Fast-Quenching Standard Comparison of two sampling and extraction protocols to perform metabolomic studies on human adherent cells

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Comparison of two sampling and extraction protocols to perform metabolomic studies on human adherent cells

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Introduction

There are numerous ways to perform metabolomics on adherent cells [1]. The main steps for sample preparation and collection are :

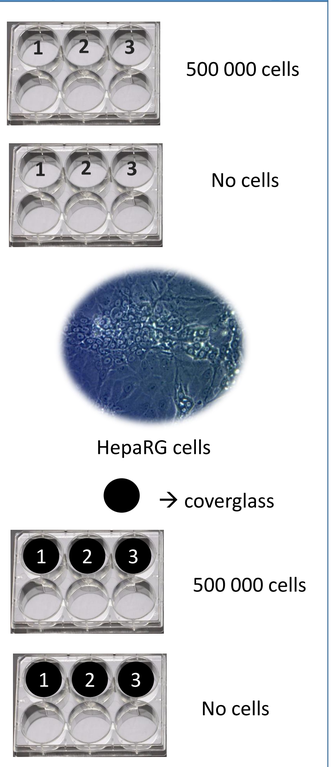
- Cell Wash → must be efficient to avoid contamination by metabolites from media.
- Quenching → metabolism arrest has to be quick and reproductible to minimize unphysiological changes of metabolites (temperature change, cell stress …)
- Cell detachment → appeared to be in favor of scrapping due to metabolites leakages with trypsination [2].
- Extraction solvent → should maximize metabolites recovery but impossible to observe the entire range of metabolites by a single extraction and analytical method [3].

1 Experimental design
2 *Sample preparation and collection*
3 Instrumental analyses
4 Data processing
5 Statistical analysis

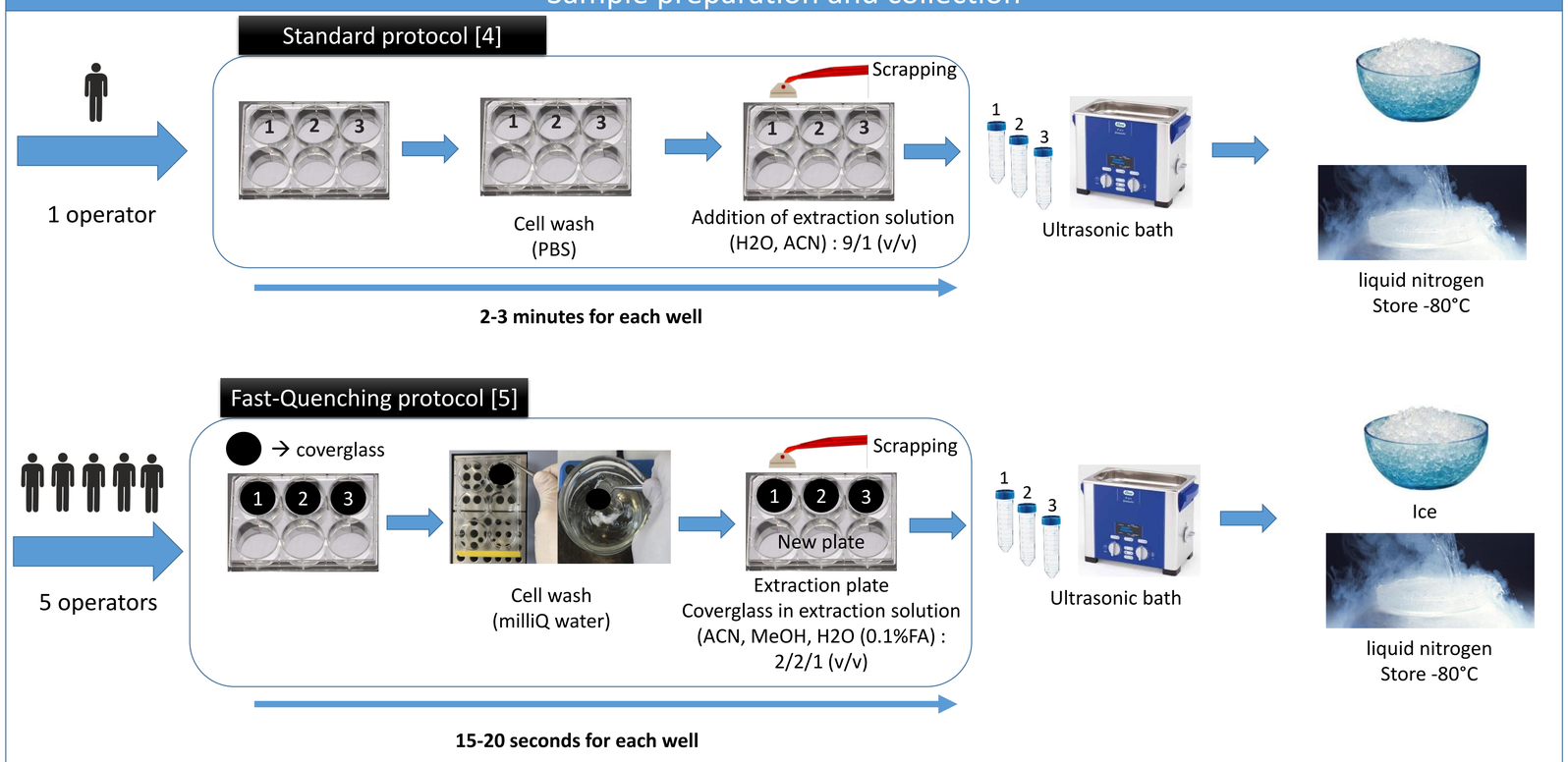
Aim → Compare two metabolites sampling methods to perform targeted and untargeted metabolomics on adherent cells.

Material and methods

Experimental design



Sample preparation and collection



Instrumental analyses

Targeted HRMS

- n= 3

Quantification with isotopic dilution standards, absolute concentration of 54 metabolites using the $^{12}C/^{13}C$ ratio were obtained :

- Amino acids (LC-MS positive ionization Qexactive) → 19 variables.
- Major carbon metabolites from central metabolism (intermediates of glycolysis, pentose phosphate pathway or tricarboxylic acid cycle) (IC-MS negative ionization LTQ orbitrap velos). → 35 variables

NMR

- n=9

- *1H*-NMR spectra were obtained on a Bruker 600 MHz spectrometer. Spectra were acquired at 300 K using NOESYPR1D (512 scan). Fixe size bucketing was performed with a 0.02 width from 0 to 10 ppm. Solvants pics were excluded from the final bucket table.

Untargeted HRMS

- n=9

- Data were generated by reverse phase liquid chromatography and using positive and negative electrospray ionization for mass spectrometry (QToF). Data were pre-processed with XCMS using W4M (detection, integration, alignment, export table). Data were then filtered using quality control samples. A total of 1099 and 696 features were obtained for positive and negative ionization respectively.

Fig. 1 Experimental design, sample collection and intrumental analyses used to compare both extraction protocols.

Results

Targeted HRMS

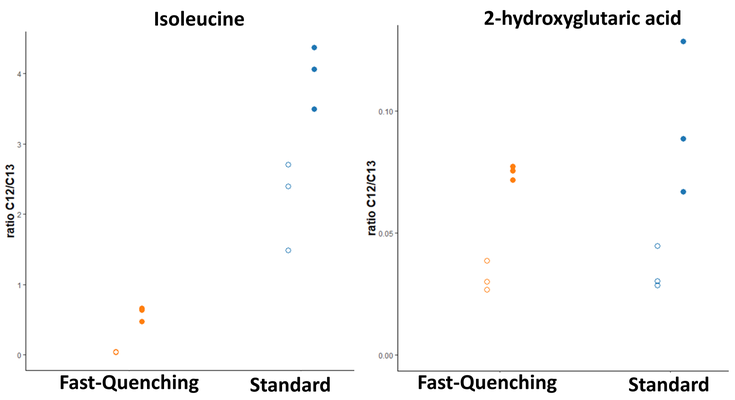


Fig. 2. Comparison of metabolites levels on blank (o) vs cell samples (•) in both protocols. $^{12}C/^{13}C$ ratio were used for these dotplots.
→ less pollution from cell culture media due to more efficient washing in fast-quenching protocol.

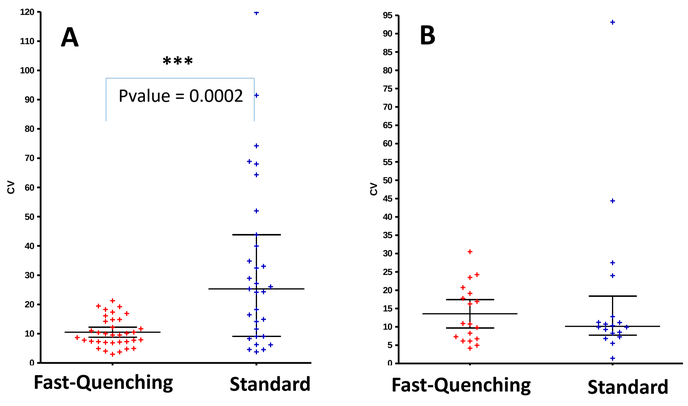


Fig. 3. Comparison of coefficients of variation obtained with absolute quantification between both protocols.
Coefficients of variation were obtained using the $^{12}C/^{13}C$ ratio for energetic metabolites (A) and for amino acids (B). Medians and interquartile range are represented for both protocols. Median were compared using a Mann & Whitney test
→ lower coefficient of variation for the fast-quenching protocol for energetic metabolites (pvalue=0.0002) (A). No significant differences for amino acids (B).

NMR

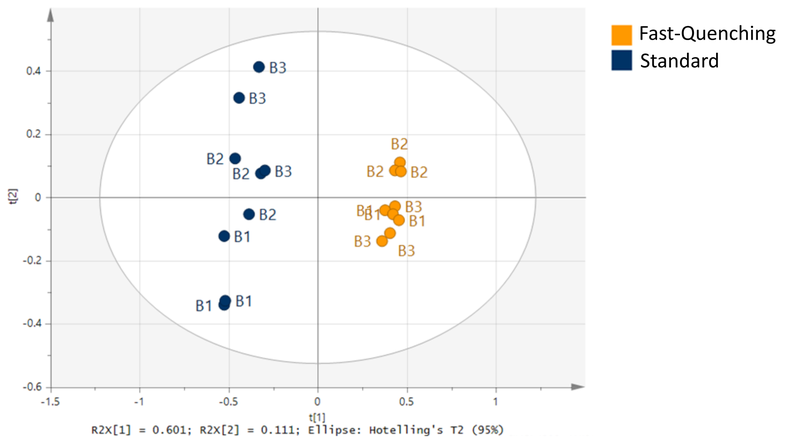


Fig. 4. PCA score plot of NMR spectra for fast-quenching and strandard protocol.
Score plot were obtained with a principle component analysis, each spectrum was normalized by the total integrated area and pareto scaling was performed for multivariate analyses. Biological batchs are represented with the label B1/B2/B3.
→ less variability for the fast-quenching protocol.

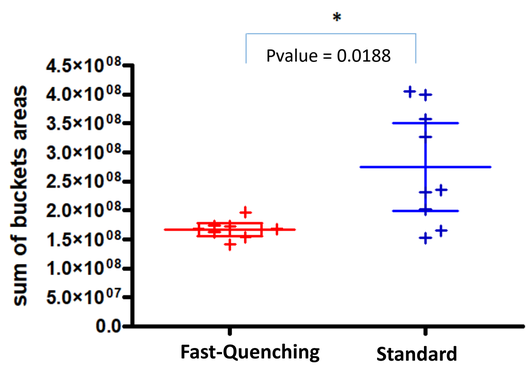


Fig. 5. Comparison of blank pollution between both protocoles
Spectra were cut with a 0.02 ppm width without normalization. Bucket areas were sumed for each spectra. Median were compared using a Mann & Whitney test).
→ less pollution in blank (pvalue = 0.0188) due to more efficient washing for the fast-quenching protocol.

Untargeted HRMS

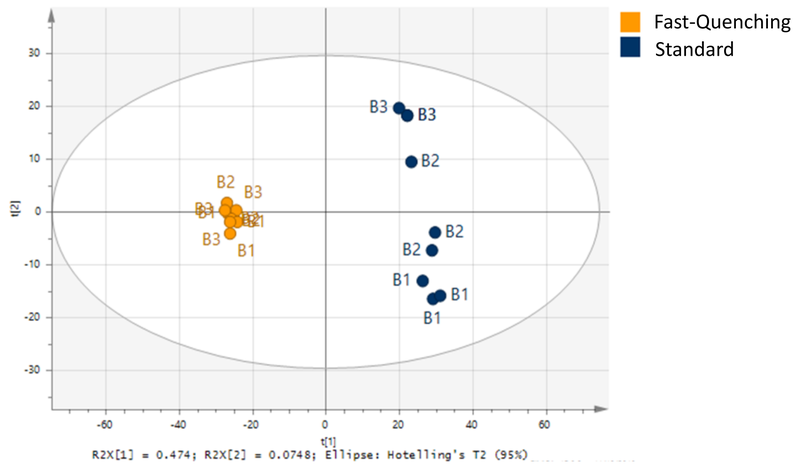


Fig. 6. PCA score plot of mass spectrometry spectra for fast-quenching and standard protocol.
Score plots were obtained using a principle component analysis in negative mode of ionization. Log-pareto scaling was perform for multivariate analyses. Biological batchs are represented with the label B1/B2/B3.
→ less variability for the fast-quenching protocol.

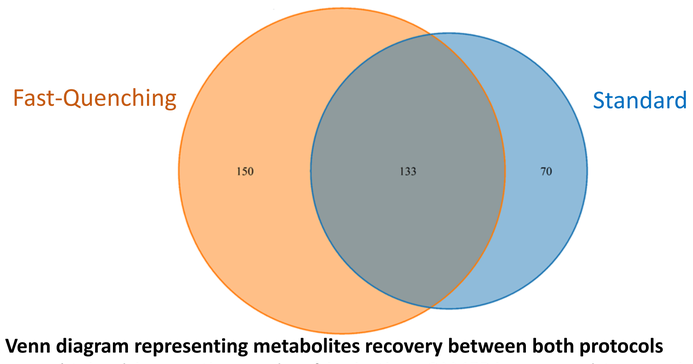


Fig. 7. Venn diagram representing metabolites recovery between both protocols
Data were obtained in negative mode of ionization. CAMERA was used to associate variables to specific metabolites adducts.
→ differences in metabolites extraction due to the use of different solvent between both protocols. More metabolites were obtained with the fast-quenching protocol.

Discussion and Conclusion

- Samples obtained with the fast-quenching method showed less variability than samples from the standard protocol (fig. 3, fig.4 and fig. 6). Regarding the score plots, Biological batchs appear to be less important in the fast-quenching protocol. This is due to the fast stop of metabolism which limits cell stress and prevents the unphysiologycal transformation of metabolites. Cells' metabolites can be observed in more physiological state.
- Samples without cells appear to be less polluted with residual metabolites from media with the fast-quenching method (fig 2. and fig. 5.). This observation is very important for targeted metabolomics to prevent overestimation of metabolite concentrations and also for untargeted metabolomics during the blank filtration in order to preserve a maximum of variables. Cell culture on coverglass allows the extraction of metabolites in a new plate with a minimum of residual metabolites from medium and more efficient washing.
- Strong separation observed in PCA score plots between both protocols (fig. 4 and fig. 6) is explained by major differences in metabolites extraction (fig. 7). These differences of metabolites recovery could be explained by the different extraction solutions used in this study. As already demonstrated in the literature, each extraction solution is linked with specific advantages and disadvantages as regards metabolites characterization, due to their different physicochemical properties [6]. However, the extraction solution can easily be adapted depending of the targeted metabolites.

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