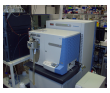
# ABSOLUTE METABOLITE QUANTIFICATION OF GLYCOLYSIS AND TREHALOSE METABOLISM FOR SYSTEMS BIOLOGY MODELLING




**Warwick Dunn, Kathleen Carroll, MCISB Consortium and Douglas B. Kell**

Manchester Centre for Integrative Systems Biology, School of Chemistry, Manchester Interdisciplinary Biocentre, University of Manchester, Manchester, M1 7ND

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## INTRODUCTION AND SUMMARY

● Glycolysis is a catabolic pathway, central to many organisms; it involves a sequence of enzymatic reactions that converts glucose to pyruvate and produces the high energy molecules ATP and NADH. In *Saccharomyces cerevisiae* the glycolysis pathway continues to anaerobic respiration and the production of ethanol. Trehalose metabolism is closely linked to glycolysis.

● Iterative forward and inverse predictive modeling of the yeast metabolic network requires the collection of experimental data representing the concentrations of metabolites and proteins. Glycolysis and trehalose metabolism which are intimately linked core pathways of energy generation and storage were chosen as an initial proof-of-concept system to set up and test the methods, software tools and modelling tools currently developed in MCISB.

● A range of quantification strategies are available, often dependant on the use of standards and isotopically labelled analogues of endogenous metabolites to compensate for experimental variability. However, these are not always commercially available. Quantification strategies appropriate for metabolites where an authentic standard (but not necessarily an appropriate internal standard) is available include external calibration and standards addition.

● Targeted analytical methods employing gas chromatography-mass spectrometry (GC-MS) and ion-pair ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) to quantify 17 metabolites related to glycolysis and trehalose metabolism has been developed and validated.

● Trehalose, glucose, fructose, glucose-6-phosphate, glucose-1-phosphate, fructose-6-phosphate, glyceraldehyde-3-phosphate, glycerate-3-phosphate, phosphoenolpyruvate, glycerol and pyruvate were detected by GC-MS with linear responses observed over 2-3 magnitudes of concentration and low µmoles.L$^{-1}$ detection limits. Reproducibility was acceptable (RSD < 12%) and recoveries calculated in the range 70-109% for most metabolites. Quantification of metabolites present in intra-cellular extracts will be performed using external calibration curves.

● The ion pairing UPLC-MS/MS detection of ATP, ADP, AMP, fructose-1,6-bisphosphate, trehalose-6-phosphate, UDP-glucose, glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate and phosphoenolpyruvate provided low µmoles.L$^{-1}$ detection limits and linear responses observed over 2-3 magnitudes of concentration . Reproducibility was measured at less than 6% with recoveries measured in the range 93-120%. Quantification of metabolites present in intra-cellular extracts will be performed with the standards addition strategy so to compensate for matrix effects observed in electrospray ionisation mass spectrometry.

● The multiple-platform detection and quantification of 17 commercially available metabolites related to glycolysis and trehalose metabolism has been successfully achieved, demonstrating that all metabolites in these pathways (except glycerate-2-phosphate and glycerol-3-phosphate where no authentic standard was available) can be quantified. This analytical approach is currently being applied to the quantification of 17 intra-cellular metabolites present in continuous cultures (where concentrations are expected to be greater than 10 µmoles.L$^{-1}$) for predictive modelling of yeast metabolism.

## EXPERIMENTAL

● Stock solutions of 17 commercially available metabolites (5-10 mmoles.L$^{-1}$) were prepared in water. Mixed standard solutions were prepared in the concentration range of 0.1-1000 µmoles.L$^{-1}$

● Trehalose, glucose, fructose, glucose-6-phosphate, glucose-1-phosphate, fructose-6-phosphate, glyceraldehyde-3-phosphate, glycerate-3-phosphate, phosphoenolpyruvate, glycerol and pyruvate were analysed by GC-MS (Agilent 6890 GC coupled with a LECO EI-TOF mass spectrometer). An injection volume of 5µl was used with a 1 in 20 split to column. Analyte separation was performed at a helium flow rate of 0.8 ml.min$^{-1}$ on a SPB50 column (Supelco, phase equivalent to a DB-17) with a 50 minute temperature program from 70 to 290°C at a rate of 5°C/minute. Quantification was performed with the calculation of peak area under a single ion chromatogram. Linear calibration ranges, limits of detection, precision (relative standard deviation calculated for replicate injections) and recoveries (quantification of metabolites in a separately prepared solution) were all calculated.

● ATP, ADP, AMP, fructose-1,6-bisphosphate, trehalose-6-phosphate, UDP-glucose, glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate and phosphoenolpyruvate were analysed by ion-pair UPLC-MS/MS (Waters ACQUITY® UPLC coupled to a ThermoFisher Scientific LTQ-Orbitrap mass spectrometer). Analyte separation was performed on a ACQUITY BEH $C_{18}$ column (1.7µm, 100 x 2.1mm) with a binary solvent gradient (A – 15mM tributylamine in water at pH 4.8, B – methanol) with a gradient elution ramp from 0-100%B over 35 minutes. This was a revised version of a previously described method (Luo *et al* J. Chromatography A 2007 1147 153-164). Integration under the peak area for a single product ion in single reaction monitoring (SRM) mode was used for quantification. Linear calibration ranges, limits of detection, precision and accuracy were all calculated.

## GC-MS QUANTIFICATION OF SUGARS, SUGAR MONOPHOSPHATES, GLYCERALDEHYDE-3-PHOSPHATE, GLYCERATE-3-PHOSPHATE, PHOSPHOENOLPYRUVATE, GLYCEROL AND PYRUVATE

● A typical chromatogram for a standard containing all of the metabolites at a concentration of 100 µmoles.L$^{-1}$ is shown (Fig 1). Metabolites detected include trehalose (TRE), glucose (GLU), fructose (FRU), glucose-6-phosphate (G6P), glucose-1-phosphate (G1P), fructose-6-phosphate (F6P), glycerate-3-phosphate (G3P), glyceraldehyde-3-phosphate (Gl3P), phosphoenolpyruvate, pyruvate (PY) and glycerol (GL). Whilst the detection of a greater number of phosphorylated metabolites is expected, a number of these were found to decompose in the heated injection system denoted by the detection of a peak corresponding to phosphate. The chromatographic separation of glucose/fructose and glucose-6-phosphate/fructose-6-phosphate has been successfully achieved (Fig 1).

● The analytical specifications for each metabolite is also shown (Table 1), including linear dynamic range, limit of detection, precision (n=6) and recovery. Responses were linear over 2-3 orders of concentration magnitude with limits of detection less than 10 µmoles.L$^{-1}$. A reproducibility of less than 12% was observed for six replicate analyses at concentrations of 25 and 200 µmoles.L$^{-1}$. Recoveries were measured in the range 70-109%. However, oxaloacetic acid and dihydroxyacetone phosphate have a reduced sensitivity with only concentrations greater than 100 µmoles.L$^{-1}$ being detected. No authentic standard was available at the time of analysis for 2-phosphoglycerate and glycerol-3-phosphate

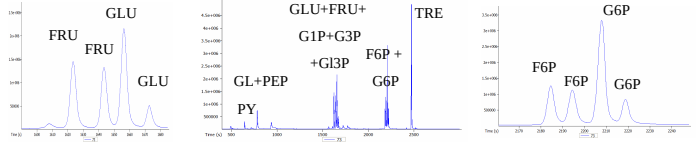


**Figure One**, a typical single ion chromatogram for mass 73 of a **100 µmoles.L$^{-1}$** standard solution and expanded regions showing the chromatographic resolution achieved

| Metabolite | Linear Calibration Range, µmoles.L$^{-1}$/(correlation coefficient) | Limit of detection (µmoles.L$^{-1}$) | RSD (n=6) 25 µmoles.L$^{-1}$ | RSD (n=6) 200 µmoles.L$^{-1}$ | Recovery 25 µmoles.L$^{-1}$ %, (n=2) |
|---|---|---|---|---|---|
| Trehalose | 0.1 - 100 (0.998) | 0.08 | 5.4 | 3.4 | 70.1 |
| Glucose-6-phosphate | 1-250 (0.998) | 0.9 | 11.2 | 2.2 | 97.5 |
| Glucose-1-phosphate | 1-250 (0.998) | 0.8 | 10.9 | 9.1 | 88.2 |
| Glucose | 1-250 (0.993) | 0.9 | 6.7 | 1.5 | 108.2 |
| Fructose-6-phosphate | 1-250 (0.998) | 0.6 | 8.1 | 4.7 | 92.6 |
| Fructose | 1-250 (0.994) | 0.9 | 5.9 | 1.4 | 82.3 |
| 3-phosphoglycerate | 10 - 1000 (0.993) | 8.3 | 11.9 | 4.7 | 104.8 |
| Phosphoenolpyruvate (PEP) | 1 - 100 (0.986) | 0.8 | 7.0 | 4.2 | 91.5 |
| Pyruvate | 0.1 - 50 (0.996) | 0.1 | 3.9 | 6.5 | 95.9 |
| Glyceraldehyde-3-phosphate | 1 - 500 (0.996) | 2.3 | 9.4 | 3.5 | 81.8 |
| Glycerol | 0.1 - 50 (0.997) | 0.1 | 4.5 | 2.4 | 103.2 |

**Table One**, a range of analytical specifications for 11 metabolites as analysed by GC-MS

## ION PAIR UPLC-MS/MS QUANTIFICATION OF ATP, ADP, AMP, FRUCTOSE-1,6-BISPHOSPHATE, TREHALOSE-6-PHOSPHATE, UDP-GLUCOSE, SUGAR MONOPHOSPHATES AND PHOSPHOENOLPYRUVATE

● Single Reaction Monitoring (SRM) was performed to increase selectivity and sensitivity. Chromatograms for 6 metabolites are shown for concentrations of 200 µmoles.L$^{-1}$ (Fig 2)

● The analytical specifications for each metabolite is shown (Table 2), including linear dynamic range, limit of detection, precision (n=3) and accuracy. Responses were linear over 2-3 magnitudes of concentration with limits of detection less than 1 µmoles.L$^{-1}$. A reproducibility of less than 10% was observed for three replicate analyses at concentrations of 100 µmoles.L$^{-1}$. Recoveries were measured in the range 93-120%.

● Glucose-1-phosphate, glucose-6-phosphate and fructose-6-phosphate were not completely chromatographically resolved, and further work is required to optimise conditions for these metabolites.

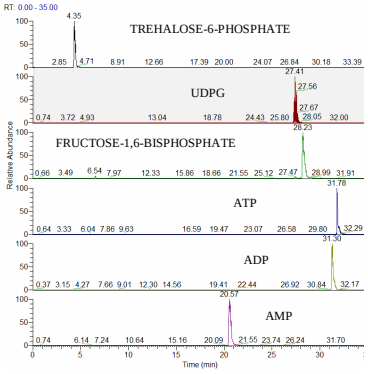


**Figure Two**, typical single reaction monitoring chromatograms for 6 metabolites

| Metabolite | Linear Calibration Range, µmoles.L$^{-1}$/ (correlation coefficient) | Limit of detection (µmoles.L$^{-1}$) | RSD (n=3) 100 µmoles.L$^{-1}$ | Recovery 250 µmoles.L$^{-1}$ %, (n=2) |
|---|---|---|---|---|
| Glucose-1-phosphate | 0.5 - 500 (0.994) | 0.4 | 2.7 | 106.1 |
| Fructose-1,6-bisphosphate | 1 - 500 (0.997) | 0.9 | 4.5 | 113.5 |
| Adenosine diphosphate | 1 - 500 (0.995) | 0.8 | 3.8 | 119.7 |
| Trehalose-6-phosphate | 0.5 - 250 (0.999) | 0.5 | 0.2 | 93.5 |
| UDP-glucose | 1 - 500 (0.999) | 0.7 | 5.5 | 103.2 |
| Adenosine triphosphate | 1 - 500 (0.998) | 0.8 | 1.7 | 96.7 |
| Glucose-6-phosphate | 1 - 500 (0.993) | 0.9 | 3.2 | 109.6 |
| Fructose-6-phosphate | 1 - 500 (0.985) | 0.9 | 3.4 | 112.2 |
| Adenosine monophosphate | 1 - 500 (0.997) | 0.9 | 3.9 | 115.4 |
| Phosphoenolpyruvate | 1 - 500 (0.983) | 0.9 | 1.8 | 112.8 |

**Table Two**, a range of analytical specifications for 10 metabolites as analysed by UPLC-MS


**FOR FURTHER INFORMATION CONTACT warwick.dunn@manchester.ac.uk or dbk@manchester.ac.uk**



**The authors wish to thank the BBSRC and EPSRC for financial support of The Manchester Centre for Integrative Systems Biology**