Title

Two strings to the systems biology bow - co-extracting the metabolome and proteome of yeast

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17 ## Abstract

18 Experimental samples are valuable and can represent a significant investment in time and resources. It is highly
19 desirable at times to obtain as much information as possible from a single sample. This is especially relevant for
20 systems biology approaches in which several 'omics platforms are studied simultaneously. Unfortunately, each
21 platform has a particular extraction methodology which increases sample number and sample volume requirements
22 when multiple 'omics are analyzed. We evaluated the integration of a yeast extraction method; specifically we
23 explored whether fractions from a single metabolite extraction could be apportioned to multiple downstream 'omics
24 analytical platforms. In addition, we examined how variations to a chloroform/methanol yeast metabolite extraction
25 regime influence metabolite recoveries. We show that protein suitable for proteomic analysis can be recovered from
26 a metabolite extraction and that recovery of lipids, while reproducible, are not wholly quantitative. Higher
27 quenching solution temperatures (-30°C) can be used without significant leakage of intracellular metabolites when
28 lower fermentation temperatures (20°C) are employed. However, extended residence time in quenching solution, in
29 combination with vigorous washing of quenched cell pellets, leads to extensive leakage of intracellular metabolites.
30 Finally, there is minimal difference in metabolite amounts obtained when metabolite extractions are performed at
31 4°C compared to extractions at -20°C. The evaluated extraction method delivers material suitable for metabolomic
32 and proteomic analyses from the same sample preparation.

33 ## Keywords:

34 Extraction, metabolome, sample preparation, proteomics, lipidomics

35

36 # 1. Introduction

37 The integration of reductionist techniques in the practice of systems biology (Kohl et al. 2010), into what has been
38 described as ensemble descriptions of the cell (Nurse and Hayles 2011) has increased the diversity of analytical
39 platforms used to obtain 'omics datasets and has, in general, increased the number and type of extraction
40 methodologies applied in any given experiment (for example see Oliver et al. 1998; Trauger et al. 2008; Weeks et al.
41 2006). Such an array of sample preparation methods not only increases experimental complexity but also the
42 required amount of sample, which can be limited depending on the system under observation and experimental scale
43 (Börner et al. 2007). Integration of 'omics extraction methods, if possible, would have several advantages including
44 reduced sample volume requirements facilitating high-resolution temporal sampling regimes for multiple platform
45 analysis and simultaneous stabilization of transcriptome, proteome and metabolome. With metabolomic analyses
46 being the most sensitive to variations in extraction conditions any attempt at integration might reasonably begin with
47 metabolite extraction as a starting point.

48 Yeast metabolite extraction methods have been the subject of intense scrutiny and ongoing review since their initial
49 development (Canelas et al. 2009; Canelas et al. 2008a; de Koning and van Dam 1992; Villas-Bôas et al. 2005;
50 Villas-Bôas and Bruheim 2007; Wittmann et al. 2004; Hans et al. 2001). Metabolite extraction is fraught with the
51 difficulty that poorly applied preparation methods can generate greater variation than originally exists within the
52 biological sample itself. Only two methods, assessed by Canelas et al.(2009), have survived the rigor of the last
53 decade to emerge as near complete, such that comprehensive metabolite extraction can be achieved with a degree of
54 confidence (at least in yeast): boiling ethanol method (Gonzalez et al. 1997) and chloroform/methanol method (de
55 Koning and van Dam 1992).

56 There are several advantages to the boiling ethanol method. It is rapid, requires minimal sample handling and it can
57 be conducted in a small volume. Small volume of extracts can be rapidly concentrated by vacuum centrifugation.
58 In contrast, chloroform/methanol extractions are more labor intensive and the relatively larger volume of metabolite-
59 containing phases take longer to concentrate. However, the chloroform/methanol method has the promising feature
60 that the extract is segregated into multiple fractions; a polar fraction (methanol/water), a non-polar fraction
61 (chloroform) and a solvent insoluble fraction. Unlike the polar fraction, containing well characterized intracellular

62 metabolites, the contents of the other fractions are only implied in earlier work evaluating yeast metabolite
63 extraction; ie. lipids in the non-polar fraction and proteins in the solvent insoluble fraction.

64 Similarities between the de Koning and van Dam (1992) metabolite extraction and the Folch (1957) method of lipid
65 extraction from fish have previously been noted (Villas-Bôas et al. 2005; Canelas et al. 2008a). The basic Folch
66 method is generally robust and gives good recovery of all major lipid classes (Iverson et al. 2001). Given the
67 similarities of the two methods, it is expected that the chloroform fraction of a de Koning and van Dam (1992)
68 metabolite extraction will contain lipids, albeit extracted with varying degrees of efficiency. However, the methods
69 have significant differences including extraction temperature, time of extraction, and the use of glass or zirconia
70 beads to reliably extract lipids from yeast (Daum et al. 1999; Ejsing et al. 2009).

71 Recent work suggests that quantitative recovery of lipid species may be possible without the use of beads to first
72 create a cell extract (Guan et al. 2010). In addition to lipids, the complete absence of fatty acids in the polar extracts
73 of Villas-Bôas et al.(2005) suggests that the chloroform fraction may also extract this metabolite class. The
74 extraction of lipids by a chloroform/methanol/water system is generally preferable to the use of ethanol, the
75 alternative metabolite extraction method, due to the propensity of ethanol to oxidize to acetaldehyde, resulting in the
76 formation of aldehyde adducts with amine containing lipids (Radin 1989).

77 The solvent insoluble material, following removal of liquid phases from a biphasic extraction, contains protein and
78 polysaccharides. The protein component has been used as a crude means of evaluating initial biomass (de Koning
79 and van Dam 1992; Villas-Bôas and Bruheim 2007). In these reports the quality of the protein component was not
80 evaluated.

81 Traditionally, yeast protein extraction for use in proteomic work is achieved using a washing, lysis and precipitation
82 regime. Washing removes components that have carried over from the growth medium, desalts the sample and,
83 when performed with trichloroacetic acid, can assist with proteome stabilization (Horvath and Riezman 1994; Grassl
84 et al. 2009; Wright et al. 1989). Protein extraction is commonly achieved using glass beads (Futcher et al. 1999;
85 Wright et al. 1989; Conzelmann et al. 1988) or French press (Lee et al. 2011) followed by precipitation with acetone
86 to further remove salts and prepare the sample for solublization in electrophoresis buffer (Picotti et al. 2009;

87 Kümmel et al. 2010). Proteome stabilization and salt removal are key functions of all protein sample preparation
88 methods.

89 Metabolite extractions also meet the requirements of protein extractions. Metabolite extractions are routinely
90 performed under extremely low temperature conditions in order to stabilize the metabolome. Quenching has already
91 been shown to stabilize the transcriptome (Pieterse et al. 2006; Martins et al. 2007) and is expected to do the same
92 for the proteome. When biphasic extractions are employed, low temperatures are combined with the use of a
93 denaturing solvent further enhancing proteome stability. In addition, a biphasic system can also quantitatively
94 remove residual salt and other growth medium components which can interfere with downstream proteomic
95 analyses (Wessel and Flügge 1984). Therefore, it is likely that the solvent insoluble material remaining after a
96 metabolite extraction would contain protein suitable for more than biomass estimation. The converse; the use of
97 protein extractions for the recovery of metabolites, is not so well matched. The use of acid as a stabilizing agent for
98 proteins can lead to metabolite loss (Villas-Bôas et al. 2005) and protein extraction reagents are not, in general,
99 compatible with direct use in mass spectrometry. Taken together these factors suggest a protocol in which
100 metabolite extraction should be followed by protein extraction and not the other way around.

101 The rigorous evaluation of metabolite extraction methods to date has made clear the necessity of extraction method
102 validation for each system to which it is applied. Therefore, this work explores how specific variations to the
103 standard chloroform/methanol metabolite-extraction method for yeast, applied to a batch-fermentation, contribute to
104 metabolite loss during the process of extraction. Specifically, the impact of quenching solution temperature,
105 washing regime and extraction temperature were evaluated. In addition, we explored whether recovery of source
106 material for proteomic and lipidomic work is possible from solvent insoluble and non-polar fractions respectively.
107 Finally we propose an extraction method that delivers material suitable for both metabolomic and proteomic
108 analyses from the same sample preparation.

109 2. Methods

110 2.1. Culture conditions

111 *Saccharomyces cerevisiae* strain AWRI1631 was obtained from the Australian Wine Research Institute
112 Microorganism culture collection (WDCM22). Cultures were maintained on 1% w/v yeast extract, 2% w/v bacto

113 peptone and 2% w/v glycerol (YPG) agar plates at 28°C. A progressive culturing regime, described below, from
114 YPD to 50% defined medium to 100% defined medium was used to generate biomass for the inoculation of
115 experimental cultures. This conditions the yeast to the defined medium environment and eliminates carry-over of
116 complex medium. Overnight starter cultures were grown in 1% w/v yeast extract, 2% w/v bacto peptone and 2%
117 w/v D-glucose (YPD) liquid broth on a rotating wheel at 28°C. YPD overnight cultures were used to inoculate 1:1
118 v/v defined medium:water, which were grown overnight in cotton-plugged Erlenmeyer flasks at 20°C. Bioreactor
119 vessels (New Brunswick Scientific, New Jersey, USA.) were inoculated from 1:1 v/v defined medium:water
120 overnight cultures to an optical density of 0.1 at 600 nm. Each bioreactor vessel contained 900 mL of defined
121 medium that had been sparged with N_2 gas until no further reduction in DO_2 was observed.

122 Defined medium composition was as follows; D-glucose (20 gL^{-1}), K_2HPO_4 (1.1 gL^{-1}), $MgSO_4.7H_2O$ (0.5 gL^{-1}),
123 $CaCl_2.2H_2O$ (0.18 gL^{-1}), NH_4Cl (0.573 gL^{-1}), trace minerals and vitamins as given in Schmidt et al.(2011) and made
124 to a pH of 3.5 with potassium hydroxide. This minimal medium composition minimized the risk of interference by
125 medium components during metabolite analysis of quenching solution and was used for all experiments except when
126 the efficacy of washing steps was evaluated. For the evaluation of washing efficiency the growth medium was
127 identical to that described above except that 100 gL^{-1} of both glucose and fructose was used. Fermentations were
128 continued until residual sugar was no longer detectable (less than 0.1 gL^{-1}). Glucose and fructose concentrations in
129 fermentations and metabolite extracts were determined enzymatically (Randox Laboratories Ltd., Crumlin, UK)
130 with modifications as described by Vermeir et al.(2007). Calibration curves relating glucose and fructose
131 concentration to absorbance were fitted by least squares regression. The majority of work reported here used
132 stationary phase cultures, sampled at a biomass concentration of 2.24 gL^{-1} dry cell weight (DCW), determined as
133 described in (Liccioli et al. 2011). When efficacy of washing was being evaluated cultures were sampled during
134 late-log phase growth from high sugar defined medium at a biomass concentration of 2.8 gL^{-1} (DCW).

135 ## 2.2. Sampling of culture, quenching of metabolites and washing of biomass

136 A diagrammatic representation of the experimental workflow is shown in Figure 1. A 10 mL sample containing
137 0.0224 g dry cell weight was transferred from the fermenter vessel using an autosampler (Medicel, Helsinki,
138 Finland) to 40 mL of 60% v/v methanol in water (quenching solution) which had been pre-cooled, to either -30°C

139 (Q-30) or -40°C (Q-40) in a Lauda ecoline RE120 (Lauda, Königshofen, Germany) with 60% v/v ethylene glycol as
140 cryo fluid and the addition of dry ice when required. The mean residence time of cell culture in sample tubing
141 during sampling was less than 4 seconds. Different internal standard concentrations were used for quenching,
142 washing and intracellular metabolite solutions such that their final concentrations were equivalent after sample
143 drying and resuspension for analysis (see below). For the purpose of evaluating metabolite leakage during
144 quenching, the quenching solution contained the following internal standards; adipic acid (0.04 µM), norvaline (0.5
145 µM) and sarcosine (0.5 µM). Yeast were collected immediately after quenching (Q-30 and Q-40) or after 20 min in
146 quenching solution (Q-30T20) by centrifugation for 5 min at 1780 × g in a Hettich Universal 32R benchtop
147 centrifuge (Tüttlingen, Germany) that had been pre-cooled to -20°C. Quenching solution was decanted into fresh
148 pre-weighed tubes and dried under nitrogen at 30°C to facilitate methanol removal prior to using a centrifugal
149 vacuum concentrator. The dried quenching solutions taken up in water (Q-40, Q-30 and Q-30T20) were used to
150 evaluate leakage of intracellular metabolites.

151 Cell pellets were washed with -40°C 60% v/v methanol containing the following internal standards: adipic acid
152 (0.4 µM), norvaline (5.0 µM) and sarcosine (5.0 µM). Two wash protocols were evaluated. Pellets were either
153 vortexed in 5 mL of -40°C wash solution and centrifuged as above (Vo) or rinsed by running 5 mL of wash solution
154 around the rim of the tube with removal by decanting into fresh tubes (Ri). The rinsing procedure was performed
155 twice on each sample. Washing solutions were dried using a centrifugal vacuum concentrator and taken up in water
156 immediately prior to analysis. Washed cell pellets were extracted immediately (see below). Cell free medium
157 samples (CF) were obtained by direct filtration as described by Mashego et al.(2003).

158 ## 2.3. Extraction of metabolites

159 Extraction of intracellular metabolites from washed cells was performed using an adaptation of the methods
160 described by de Koning and van Dam (1992). Yeast cell pellets isolated as described above were resuspended by
161 vortexing for 30 s in 5 mL chloroform containing lysophosphatidylcholine (LPC, 0.2 µM) and cholesterol esters
162 (CE, 0.2 µM) as internal standards for the non-polar fraction. Methanol (2.5 mL) was added to the chloroform
163 suspended cells, mixed again by vortexing and the chloroform/methanol suspension transferred to a fresh, pre-
164 weighed, 10 mL polypropylene centrifuge tube. Pre-cooled water (4°C), containing 20 µM ribitol, 20 µM sarcosine,
165 1.5 µM adipic acid and 20 µM nor-valine as internal standards, was added to the chloroform/methanol suspension

166 and mixed by shaking at 200 r.p.m. for 45 min at either -20°C or 4°C on a Ratek RM2 reciprocating mixer (Boronia,
167 Victoria, Australia). The water/methanol and chloroform phases were separated by centrifugation for 5 min at 1,780
168 × g and -20°C. The upper water-methanol phase was removed (intracellular polar metabolite fraction – PM) leaving
169 the non-polar lower phase (intracellular non-polar metabolite fraction - NpM) and protein containing interface. The
170 chloroform lower phase was back extracted by the addition of a further 2 mL methanol and 2 mL water followed by
171 vortexing for 30 s to create an emulsion and centrifuged as described above. The upper methanol/water phase was
172 again removed and combined with the upper phase from the first centrifugation (PM). Methanol (5 mL) was added
173 to the chloroform lower phase, samples were mixed by vortexing for 30 s and solvent insoluble material was
174 precipitated by centrifugation at 1,780 × g for 5 min at -20°C. The supernatant was removed to a fresh pre-weighed
175 tube (intracellular non-polar metabolite fraction - NpM). The pellet was washed with 5 mL methanol and again
176 pelleted by centrifugation as described above. The methanol wash was discarded and the pellet was dried under a
177 stream of N_2 gas until just dry. Tubes were sealed and pellets were stored at -80°C (solvent insoluble fraction - SIF).
178 Intracellular polar (PM) and non-polar (NpM) metabolite fractions were dried using a centrifugal vacuum
179 concentrator (Labconco, Kansas city, USA.) as recommended by Villas-Bôas et al.(2005). Dried PM and NpM
180 fractions were taken up in water and butanol:methanol (50:50) respectively prior to analysis.

181 A monophasic lipid extraction method as described previously (Bligh and Dyer 1959) was used for comparison.
182 Briefly, 2 mL of chloroform were added to quenched and washed cells and vortexed to resuspend the pellet. Then 4
183 mL of methanol and 2 mL of water were added and the cells were extracted by shaking at shaking at 200 r.p.m. for
184 45 min on a Ratek RM2 reciprocating mixer at either 4°C (ME4) or 20°C (ME20).

185 2.4. Protein solublization from yeast pellets and solvent extracted material

186 Proteins were extracted from solvent insoluble fractions (SIF) as follows; 1 mL of 2D buffer (7 M urea, 2 M
187 thiourea, 20 mM tris and 4% w/v 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)) was
188 added to SIF fractions, probe sonicated for 2 × 10 s on ice using a probe sonicator (Branson sonifier 450, John
189 Morris Scientific, Chatswood, Australia) and then water bath sonication (Transsonic 700/H, Elma GMBH, Singen,
190 Germany) for 15 min. The supernatant (SIFE1) was collected by centrifuging at 20,000 × g for 10 min and pelleted
191 material was re-extraction as above (SIFE2). Both SIFE1 and SIFE2 were pooled, desalted and concentrated by
192 using 3 kDa cut off concentrators (Sartorius AG, Goettingen, Germany). Proteins were also extracted directly from

193 washed yeast cell pellets using the method of Herbert et al.(2006) to create a reference protein extract (Ref Ex).
194 Following protein extraction, supernatants were precipitated by addition of 9 volumes of acetone, incubation at -20C
195 for 1h and centrifugation at 4,000 × g for 15 min. Pellets were solubilized with 7M urea, 2M thiourea, 4% CHAPS
196 and 20mM Tris. For 2-D gel electrophoresis, conductivity was kept at, or below 300 μScm^{-1}, measured using
197 TwinCond conductivity meter B-173 (Horiba, Kyoto, Japan). Protein concentrations were determined with a
198 Bradford protein assay kit (Sigma, St Louis, USA) using BSA as a standard.

199 **2.5. Separation of protein fraction by 2-D electrophoresis**

200 Prior to protein separation by 2-D electrophoresis, extracted proteins were reduced with 5 mM Tributyl phosphine
201 (TBP) and alkylated with 15 mM acrylamide for 90 min at room temperature. Following reduction and alkylation,
202 100 µg of protein were loaded on a 17 cm pH 5-8 linear IPG strip (Bio-Rad, Regents Park, Australia). First
203 dimension isoelectric focusing was performed on an Ettan IPGphor II (GE Health Care, Uppsala, Sweden) for ~ 20
204 h at 20°C using the following program: 300 V for 4 h, linear increase from 300 V to 8000 V over 8 h and hold at
205 8000 V until approximately 100 kVh had been accumulated. The focused IPG strip was equilibrated for
206 approximately 2 x 15 min in equilibration buffer (6 M urea, 2% v/v SDS, 20% w/v glycerol, 0.375 M tris-HCl
207 buffer). Equilibrated IPG strips were overlaid onto 8 -18 % w/v SDS-polyacrylamide gradient gel (180 mm x
208 190 mm) and sealed with 0.5% w/v agarose solution containing 0.1% w/v bromophenol blue (Bio-Rad, Regents
209 Park, Australia). The SDS-polyacrylamide gradient gels were cast in our laboratory using ProteanII xi Multi-Gel
210 Casting Chamber (Bio-Rad, Regents Park, Australia) according to manufacture's instructions. Molecular weight
211 standards (Bio-Rad, Regents Park, Australia) were loaded at the edge of the cast gradient gel. IEF separated proteins
212 were then resolved in the second dimension at 5 mA/gel overnight followed by 40 mA/gel with a constant
213 temperature of 4°C until the bromophenol blue dye front had just run off the bottom of the gel. The gel was fixed in
214 10% v/v methanol and 7% v/v acetic acid for 4 h and stained with SYPRO® Ruby staining solution at room
215 temperature overnight. The gel was then destained twice with 10% methanol and 7% acetic acid for 4 h and 1 ×
216 with 1% v/v acetic acid. Stained gels were imaged at 100 µm resolution using a Typhoon Trio 9400 variable mode
217 laser scanner (GE Life Sciences, Rydalmere, Australia) with 457 nm excitation and 610 nm BP 30 emission filter.

218 **2.6. Protein identification**

219 Protein spots on 2-D gel were excised using an ExQuest Robotic fluorescent spot cutter equipped with a CCD
220 camera and 1 mm diameter cutting head (Bio-Rad, Regents Park, Australia). The gel plugs were washed 3 times
221 with 25 mM ammonium bicarbonate in 50% v/v acetonitrile (ACN) for 15 minutes at 37ºC with gentle agitation,
222 dehydrated with 100% ACN for 10 min at room temperature and incubated at 37ºC until the gel plugs were
223 completely dry. Dry gel plugs were rehydrated with trypsin solution (5 ng/µL, Sigma, St Louis, USA) for 1 h at 4ºC.
224 The excess trypsin solution was removed and 25 mM ammonium bicarbonate was added to cover the gel plugs
225 which were incubated overnight at 37ºC for digestion. Tryptic digestion was quenched by adding 0.1%
226 trifluoroacetic acid (TFA) and peptides were extracted from gel plugs by water bath sonication (Transsonic 700/H,
227 Elma GMBH, Singen, Germany). The peptides were desalted and concentrated using Perfect Pure C18 zip-tip
228 (Eppendorf, North Ryde, Australia) and were spotted onto a Matrix-assisted laser desorption/ionization (MALDI)
229 sample plate with 1 µL of matrix (α-cyano-4-hydroxycinnamic acid, 4 $mgmL^{-1}$ in 90% v/v ACN, 0.1% v/v TFA) and
230 allowed to air dry.

231 MALDI mass spectrometry was performed with an Applied Biosystems 4800 *Plus* MALDI TOF/TOF™ Analyser
232 (Carlsbad, USA). A neodymium-doped yttrium aluminium garnet laser (355 nm) was used to irradiate the sample.
233 Spectra were acquired in reflectron mode in the mass range 700 to 3500 Da and were externally calibrated using
234 known peptide standards (bradykinin, neurotensin, angiotensin and adrenocorticotropic hormone). The eight
235 strongest peptides from the MS scan were isolated and fragmented in tandem time of flight mode (by collision-
236 induced dissociation using filtered laboratory air), then re-accelerated to measure their masses and intensities. A near
237 point calibration was applied to give a typical mass accuracy better than 50 ppm. The peptide peak list was used to
238 interrogate the Mascot database (Matrix Science Ltd, London, UK). All samples were searched against
239 *Saccharomyces cerevisiae* (baker's yeast) in SwissProt with the following search parameters; type of search: PMF
240 and MSMS, enzyme: trypsin, variable modifications: methyl (DE), oxidation (M), propionamide (C), mass values:
241 monoisotopic, protein mass: unrestricted, peptide mass tolerance: ± 50 ppm, fragment mass tolerance: ± 0.8 Da,
242 peptide charge state: 1+, max missed cleavages: 1). High MOWSE scores in the database search indicated a likely
243 match, confirmed by manual inspection. Percentage sequence coverage, the difference between calculated and

244 observed peptide masses, the number of missed cleavages was taken into account for positive identification in
245 addition to how well the MW and pI of the identified protein matched other experimental data such as 2-DE.

246 **2.7. Quantification of intracellular and extracellular metabolites**

247 γ-Aminobutyric acid and 20 proteinogenic amino acids in the cell extract as well as the quenching solution, washing
248 solutions and the culture broth were quantified using high performance liquid chromatography (HPLC). The
249 protocol employed precolumn o-phtalaldehyde derivatization followed by fluorescence detection and is described
250 elsewhere (Dietmair et al. 2010). Norvaline and sarcosine were used as internal standards for primary and secondary
251 amines, respectively. The concentration of internal standard in all samples was 250 μM. This was achieved by
252 appropriate concentration / dilution steps.

253 Analyses of 32 intracellular metabolites (18 glycolysis and TCA intermediaries, 11 nucleotides phosphate and 3
254 nicotinamide adenine dinucleotides) were conducted on a Dionex UltiMate 3000 liquid chromatography system
255 (Thermo Fischer, Scoresby, Australia) coupled to an ABSciex 4000 QTRAP mass spectrometer (Waverly,
256 Australia). Chromatographic separation was achieved on a Gemini-NX C18 150 × 2 mm I.D., 3 μm, 110 Å particle
257 column (Phenomenex, Aschaffenburg, Germany) operated at 55°C. Mobile phase adapted from Luo et al.(2007)
258 was 7.5 mM tributylamine aqueous solution adjusted to pH 4.95 with glacial acetic acid (eluent A) and acetonitrile
259 (eluent B). The linear HPLC gradient profile was 0% B for 8 min, 0-20% B for 12 min, 20-27% B for 10 min, 27-
260 100% B for 1 min, followed by a column wash at 100% B for 3 min and re-equilibration back to initial conditions
261 for 16 min, all at 0.3 $mLmin^{-1}$. Injection volumes of 10 μL were used. Mobile phase was directly introduced into
262 the mass spectrometer.

263 The mass spectrometer was equipped with a TurboV electrospray source operated in negative ion mode. Infusions
264 of analyte standards dissolved in purified water were used for tuning compound dependent MS parameters.
265 Declustering potential (DP), collision energy (CE) and collision cell exit potential (CXP) of each transition were
266 optimized and are shown in Online Resource 1. Entrance potential (EP) was fixed to −10 V for all transitions. To
267 tune source dependent parameters an analyte standard mix was infused along with mobile phase (7.5 mM
268 tributylamine : acetonitrile 50:50 (v/v), 100 μL/min) connected by a T-port. A combination of parameters was
269 chosen based on highest abundance for the majority of analytes. These optimized parameters were ionspray voltage

270 −4500 V, nebulizer (GS1), auxiliary (GS2), curtain (CUR) and collision (CAD) gases were 60, 60, 20 and medium
271 (arbitrary units), respectively, being generated from pressurized air in a N300DR nitrogen generator (Peak
272 Scientific, Massachusetts, USA). The auxiliary gas temperature was maintained at 350°C. To obtain adequate
273 selectivity and sensitivity, the mass spectrometer was set to unit resolution and scheduled Multiple Reaction
274 Monitoring mode after determining the retention time of each analyte under the chromatographic conditions
275 described above. The acquisitions were made with a Target Scan Time setting of 1 s. Peaks varied in width with the
276 narrowest peak being 0.3 minutes at the baseline. During acquisition, the number of points acquired across this
277 particular peak was 18. For peaks with a greater peak width, the number of points defining the peak would increase.

278 Calibration curves were constructed by plotting peak area of the analyte against the concentration of the compound
279 from reference standards. The relative concentration of each metabolite in the cell extract sample was determined
280 by back-calculating the metabolite peak area against the calibration curve.

281

282 **2.8. Quantification of cellular lipids**

283 Dried chloroform or monophasic cellular extracts were resuspended in butanol/methanol (1:1, v/v) containing 5 µM
284 ammonium formate. Cellular lipids were separated by injecting 5 µL aliquots onto a 50 mm × 2.1 mm × 2.7 µm
285 Ascentis Express RP Amide column (Supelco, Sigma, St Louis, USA) at 35 °C using an Agilent LC 1200
286 (Mulgrave, Australia). Lipids were eluted at 0.2 $mLmin^{-1}$ over a 5 min gradient of water/methanol/tetrahydrofuran
287 (50:20:30, v/v/v) to water/methanol/tetrahydrofuran (5:20:75, v/v/v), with the final buffer held for 3 min. Lipids
288 were analysed by electrospray ionisation-mass spectrometry (ESI-MS) using an Agilent Triple Quad 6460
289 (Mulgrave, Australia). Lipid species from each lipid class were identified using precursor ion scanning from 100 -
290 1000 m/z , in positive ion mode, phosphatidylcholines (PC, precursors of m/*z* 184.1), sphingomyelins (SM, m/*z*
291 184.1), ceramides (CER, m/*z* 264.6), cholesterol esters (CE, m/*z* 369.4), phosphatidylglycerols (PG, m/*z* 189) and in
292 negative ion mode phosphatidylinositols (PI, m/*z* 241). Neutral loss scanning was used to identify
293 phosphatidylethanolamines (PE, in positive ion mode, neutral loss of m/*z* 141) and phosphatidylserines (PS, negative
294 ion mode, m/*z* 87). Identified lipid species were quantified using multiple reaction monitoring (MRM) with a 50 ms
295 dwell time for the simultaneous measurements of ~20 to 50 compounds and the chromatographic peak width of 30
296 sec to 45 sec, a minimum data points collected across the peak was 12 to 16. Optimised parameters for capillary,

297 fragmentor, and collision voltages were 4000 V, 140 - 380, and 15–60 V, respectively. In all cases, the collision gas
298 was nitrogen at 7 $Lmin^{-1}$. ESI-MS data was processed using Agilent Mass Hunter (Mulgrave, Australia). Lipid
299 standards (Avanti Polar Lipids, Alabaster, USA) were prepared by combining equal volumes of individual lipid
300 stock solutions. The standard solution was then diluted to provide a set of calibration solutions ranging in
301 concentration from 0.1 to 10 µM. Calibration curves were constructed by least squares linear regression, fitting
302 reverse phase peak area of the analyte against the concentration of the lipid in the reference standards. The
303 concentration of each lipid species in the cell extract sample was estimated by normalizing the lipid peak area
304 against the internal standard (LPC) and then using the regression model to convert normalized peak area to lipid
305 concentration. Detected lipid species, 55 in total from 8 lipid classes, were annotated as follows; lipid class (sum of
306 carbon atoms in the two fatty acid chains:sum of double bonds in the fatty acid chains).

307 ### 2.9. Statistical Methods

308 All samples were taken from the same bioreactor vessel (no biological replication). Each treatment or extraction
309 condition (ie. quenching at -30°C or -40°C) was performed in triplicate (3 technical replicates for each treatment). A
310 2-tailed pairwise t-test at $p < 0.05$ was used to make comparisons of amounts obtained between two treatments.
311 Comparison of more than two treatments was made using ANOVA ($p < 0.05$). Dunnet's multiple comparison post
312 test was applied when multiple comparisons were made to a control sample. All results are expressed as means ±
313 standard deviation. Statistical analyses were performed using Graphpad PRISM (GraphPad Software Inc. LaJolla,
314 CA, USA). The degree of difference between treatments was explored by calculating the percent difference (% diff
315 $= ([m_1 - m_2]/[(m_1 + m_2)/2)]) \times 100$) where m = mean metabolite concentration.

316 ## 3. Results and Discussion

317 ### 3.1. Effect of quenching solution temperature on metabolite leakage

318 Evaluations of quenching solution temperature by a number of laboratories have indicated that sub -40°C
319 temperatures are necessary in order to limit wholesale leakage of intracellular metabolites during sample
320 preparation. This temperature requirement is based, in part, on the maximal permissible temperature of the
321 quenching solution following addition of a warm sample, such that leakage of intracellular metabolites is minimized.
322 In two comprehensive evaluations of quenching conditions (Canelas et al. 2008a; Villas-Bôas et al. 2005) the

323 cultures being evaluated were grown at 30°C which, once added to quenching solution, resulted in temperatures at
324 around -10°C. We routinely perform fermentations at 20°C. Therefore we tested whether lower sample
325 temperatures permitted the use of higher quenching solution temperatures.

326 Quenching solution temperature following addition of 20°C sample did not exceed -17°C irrespective of whether the
327 quenching solution was -30°C or -40°C. Nanomole recoveries of amino acids from the sub -40°C quenched sample
328 (Q-40) were compared with all other treatments and amino acids recovered from cell free media (CF). The higher
329 quenching solution temperature (Q-30) did not result in significantly increased leakage of amino acids into the
330 quenching solution (Table 1). The low level of leakage observed agreed with some reports (Gonzalez et al. 1997;
331 Bolten and Wittmann 2008) but differed to some degree with others (Canelas et al. 2008a; Tredwell et al. 2011).
332 The losses of glutamate (>50%) reported by Canelas et al.(2008a) or the losses of alanine, tyrosine and isoleucine
333 reported by Tredwell et al. (2011) in otherwise identical conditions were not observed in this study. Extensive
334 leakage of amino acids occurred during 20 min incubation at -30°C (Q-30T20), demonstrating the importance of
335 sample processing soon after quenching.

336 **3.2. Choice of yeast cell washing regime can increase metabolite leakage**

337 Washing of biomass is a critical and at times overlooked component of metabolite preparation. It is especially
338 important when analysis of batch cultures or cultures growing in complex media is being undertaken, not only
339 because leakage into the washing solution can contribute to variations in metabolite estimation but because medium
340 carryover can interfere with post extraction analytical methods. Two cell pellet washing procedures are described
341 in reports in which the washing step is explicitly given: vortexing of the pellet in cold methanol followed by
342 centrifugation (Castrillo et al. 2003) or methanol addition to the pellet and decanting (Canelas et al. 2008a). Canelas
343 et al.(2008a) reported that a small but significant proportion of metabolites could be recovered from the washing
344 solution.

345 The above two washing regimes (vortexing and rinsing) were evaluated following quenching at -30°C. A
346 significant increase in amino acid leakage into wash solution was observed when cells were washed by vortexing
347 rather than rinsing (Table 2). The efficiency of washing, i.e. how well each washing regime removed medium
348 components, was also evaluated. This was achieved through a separate extraction of early exponential phase cells

349 growing in defined medium containing 165 gL^{-1} of glucose and fructose at the time of sampling. Measures of
350 culture medium removal efficiency by each washing regime were based on the recovery of glucose and fructose in
351 the polar phase of a biphasic extraction. Intracellular metabolite extracts from cells that had been washed by
352 vortexing had 2.5 fold less sugar than when cells were washed by rinsing (Table 2). Despite the higher sugar
353 concentrations in metabolite extracts of 'rinsed' cells, rinsing still achieved an 850-fold reduction in medium
354 component concentrations, which ultimately did not interfere with mass spectral analysis of intracellular
355 metabolites.

356 **3.3. Metabolite extraction temperature does not effect estimation of intracellular**
357 **metabolite concentrations**

358 Biphasic extraction of metabolites from yeast usually involves extended shaking at -20°C (de Koning and van Dam
359 1992) or more recently at -40°C (Canelas et al. 2009). Such cryo-extractions require specialized equipment or
360 extensive modification of existing facilities. Extractions at 4°C have been reported (Fairchild et al. 2010; t'Kindt et
361 al. 2009) but the effect of this extraction temperature on metabolite recoveries were not compared to lower
362 temperature extractions. We assessed if the use of higher extraction temperature compromised the recovery of
363 intracellular metabolites from yeast.

364 The effect of temperature on the extraction of 53 intracellular metabolites was evaluated; nanomole amounts
365 obtained from extractions performed at either 4°C or -20°C of quenched and washed yeast cell pellets are reported in
366 Table 3. For the majority of metabolites the amounts obtained were indistinguishable, irrespective of extraction
367 temperature. Of the 8 metabolites that were significantly different, we observed increased amounts of ATP, GTP,
368 CTP, UTP, GDP, malate and E4P but decreased amounts of NADH and FBP when extractions were performed at
369 4°C. Percent differences were greater than 15% for nine metabolites, most notably NADH (-51%) and GTP (42%).
370 NADH and FBP have previously been shown to be unstable during extraction (Klimacek et al. 2010). However,
371 instability does not explain the increased amounts obtained from 4°C extractions of all the nucleoside triphosphates,
372 which were also shown to be unstable in the same study. An alternative explanation for the decreased quantities of
373 NADH and FBP obtained during extraction at 4°C is that these differences reflect both measurement variation and
374 comparatively small intracellular concentrations. The large increase in GTP obtained by extraction at 4°C is

375 consistent with increased amounts of other nucleoside triphosphates and point towards either more favourable
376 conditions for the extraction of nucleoside triphosphates or metabolite conversion due to residual enzymatic activity.

377 Mean metabolite specific yields expressed as μmol/gDCW are given in the last column of Table 3 in order to
378 compare with the work of others. While direct comparison is difficult due to the differing experimental
379 circumstances (such as strain, growth medium composition, oxygen status, growth phase, etc.) the recoveries of
380 intracellular metabolites presented in Table 3 are, in general, consistent with the intracellular metabolite
381 concentrations in *S. cerevisiae* estimated using varying extraction methods (Canelas et al. 2008b; Canelas et al.
382 2009; Gonzalez et al. 1997).

383 3.4. Overall contributions of sample preparation method to metabolite loss

384 Figure 2 shows total amino acid amounts obtains from all stages of the metabolite processing pipeline. Less than
385 2% of the total recoverable molar amount of each amino acid was lost during quenching regardless of the quenching
386 temperature. After a 20 min incubation in quenching solution amino acid leakage into the quenching solution
387 increased from an average of 2% to between 10% and 20% for many of the amino acids in Table 1. The increase in
388 metabolite leakage with increasing residence time in quenching solution shows that time in quenching solution is of
389 significantly greater importance than quenching solution temperature, at least when lower culture temperatures are
390 used. Increased metabolite leakage was also reported in a study exploring differences in extraction of intracellular
391 metabolites following extended incubation in quenching solution at different temperatures (Villas-Bôas and
392 Bruheim 2007).

393 Leakage of amino acids during a vortex wash resulted in an additional average loss of 2% of the total. Leakage of
394 glycine (6.8%) and alanine (5.2%) into wash solution were higher than leakage of other amino acids and represented
395 significant fractions of the total amounts obtained of those amino acids. Four of the top five amino acids with the
396 greatest loss during washing correspond to amino acids with low molecular weights (less than 120 Da) indicating
397 that loss of small metabolites could be exacerbated by the use of a vortex washing regime. Losses incurred during
398 both quenching and washing were on average less than 1.5 % (max 3.9 %) when rapid quenching was combined
399 with washing by rinsing (Figure 2B). Therefore, if washing is required and if the residual concentration of medium
400 components can be accommodated in the analytical platform used for intracellular metabolite analysis, then it would

401 be preferable to rinse cell pellets at least twice rather than using the more vigorous vortex and centrifugation
402 method. If extensive cell pellet washing were to be combined with an extended residence time in quenching
403 solution, extensive leakage of intracellular metabolites should be anticipated (Figure 2C).

404 3.5. Biphasic extraction yields protein suitable for proteomic analysis

405 Standard yeast protein extraction protocols for proteomic analysis generally utilize TCA or thermal pre-treatment in
406 order to stabilize proteins prior to storage and extraction (Grassl et al. 2009) or extract directly into lysis buffer
407 containing protease inhibitors and denaturants (Futcher et al. 1999). The quenching step of metabolite preparation
408 should effectively stabilize the proteome in preparation for extraction. The subsequent biphasic extraction removes
409 medium components that may interfere with subsequent electrophoretic analysis or proteolytic treatments as
410 discussed by Wessel and Flügge (1984). That protein for use in proteomic work can be derived using sequential
411 extraction methodologies has been shown for systems other than yeast (Weckwerth et al. 2004). We evaluated the
412 quality of protein in metabolite extracted insoluble material using our preferred extraction method (sample
413 quenching at -30°C, rinsing of the cell pellet and metabolite extraction at 4°C) by comparison with a reference
414 protein extraction method.

415 Figure 3 shows the equivalence of general protein profiles between (A) protein extracted using a reference protein
416 extraction method (Herbert et al. 2006) and (B) protein from metabolite extracted yeast. One of the features of the
417 metabolite extraction method described in this work is centrifugation at 1,575 × g as opposed to centrifugation at
418 10,000 × g that is commonly used to isolate protein. Comparison of the two gels in figure 3 shows no additional
419 recovery of small molecular weight proteins or alteration in overall protein profile when a higher centrifugation
420 speed is used. We did observe an increased overall amount of protein extracted per gram dry cell weight using the
421 reference method (data not shown).

422 Subsequently, proteome stability was evaluated using 2-D PAGE (Figure 4) and MALDI-MS/MS. Resolution in
423 2D-PAGE stained with sypro ruby was comparable with previous reports (Grassl et al. 2009). One hundred and
424 ninety protein spots were excised and identified using MALDI MS/MS. We observed little evidence of large scale
425 degradation, with most proteins running in accordance with their predicted molecular weights (Online Resource 2).
426 Only 9 spots were identified as proteins whose predicted molecular weights were higher than was observed on the

427 gel. Spots 8 and 9 were identified as VMA1, a protein known to undergo post-translational intein-mediated splicing
428 to generate a 69 kDa subunit of the vacuolar adenosine triphosphatase (Kane et al. 1990), which concurs with the
429 observed molecular weight. Spot 164 was identified as CCP1, a mitochondrial protein that undergoes processing to
430 remove a mitochondrial import signal resulting in a 33 kDa mature protein (Maccecchini et al. 1979), also in
431 accordance with observation. Fragments of Eno1 (spot 181), Eno2 (spots 179, 180) and SSA2 (spots 168 and 169)
432 were detected at sizes that could not be explained by cellular protein processing events and were detected at very
433 low abundance, indicating limited degradation of those proteins (circled, Figure 3). However, we cannot rule out
434 that these fragments arise as a result of normal protein turnover rather than artifacts of sample isolation. This
435 protein survey demonstrates that solvent insoluble material remaining after a biphasic metabolite extraction of yeast
436 is capable of yielding intact protein of a quality suitable for proteomic work.

437 **3.6. Effectiveness of lipid extraction is dependent on extraction temperature**

438 It has been suggested that the lower chloroform fraction may be of use for the evaluation of lipid metabolism
439 (implied in Canelas et al. 2009; Smart et al. 2010). Although it has previously been shown that lipid recovery can be
440 highly variable when using the Folch method without prior cell disruption (Hanson and Lester 1980) we evaluated
441 lipid recoveries from biphasic metabolite extractions (BPE) of stationary phase yeast, at -20°C or 4°C compared
442 with monophasic Bligh and Dyer (1959) extractions (ME) at 4°C or 20°C.

443 There were no significant differences in amounts obtained from biphasic extractions performed at either -20°C
444 (BPE-20) or 4°C (BPE4) of either whole lipid classes (Online Resource 3) or individual lipid species (Online
445 Resource 4), with the exception of PC(26:0), PC(26:1) and PC(38:1). BPE4 resulted in slightly higher recovery of
446 these lipid species. Monophasic extraction at 4°C (ME4) gave significantly lower yields of total LPG, PC and PE
447 lipid classes but significantly higher yields of total PI and PS than BPE4. This pattern of recovery was true for all
448 species within each class. When monophasic extractions were performed at 20°C, yields of total LPC, LPG, PC, PE
449 and PG lipid classes were no different than yields from BPE4 extractions. However, total yields of PI, PS and
450 ergosterol were significantly higher than for any other extraction condition including ME4. These lipid classes are
451 both apolar and polar and individually represent significant proportions of total cellular lipid. Much of the increase
452 in yield was recovered during a second extraction step that was performed as part of the ME protocol (data not
453 shown).

454 A broad range of lipids were recovered from the chloroform phase of a biphasic extraction, which is commonly used
455 for the preparation of intracellular metabolites from yeast. However, yields of individual lipids can vary with
456 extraction temperature and extraction method. Specifically, extraction at 20°C favoured recovery of serine, inositol
457 and sterol-based lipids. Additional optimization will be required before quantitative extraction of lipids is achieved
458 from an integrated metabolite extraction protocol. Re-extraction with the same solvent system is beneficial and, in
459 combination with the use of increasingly hydrophobic solvents as outlined by Guan et al.(2010), may provide the
460 necessary improvement in lipid recoveries.

461 4. Concluding remarks

462 Experimental samples are valuable and can represent a significant investment in time and resources. It is therefore
463 desirable to extract as much information as possible from a single experiment. Specifically, volume limitation either
464 due to culture miniaturization or multiple sequential sampling from batch systems, can lead to situations where
465 additional biomass for parallel 'omics extractions is not viable. For example, microtiter plate cultures provide
466 enough cell biomass for a single metabolite extraction but unfortunately there is not enough biological material left
467 for other analyses. Additionally, sequential sampling from individual batch cultures in order to derive temporal
468 'omic datasets can decrease dramatically residual volume and therefore affect growth kinetics and cell metabolism.
469 This is exaggerated at early time point samples, when biomass concentration is low and large sample volumes are
470 required. We have shown that additional value can be obtained from yeast metabolite extractions through the
471 recovery of material from fractions that are often discarded.

472 Solvent insoluble material recovered following a standard biphasic chloroform/methanol extraction of polar
473 metabolites contains protein of a quality that is suitable for proteomic analysis. Solubilization in an appropriate
474 buffer can deliver protein preparations for either electrophoretic or mass spectral proteomic work. The metabolite
475 extraction procedure has the additional advantage that the proteome is rapidly stabilized through cold and
476 chloroform denaturation and that interfering environmental contaminants are removed. As expected the chloroform
477 fraction obtained during this procedure contained lipids that could be quantitatively and reproducibly recovered.
478 However, overall lipid yields were significantly less compared with lipids extracted using a monophasic system at
479 higher temperature. It is possible that repeated extractions of insoluble material at low temperature may lead to
480 increased recoveries.

481 A comprehensive characterization of the yeast metabolite extraction method reported here, which was performed at
482 higher temperatures than usually recommended, revealed minimal metabolite leakage during quenching. It is likely
483 that this was the result of the lower fermentation temperature. However, considerable metabolite leakage was
484 observed when quenched cells were vigorously washed, even when very cold quenching solution was used.
485 Therefore, washing of yeast cells is generally counter productive and should not be undertaken in situations when
486 the analytical platform can withstand the associated introduction of environmental material. Even when very high
487 concentrations of environmental components are present a simple rinse of tube walls is generally sufficient to reduce
488 contaminating components to levels compatible with mass spectral analyses.

489 Finally, very little difference was found in metabolite yields from extractions performed at either 4°C or -20°C.
490 Even in those instances when differences were statistically significant it is not entirely clear that the magnitude of
491 those differences were biologically meaningful. Based on our results metabolite extraction at 4°C is sufficient both
492 qualitatively and quantitatively when facilities for very low temperature extractions are not available.

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635 **Table 1 Effect of quenching temperature on loss of amino acids from *S. cerevisiae***

636 Nanomoles of amino acids obtained from 60% v/v methanol quenching solution at -40°C (Q-40) were compared to
637 amounts obtained from -30°C quenching solution (Q-30), following extended incubation (20min) in -30°C
638 quenching solution (Q-30T20) and filtered culture supernatant (CF). Comparisons were made by ANOVA (n=3 for
639 all samples).

	Q-40	*Q-30*		*Q-30T20*		*Culture filtrate (CF)*	
	Mean ± SD	*Mean ± SD*	*P < 0.05*	*Mean ± SD*	*P < 0.05*	*Mean ± SD*	*P < 0.05*
ASP	2.17 ± 0.06	2.47 ± 0.46	ns	11.60 ± 0.36	*	nd	*
GLU	4.63 ± 0.11	5.73 ± 1.94	ns	63.42 ± 3.84	*	1.72 ± 1.58	ns
ASN	2.65 ± 0.08	3.04 ± 0.70	ns	18.97 ± 0.76	*	1.45 ± 0.15	ns
SER	3.36 ± 0.23	4.11 ± 1.32	ns	31.26 ± 0.97	*	nd	*
GLN	5.57 ± 0.19	7.45 ± 3.38	ns	92.89 ± 3.74	*	2.46 ± 0.86	ns
HIS	1.16 ± 0.91	1.26 ± 0.83	ns	13.29 ± 0.78	*	nd	ns
GLY	10.37 ± 0.51	13.22 ± 5.38	ns	83.15 ± 2.16	*	2.62 ± 0.35	*
THR	2.00 ± 0.05	2.49 ± 0.84	ns	23.86 ± 0.94	*	nd	*
ARG	9.41 ± 0.46	11.18 ± 3.33	ns	176.76 ± 10.28	*	2.24 ± 2.05	ns
ALA	7.40 ± 0.19	9.18 ± 3.24	ns	65.69 ± 1.78	*	1.05 ± 0.26	*
GABA	1.29 ± 0.03	1.30 ± 0.04	ns	3.14 ± 0.08	*	1.40 ± 0.47	ns
TYR	0.82 ± 0.04	0.72 ± 0.19	ns	3.71 ± 0.17	*	nd	*
VAL	nd	nd	-	nd	-	nd	-
MET	nd	nd	-	0.69 ± 0.01	*	nd	-
TRP	nd	nd	-	nd	-	nd	-
PHE	0.34 ± 0.05	0.39 ± 0.06	ns	3.20 ± 0.12	*	nd	*
ILE	4.49 ± 0.10	4.98 ± 0.91	ns	37.78 ± 1.74	*	1.50 ± 1.31	*
ORN	nd	3.42 ± 5.92	ns	14.66 ± 0.89	*	13.64 ± 1.76	*
LEU	0.89 ± 0.19	1.00 ± 0.18	ns	8.36 ± 0.35	*	nd	*
LYS	2.23 ± 0.28	2.30 ± 0.37	ns	14.63 ± 0.97	*	nd	*
PRO	1.42 ± 0.07	2.33 ± 1.52	ns	12.35 ± 0.78	*	2.60 ± 0.69	ns

640 * Metabolites for which amounts obtained were significantly different from Q-40, determined using Dunnett's
641 Multiple Comparison Test ($P < 0.05$, df = (3,11)); ns = not significantly different; nd = not detected.

642

643 **Table 2 Effect of cell washing regime on loss of amino acids from *S. cerevisiae* and washing efficacy.**

644 Amino acid leakage from *S. cerevisiae* during two washing treatments, rinsing and vortexing, was evaluated.

645 Amounts of amino acid obtained from triplicate wash treatments are given as mean nanomoles of amino acid ± SD

646 (n=3 for all samples). Total reducing sugars present in polar metabolite extracts of late exponential phase cells

647 following application of either of the two washing regimes is given as an indicator of washing efficacy.

	Rinse	***Vortex***	***% diff***
ASP	1.10 ± 0.10	3.40 ± 1.90	-102.2
GLU	7.62 ± 1.02	17.76 ± 7.08	-79.9
ASN	1.45 ± 0.11	4.19 ± 1.17	-97.4
SER	2.15 ± 0.07	9.80 ± 3.47	-128.1
GLN	8.95 ± 0.89	29.33 ± 10.47	-106.5
HIS	1.14 ± 0.24	1.00 ± 0.46	13.2
GLY	4.77 ± 0.09	28.94 ± 7.20	-143.4
THR	1.71 ± 0.13	6.14 ± 2.16	-112.8
ARG	16.76 ± 3.30	19.92 ± 8.07	-17.2
ALA	4.04 ± 0.05	24.09 ± 6.49	-142.6
GABA	0.19 ± 0.00	0.74 ± 0.14	-120.2
TYR	0.31 ± 0.04	1.19 ± 1.23	-117.2
VAL*	2.23 ± 0.20	6.87 ± 2.29	-101.9
MET	nd	0.18 ± 0.10	-200.0
TRP	nd	0.02 ± 0.03	-200.0
PHE	0.32 ± 0.03	1.04 ± 0.55	-105.0
ILE	3.52 ± 0.39	8.33 ± 2.76	-81.3
ORN	1.97 ± 0.22	2.65 ± 1.16	-29.2
LEU	0.74 ± 0.06	2.37 ± 0.98	-104.4
LYS	1.68 ± 0.33	2.07 ± 0.92	-20.8
PRO	2.36 ± 0.17	4.10 ± 1.23	-53.8
†Total Sugar*	0.194 ± 0.011	0.075 ± 0.011	200

648 * Metabolites for which amounts obtained from wash solutions were significantly different, determined using a

649 pairwise t-test ($P < 0.05$, df = 4); nd = not detected.

650 † Concentration in polar phase of metabolite extract (PM), given in gL^{-1}.

651

652 **Table 3 Effect of extraction temperature on amounts of intracellular polar metabolites obtained from *S.***

653 ***cerevisiae*.**

654 Equal volumes of yeast cell culture were quenched at -40°C, washed by vortexing and intracellular polar metabolites

655 were extracted from yeast cell pellets by shaking at either 4°C or -20°C for 40 min. Extractions were performed

656 using a biphasic chloroform/methanol/water method. Following extraction the aqueous phase was separated from

657 the polar phase and insoluble material and dried. Metabolite amounts for each extraction are givens as average

658 nanomoles of metabolite ± SD from triplicate extractions. Since there was no statistical different between the two

659 treatments for the majority of metabolites the data from both extractions was combined to give a mean amount

660 extracted which is the average of all extractions (4°C and 20°C) normalized to dry cell weight (DCW).

		Extraction Temperature			*Mean amount extracted*
Metabolite		***4°C***	***-20°C***	***% diff***	***µmol/gDCW***
D-glucose-6-phosphate		16.0 ± 1.9	15.7 ± 0.6	1.8	0.71 ± 0.06
D-fructose-6-phosphate		1.8 ± 0.4	2.0 ± 0.1	-10.7	0.08 ± 0.01
D-fructose 1,6-bisphosphate	*	7.0 ± 0.4	8.3 ± 0.2	-17.3	0.34 ± 0.04
2- and 3-phosphoglyceric acid		27.7 ± 2.8	27.6 ± 1.5	0.3	1.24 ± 0.09
Phosphoenolpyruvate		8.0 ± 0.3	7.7 ± 0.4	2.9	0.35 ± 0.01
Pyruvate		2.0 ± 0.0	2.1 ± 0.2	-8.3	0.09 ± 0.01
Acetyl-CoA		0.5 ± 0.1	0.4 ± 0.0	12.3	0.02 ± 0.00
Isocitrate and Citrate		451.3 ± 73.1	360.8 ± 62.1	22.3	18.26 ± 3.52
Aconitrate		3.7 ± 0.2	3.8 ± 0.2	-2.9	0.17 ± 0.01
α-ketoglutarate		9.2 ± 0.3	9.3 ± 0.4	-0.8	0.41 ± 0.01
Succinic acid		256.3 ± 15.2	284.3 ± 16.7	-10.4	12.15 ± 0.94
Fumaric acid		2.4 ± 0.0	2.3 ± 0.1	1.4	0.11 ± 0.00
Malic acid	*	115.9 ± 6.0	103.9 ± 2.1	10.9	4.94 ± 0.35
Oxaloacetic acid		11.1 ± 0.7	11.6 ± 0.7	-3.7	0.51 ± 0.03
Lactic acid		8.0 ± 1.6	8.2 ± 1.7	-2.1	0.36 ± 0.07
D-glucose-1-phosphate		2.5 ± 0.3	2.8 ± 0.2	-12.1	0.12 ± 0.01
UDP-D-Glucose		267.1 ± 9.2	264.9 ± 5.6	0.8	11.96 ± 0.31
D-erythose-4-phosphate	*	1.3 ± 0.0	1.2 ± 0.0	7.8	0.06 ± 0.00
CMP		1.2 ± 0.0	1.3 ± 0.1	-12.1	0.06 ± 0.01
UMP		8.6 ± 0.6	10.7 ± 1.4	-21.3	0.43 ± 0.07
AMP		9.8 ± 0.6	10.4 ± 0.6	-6.4	0.45 ± 0.03
GMP		4.1 ± 0.2	4.4 ± 0.3	-7.1	0.19 ± 0.01
UDP		10.1 ± 0.4	10.3 ± 1.1	-1.3	0.46 ± 0.03
ADP		200.2 ± 29.1	171.9 ± 27.5	15.2	8.36 ± 1.34
GDP	*	16.3 ± 0.7	14.8 ± 0.4	9.5	0.70 ± 0.04
CTP	*	58.8 ± 1.2	49.8 ± 1.2	16.6	2.44 ± 0.23
UTP	*	136.7 ± 8.7	114.5 ± 10.6	17.6	5.65 ± 0.67
ATP	*	386.4 ± 2.5	328.6 ± 14.2	16.2	16.08 ± 1.48
GTP	*	187.7 ± 7.5	122.9 ± 17.5	41.8	6.98 ± 1.69
NAD		218.3 ± 6.9	214.5 ± 8.5	1.8	9.73 ± 0.32
NADH	*	3.2 ± 0.3	5.4 ± 0.8	-49.9	0.19 ± 0.06
NADP		8.5 ± 0.5	8.2 ± 0.2	2.5	0.38 ± 0.02
ASP		90.4 ± 3.4	89.5 ± 3.0	1.0	4.05 ± 0.13
GLU		1376.3 ± 39.3	1424.2 ± 32.5	-3.4	62.96 ± 1.87
ASN		214.5 ± 10.3	225.2 ± 6.5	-4.8	9.88 ± 0.44
SER		251.8 ± 11.7	262.7 ± 9.2	-4.2	11.57 ± 0.50
GLN		7750.8 ± 110.2	7834.2 ± 264.2	-1.1	350.38 ± 8.40
HIS		208.3 ± 4.3	208.7 ± 4.4	-0.2	9.38 ± 0.18
GLY		375.5 ± 20.3	381.3 ± 24.6	-1.5	17.02 ± 0.92
THR		228.0 ± 6.6	232.8 ± 8.1	-2.1	10.36 ± 0.32
ARG		2064.6 ± 77.8	2146.4 ± 56.9	-3.9	94.67 ± 3.40
ALA		423.0 ± 18.0	430.1 ± 22.2	-1.7	19.18 ± 0.83
GABA		26.5 ± 0.9	26.6 ± 0.9	-0.4	1.20 ± 0.04
TYR		49.9 ± 0.8	51.0 ± 1.0	-2.2	2.27 ± 0.05
VAL		403.0 ± 12.0	413.6 ± 12.4	-2.6	18.36 ± 0.56
MET		6.1 ± 0.4	6.7 ± 0.1	-9.2	0.29 ± 0.02

TRP	8.1 ± 0.1	8.2 ± 0.2	-1.1	0.37 ± 0.01
PHE	45.1 ± 1.2	46.1 ± 1.4	-2.3	2.05 ± 0.06
ILE	1037.8 ± 66.2	1030.8 ± 43.7	0.7	46.51 ± 2.26
ORN	287.3 ± 6.8	280.1 ± 7.1	2.5	12.76 ± 0.33
LEU	96.3 ± 2.4	98.1 ± 3.0	-1.9	4.37 ± 0.12
LYS	336.0 ± 7.4	334.3 ± 8.0	0.5	15.07 ± 0.31
PRO	123.1 ± 4.2	126.1 ± 5.8	-2.4	5.60 ± 0.22

661 * Metabolites for which amounts obtained were significantly different between treatments ($P < 0.05$, df = 4).

662

663 # Figure Legends

664 **Figure 1 Outline of experimental workflow used for preparation of yeast cellular material prior to metabolite**
665 **and protein extraction.**

666 **Figure 2 Contribution of different processing methods to total loss of metabolites during extraction from *S.***
667 ***cerevisiae*.**

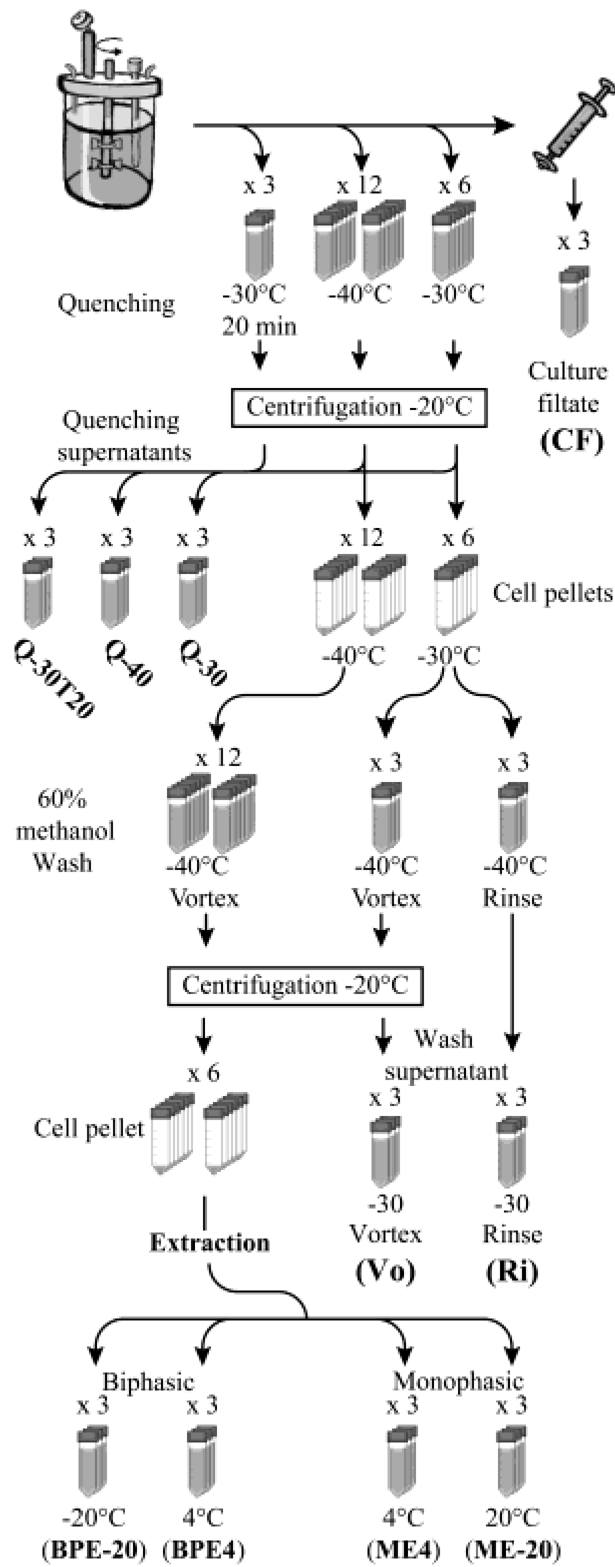
668 The molar amounts of amino acids obtained from quenching solution (Q), washing solution (Vo and Ri) and
669 metabolite extract (EX) were summed and the total number of moles obtained for each process combination was
670 regarded as 100%. The molar amount obtained individually from quenching solution, washing solution and polar
671 extract is then expressed as a percentage of that total. A; Quenched at -30°C, washed by vortexing and extracted at
672 4°C (Q-30:Vo:EX4), B; Quenched at -30°C, washed by rinsing and extracted at 4°C (Q-30:Ri:EX4), C; . Quenched
673 at -30°C for 20min, washed by vortexing and extracted at 4°C (Q-30T20:Vo:EX4).

674 **Figure 3 Comparison of protein recoveries from yeast cultures at different growth phases**

675 Protein from stationary phase cultures was extracted from whole cells (A) using method of Herbert et al. (2006), and
676 from solvent insoluble material remaining following a biphasic chloroform/methanol extraction of metabolites (B)
677 as described in the materials and methods. Extracted proteins were separated in the first dimension using pH 5-8
678 immobilized gradient isoelectric focusing strips and in the second dimension by 8 – 16% SDS-PAGE. Separated
679 proteins were stained with Sypro ruby.

680 **Figure 4 2-D PAGE separation of proteins recovered from solvent-insoluble material.**

681 Protein was solubilized from solvent-insoluble material following a biphasic metabolite extraction of a stationary
682 phase culture. Separated proteins were stained with Sypro-ruby and the identity of individual spots (numbered) was
683 determined by MALDI-MS/MS. Highlighted numbers indicate protein identifications that do not correspond to
684 predicted molecular weights.



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