectrometry

Hans F.N. Kvitvang, Per Bruheim*

Department of Biotechnology, NTNU Norwegian University of Science and Technology, Trondheim, Norway

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ABSTRACT

Capillary ion chromatography (capIC) is the premium separation technology for low molecular phosphometabolites and nucleotides in biological extracts. Removal of excessive amounts of salt during sample preparation stages is a prerequisite to enable high quality capIC separation in combination with reproducible and sensitive MS detection. Existing sampling protocols for mammalian cells used for GC–MS and LC–MS metabolic profiling can therefore not be directly applied to capIC separations. Here, the development of a fast filtration sampling protocol for mammalian suspension cells tailored for quantitative profiling of the phosphometabolome on capIC–MS/MS is presented. The whole procedure from sampling the culture to transfer of filter to quenching and extraction solution takes less than 10 s. To prevent leakage it is critical that a low vacuum pressure is applied, and satisfactorily reproducibility was only obtained by usage of a vacuum pressure controlling device. A vacuum of 60 mbar was optimal for filtration of multiple myeloma Jjn-3 cell cultures through 5 μm polyvinylidene (PVDF) filters. A quick deionized water (DI-water) rinse step prior to extraction was tested, and significantly higher metabolite yields were obtained during capIC–MS/MS analyses in this extract compared to extracts prepared by saline and reduced saline (25%) washing steps only. In addition, chromatographic performance was dramatically improved. Thus, it was verified that a quick DI-water rinse is tolerated by the cells and can be included as the final stage during filtration. Over 30 metabolites were quantitated in JJN-3 cell extracts by using the optimized sampling protocol with subsequent capIC–MS/MS analysis, and up to 2 million cells can be used in a single filtration step for the chosen filter and vacuum pressure. The technical set-up is also highly advantageous for microbial metabolome filtration protocols after optimization of vacuum pressure and washing solutions, and the reduced salt content of the extract will also improve the quality of LC–MS analysis due to lower salt adduct ion formation.

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1. Introduction

Mass spectrometric (MS) metabolite profiling has become an important tool in mapping and understanding biological systems at the metabolome level [1–3]. MS-based approaches are most frequently used in combination with a separation step (e.g. gas- and liquid chromatography, capillary electrophoresis), since biological matrices are often too complex to analyze directly on a MS instrument [4]. Also, since the diversity in physico-chemical properties of the various metabolites of the metabolome is vast, a single MS method cannot be used to analyze the complete set of metabolites present in a single cell or biological system [5]. Several MS-based analytical platforms are therefore needed to cover the whole metabolome [6].

Sample preparation is one of the key steps in MS-based metabolomics and consists of several consecutive steps which include: washing and removal of medium constituents, quenching (i.e., “cool down”/ inactivation of metabolism), extraction, and further processing such as removal of proteins and concentration of metabolites. The ideal sampling protocol should be user-friendly and easy to reproduce between laboratories, cover the whole metabolome, fast with immediate quenching of metabolism, independent upon analytical protocol, and no metabolite loss and degradation during concentration and reconstitution of sample. However, it is well known that due to the large complexity of the metabolome, sampling protocols need to be optimized with regard to the biological model system under study and the choice of analytical method [7–10].

* Corresponding author. Fax: +47 73591283.
E-mail address: Per.Bruheim@ntnu.no (P. Bruheim).

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Sampling protocols for MS-based metabolomics of mammalian cells need to consider that these cells are more fragile and easier to disrupt than bacteria, plant tissue and yeast. Nonetheless, several protocols have been developed recent years, either for adherently growing cells or for suspension cells. Adherently growing cells can either be washed while attached [11,12] or be enzymatically detached with trypsin prior to washing and quenching [13,14]. Enzymatic detachment is not recommended due to an increased chance of changing the metabolite profile; caused by the prolonged time used during sampling [10] or formation of artifacts [15]. Lorenz and co-workers applying a wash of attached cell protocol showed that adherently growing cells tolerated a fast water rinse prior to extraction [16]. This is highly beneficial for the subsequent MS analysis and they reported increased sensitivity for 26 out of the 27 detected metabolites for the adherent cell line INS-1 when compared to washing with Krebs-Ringer-HEPES buffer only. Suspension cells on the other hand must either be centrifuged [17] or filtered [18–20] for separation of the cells from growth medium. Centrifugation takes several minutes including washing, and thereby the metabolome composition will likely undergo major changes prior to quenching and extraction. Filtration is much faster but needs careful optimization and controlling of process parameters as cell leakage during washing is likely to occur [17]. Metabolite leakage can however be prevented by controlling and minimizing the applied vacuum pressure [18–20].

Several quenching solutions have been tested for mammalian cells and there is some contradicting reporting. Dietmair and colleagues performed a thorough evaluation of twelve different extraction solvents with different temperature regimes of a suspension grown Chinese Hamster Ovary (CHO) cell line [17]. They reported 50% ACN in water to be superior to all other extraction solvents.

Taking the above mentioned aspects into account we have further developed a sampling method for suspension growing cells tailored for analysis with a capillary ion chromatograph (capIC) coupled to tandem mass spectrometry (MS/MS). CapIC–MS/MS is the premium choice for quantitative phosphometabolome profiling [21,22]. Present protocols employ saline or phosphate buffered saline as final washing solutions prior to extraction, but these are not compatible with capIC–MS/MS analysis. Here, we present the development of a capIC–MS/MS compatible sampling protocol for suspension cells by introducing a fast water rinse step.

2. Materials and methods

2.1. Cultivation

Multiple myeloma cells, cell line *Jjn-3* (ACC 541, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) was grown in 175 cm^2 cell culture flask (#159910, Nunc) containing 30 mL RPMI 1640 (#R8758, Sigma–Aldrich), supplemented with 10% Fetal Bovine Serum (#F7524, Sigma–Aldrich), 100 μg/mL of gentamycin (#G1272, Sigma–Aldrich), 2.5 μg/mL Amphotericin B (#A2942, Sigma–Aldrich) and 2 mM L-Glutamine (#K0283, Biochrom). The cells were grown at 37 °C in a humidified atmosphere with 5% CO_2 in four replica bottles for about 72 h. Further, the bottles were pooled into one bottle for cell concentration determination with a Moxi^z cell counter type M cassette and diluted to 10^6 cells/mL in fresh growth medium.

2.2. Sample preparation

The pooled bottle was constantly kept in an upright position in a water bath holding 37 °C during the sampling. The cell culture were harvested onto 5 μm Nylon SVPP 47 mm filters (Merck Millipore, #SVLP04700) and fast filtered with 60 mbar vacuum pressure below the ambient pressure. The vacuum pressure was controlled by CVC 3000 and VSK 3000 units (Vacuubrand Gmbh).

First, determination of optimal rinsing solution and temperature was done by harvesting triplicates – one by one – of 5 mL cell culture containing total 5 million cells onto their respective filters. Washing of residual medium with 10 mL saline (0.9% NaCl solution) holding either 0 °C or 37 °C was immediately performed when 90% of the growth medium was filtered away. The filters were rinsed, to get rid of excess salt, with either 5 mL 25% saline (0.225% NaCl solution) or deionized water (DI-water) with the same temperature profiles, resulting in four different washing regimes and in total 12 filters, respectively. All filters were immediately transferred into 50 mL tubes containing 10 mL 50/50 ACN/DI-water (v/v) (0 °C), and next transferred to a liquid nitrogen (LN_2) bath for complete quenching.

Second, robustness of DI-water rinse volumes was tested by utilizing 5, 10 and 20 mL DI-water to filter when 3 mL cell culture containing 3 million cells were added to the filter (5 resamplings). Saline wash and further treatment was executed in the same way as mentioned above. Third, optimal cell number per filter was determined by adding cell culture suspension containing 1–3 and 6 million cells per filter (5 resamplings), followed by washing with saline and rinsing with 5 mL DI-water at 0 °C. Complete quenching was performed as mentioned above for all experiments.

Extraction was performed by three repeated freeze-thaw cycles between an ice-cold water bath and LN_2 bath, including vigorously mixing between thawing and freezing. The filters were removed after completed extraction with a forceps, and the 50 mL tubes were centrifuged for 10 min at 5000 rpm at 0 °C. The supernatant was divided into three separate 50 mL tubes – 3 mL in each, snap-frozen in LN_2, and concentrated by freeze-drying.

2.3. capIC–MS/MS and data analysis

Dried samples were dissolved in 400 μL 50% ACN/DI-water and centrifuged at 5000 rpm for 10 min. The supernatant was carefully transferred to 3 kD molecular cutoff filters (#516-0228, VWR) and centrifuged at 14.000 rpm for 10 min. The peptide and protein free supernatant was then transferred into LC–MS vial inserts and run in a randomized order on a Dionex ICS-4000 capillary ion chromatograph coupled to a Waters Xevo TQ-S MS/MS according to Kvitvang et al. [21]. The data was analyzed in MassLynx V4.1.

3. Results and discussion

3.1. Filtration apparatus set-up and considerations

During the development of the capIC–MS/MS method for phosphometabolome profiling it was observed that saline washed extracts were not compatible with capIC separation (i.e., large salt peak for the 10–15 first minutes, peak splitting, less stable retention times, and frequent replacement of expensive instrument parts such as column and suppressor), but this was successfully solved for adherently growing cells by including a fast DI-water rinse step that was proven tolerable by the cells [21]. Filtration has to be considered a tougher treatment for human cells as cells entrapped in filter materials are exposed to higher shear forces and faster osmotic changes than the washing of adherent cells. Thus, it needs careful validation that a fast DI-water rinse step is also tolerated by suspension cells during a filtration sample preparation procedure.

Based on the literature (see e.g. [17]) and our own experimentation (data not shown) 50% ACN in water was chosen as the extraction solvent for phosphometabolome analysis. Of the tested filter materials not disintegrating in ACN was polyvinylidene fluoride (PVDF) filters chosen due to available sizes, pore sizes and

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