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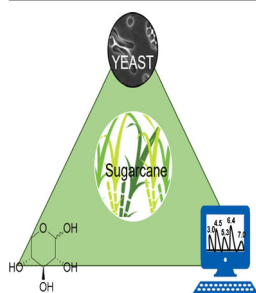
Abstract. Xylose fermentation is a bottleneck in second-generation ethanol production. As such, a comprehensive understanding of xylose metabolism in naturally xylose-fermenting yeasts is essential for prospection and construction of recombinant yeast strains. The objective of the current study was to establish a reliable metabolomics protocol for quantification of key metabolites of xylose catabolism pathways in yeast, and to apply this protocol to *Spathaspora arborariae*. Ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) was used to quantify metabolites, and afterwards, sample preparation was optimized to examine yeast intracellular metabolites. *S. arborariae* was cultivated using xylose as a carbon source under aerobic and oxygen-limited conditions. Ion pair chromatography (IPC) and hydrophilic interaction liquid chromatography-tandem mass spectrometry (HILIC-MS/MS) were shown to efficiently quantify 14 and 5 metabolites, respectively, in a more rapid chromatographic protocol than previously described. Thirteen and eleven metabolites were quantified in *S. arborariae* under aerobic and oxygen-limited conditions, respectively. This targeted metabolomics protocol is shown here to quantify a total of 19 metabolites, including sugars, phosphates, coenzymes, monosaccharides, and alcohols, from xylose catabolism pathways (glycolysis, pentose phosphate pathway, and tricarboxylic acid cycle) in yeast. Furthermore, to our knowledge, this is the first time that intracellular metabolites have been quantified in *S. arborariae* after xylose consumption. The results indicated that fine control of oxygen levels during fermentation is necessary to optimize ethanol production by *S. arborariae*. The protocol presented here may be applied to other yeast species and could support yeast genetic engineering to improve second generation ethanol production.
Keywords: Xylose fermentation, Mass spectrometry, UHPLC-MS/MS, Metabolomics, *Spathaspora arborariae*

Introduction

The increased demand for alternative energy sources together with concerns about the environmental impacts of fossil fuels has motivated studies into the production of biofuels from lignocellulosic biomass such as second generation ethanol (Ethanol 2G) [1, 2]. Currently, a few pilot plants worldwide are producing ethanol 2G from different types of biomass, such as feedstock, however, high-capacity ethanol

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production using current technologies is not fully achievable, and some improvements are still required. In the fermentation process, for example, xylose, the second most abundant sugar in sugarcane bagasse [3], is not converted to ethanol by *S. cerevisiae*, the yeast used in commercial ethanol production [4, 5]. Therefore, recombinant *S. cerevisiae* strains capable of fermenting xylose have been produced by the expression of xylose-catabolic pathways from naturally xylose-consuming microorganisms [6, 7]. In addition, naturally xylose-fermenting yeasts have been identified and evaluated for industrial ethanol production. Despite this, the relative success in obtaining strains able of producing ethanol through these xylose-conversion strategies is still limited by our understanding of the constraints in xylose metabolism by yeast [7]

Naturally xylose-fermenting yeasts or recombinant strains obtained by overexpression of xylose catabolic pathways are capable of producing ethanol, though with low yield, due to xylitol formation. This happens because xylose is first reduced to xylitol by a NAD(P)H-dependent xylose reductase (XR), followed by xylitol oxidation to xylulose by the NAD$^+$-dependent xylitol dehydrogenase (XDH) [7, 8]. Thus, other modifications have been proven to be necessary in order to increase process productivity [9–14]. These strategies involve the collection, analysis, and quantitative integration of biological data on a large scale through OMICs tools (genomics, transcriptomics, proteomics, fluxomics, and metabolomics). This may help in the construction of more relevant and predictive models to identify limiting steps in xylose metabolism [12].

Metabolomics analysis allows qualitative and quantitative analysis of metabolites [15, 16]. Targeted metabolomics has been widely used to quantify metabolites in specific pathways, providing kinetic information regarding production and consumption rates that could be further used in metabolic engineering [17, 18]. However, to get a reliable "metabolite picture" of a system using metabolomics, certain steps should be carefully followed: (1) careful choice of material, (2) sample preparation and extraction, (3) analytic methods, (4) data processing, and (5) data analysis and interpretation [19, 20]. An important challenge in metabolomics studies is choosing the ideal analytical method, where specific analytic tools capable of simultaneously analyzing a large number of compounds with high sensitivity and selectivity are required, especially for biological samples containing a large variety of low-abundance metabolites [21, 22]. In this context, liquid chromatography coupled to mass spectrometry has been widely used in targeted metabolomics of polar compounds [23, 24].

Furthermore, ultra-high performance liquid chromatography-mass spectrometry (UHPLC-MS) allows high-throughput efficient analysis, reduces solvent use, improves peak resolution, and consequently, metabolite separation, resulting in better quantification analysis [25]. There is a wide variety of chromatographic separation methods available to separate specific classes of compounds, such as polar, non-polar, and ionic compounds. LC-MS/MS methods based on ion pair chromatography (IPC) have been described in the literature for quantifying metabolites of glycolysis, the pentose phosphate pathway (PPP), and the tricarboxylic acid (TCA) cycle [26–28]. However, they are limited to phosphorylated compounds, carboxylic acids, nucleotides, and coenzyme-A esters. Moreover, high concentrations of tributylamine (TBA) are used in the mobile phase as an ion pair reagent, which results in mass spectrometer contamination [26]. Hydrophilic interaction liquid chromatography-tandem mass spectrometry (HILIC-MS/MS) allows the use of a range of stationary phases for analyzing polar molecules that are weakly retained in the reverse phase [29]. BEH amide column-based methods are used to quantify sugars [30] but they still require improvements in order to perform faster chromatographic runs with better peak resolution.

Here, we present two complementary chromatographic methods, IPC and HILIC, coupled to tandem mass spectrometry (MS/MS), which together allow complete separation and quantification of 19 intracellular metabolites from central carbon metabolism. Furthermore, sample preparation methods were optimized for the quenching of cellular metabolites and for metabolite extraction steps for yeast sample preparation. The final metabolomics protocol developed here was successfully applied for metabolite quantification in the naturally xylose-fermenting yeast *S. arborariae* during cultivation on xylose as the sole carbon source, under aerobic and oxygen-limited conditions. The metabolomics protocol presented here may be applied to other yeast strains.

Experimental

Standards and Chemicals

All metabolite standards were purchased from Sigma-Aldrich (St. Louis, MO, USA) with purity superior to 95%: acetyl coenzyme-A (ACCOA), acetaldehyde (Acald), alpha ketoglutaric acid (AKG), D-malic acid (L-MAL), oxaloacetic acid (Oaa), D-(+)-glucose, xylose, glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), dihydroxy acetone phosphate (DHAP), erythrose-4-phosphate (E4P), glyceraldehyde-3-phosphate (GAP), glycerol-3-phosphate (GLY3P), ribose-5-phosphate (R5P), ribulose-5-phosphate (RU5P), xylulose (Xylu), phospho(enol)pyruvate (PEP), glycerol, sodium pyruvate (PYR), sedoheptulose-7-phosphate (S7P) and xylitol. Solvents used for development of chromatography and mass spectrometry, such as formic acid, ammonium formate, tributylamine, triethylamine, acetonitrile, and methanol, were also purchased from Sigma-Aldrich (St. Louis, MO, USA) at the highest available purity. Ammonium acetate was acquired from Vetec (St. Louis, MO, USA) and ammonium hydroxide from Fluka (St. Louis, MO, USA). Deionized water (18.2 MΩ) was obtained from a Direct 16 Milli-Q purification system (Millipore, Bedford, MA, USA).

Biological Material

The xylose-fermenting yeast *Spathaspora arborariae* NRRL Y-48658 was grown on a YPD plate overnight, and then one single colony was transferred to pre-inoculum containing 50

mL of mineral medium, 2.5 times concentrated, and supplemented with 40 g/L of xylose as the carbon source [31]. For the fermentation process, a bioreactor was used (Multifors 2 Infors) with working volume of 500 mL of the same mineral medium used in the pre-inoculum. Fermentation was carried out under aerobic and oxygen-limited conditions. The cultures were grown at 28 °C, with agitation (400 rpm), and pH 5.5 adjusted with 3M KOH. Yeast samples were collected in triplicate, during the exponential growth phase between 20 h of aerobic and 40 h oxygen-limited fermentations. Ideal growth rates were estimated using optical density (OD_{600}).

Quenching and Extraction Steps

The quenching of cellular activity and metabolite extraction procedures were optimized for the yeast samples based on previously described protocols [32, 33].

For the quenching step, 2 mL of cell culture were added to 8 mL of 60% (v/v) methanol buffered with 10 mM ammonium acetate (pH 7.4), in a –40 °C thermostatic bath (FP 50-MA, Julabo, Germany). The samples were then centrifuged at 5000 rpm, –9 °C for 5 min. The supernatant was discarded and the resulting pellet was immediately frozen in liquid nitrogen and stored at –80 °C until the extraction step. Frozen cell pellets were kept in a thermostatic bath at –40 °C for 5 min before the extraction procedure.

The boiling ethanol method was used for metabolite extraction [33]. A total of 2 mL of 75% (v/v) ethanol buffered with 10 mM ammonium acetate (pH 7.4) solution at 85 °C was added to the pellet. The samples were homogenized in a vortex and then transferred to a 2 mL tube for 3 min incubation at 85 °C with constant shaking (Thermoximer comfort, Eppendorf). Subsequently, cells were cooled at –40 °C using a thermostatic bath and centrifuged (5424-R, Eppendorf) at 5000 rpm, –9 °C for 3 min. The supernatant was collected in a 2 mL tube and dried under vacuum (Centrivap DNA concentrator, Labconco). Samples were storage at –80 °C until UHPLC-MS/MS analysis.

UHPLC-MS/MS

All experiments were performed using an Acquity UPLC system (Waters, Milford, MA, USA) coupled to a triple quadrupole mass spectrometer (Xevo TQD, Waters) equipped with an electrospray ionization source. Data were acquired and processed with MassLynx 4.1 software (Waters). The MS was operated in the negative ionization mode, ESI(–)-MS, using multiple reaction monitoring (MRM). The instrument parameters were as follows: capillary voltage 3500 V, desolvation temperature: 450 °C, source temperature: 130 °C, cone gas flow: 20 L/h, and desolvation gas flow: 700 L/h. MRM transition channels and collision cell voltages were optimized for each metabolite after direct infusion into the MS.

Hydrophilic interaction liquid chromatography (HILIC) was performed using a BEH amide column (2.1 × 150 mm × 1.7 µm) (Waters) at 50 °C, with eluent A (0.1% ammonium hydroxide aqueous solution) and eluent B (acetonitrile with 0.1% ammonium hydroxide). Five metabolites were analyzed

by this method: glucose, xylose, glycerol, xylitol, and xylulose. Ion pair chromatography (IPC) was performed using a reverse phase column, HSS-T3 (2.1 × 150 mm × 1.8 µm) (Waters) at 45 °C, with eluent A (5 mM tributylamine, 10 mM acetic acid, and 5% (v/v) methanol, pH 4.8), and eluent B (methanol). Fourteen metabolites were analyzed by this method: ACCOA, AKG, L-MAL, G6P, F6P, DHAP, E4P, GAP, GLY3P, R5P, RU5P, PEP, PYR, S7P. The gradient flow rates for both methods are shown in Tables 1 and 2, respectively.

Quantification of Targeted Metabolites

A standard solution (SS) was prepared for each metabolite (1 mg/mL in water). Then, dilutions of SS, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10, and 50 µg/mL were used for calibration curve construction and quality control (QC) experiments. The limit of detection (LOD) and quantitation (LOQ) were established based on signal/noise ratio of 3:1 and 10:1, respectively [34]. The definitive analytical calibration curve was constructed with six levels of standard mixture solutions and obtained by plotting the area against the concentration of each compound, using second-order polynomial regression. Calibration curves and samples of exponential growth phases were analyzed in triplicate, using UHPLC-MS/MS.

Results and Discussion

A total of 19 commercially available metabolites of central carbon metabolism (Figure 1) related to the glycolysis, PPP, and TCA pathways were used for method development. Although these are not the only metabolites involved in these metabolic pathways, each molecule analyzed is key to at least one pathway, and therefore they together represent the metabolic flux of conversion of xylose to ethanol in yeast.

UHPLC-MS/MS

Mass spectrometry is a fast and simple method for detecting metabolites. However, prior chromatographic separation is required in order to avoid ion suppression effects and to identify isomers. A highly sensitive and selective method based on mass spectrometry, the multiple reactions monitoring (MRM) strategy, was used in this study. To perform UHPLC-MS/MS using MRM, first, mass spectrometer parameters such as ionization source and mass analyser (Q1 and Q3 channels) were

Table 1. UHPLC Conditions Using HILIC Mode with Mobile Phase (Eluent) A: 0.1% Ammonium Hydroxide, and B: Acetonitrile with 0.1% Ammonium Hydroxide, Applied to Glycerol, Xylulose, Xylose, Xylitol, and Glucose Analyses

Time (min)	Flow (mL/min)	Eluent A (vol. %)	Eluent B (vol. %)
0.0	0.2	15	85
6.5	0.2	50	50
7.5	0.4	50	50
8.0	0.2	15	85
12.0	0.2	15	85

Table 2. UHPLC Conditions Using IPC Mode with Mobile Phase (Eluent) A: 5 mM Tributylamine, 10 mM Acetic Acid, and 5% (v/v) Methanol, and B: Methanol, Applied to Glucose-6-Phosphate, Fructose-6-Phosphate, Ribose-5-Phosphate, Ribulose-5-Phosphate, Sedoheptulose-7-Phosphate, Glycerol-3-Phosphate, Erytrose-4-Phosphate, Glyceraldehyde-3-Phosphate, Dihydroxyacetone Phosphate, Sodium Pyruvate, Malic Acid, Alpha Ketoglutaric Acid, Phospho(enol)pyruvate, and acetyl co-enzyme-A Analyses

Time (min)	Flow (mL/min)	Eluent A (vol. %)	Eluent B (vol. %)
0.0	0.4	100	0
10.0	0.4	89.5	10.5
18.0	0.4	47.4	52.6
19.0	0.4	47.4	52.6
20.0	0.4	100	0
26.0	0.4	100	0

optimized, followed by chromatographic parameters, such as the mobile phase.

Mass Spectrometry Direct infusion mass spectrometry (DIMS) was used to optimize the ionization source and

collision cell voltages for each metabolite. Although ESI(+)-MS and ESI(−)-MS were tested, all metabolites were detected with better sensitivity using ESI(−)-MS, which was therefore selected for further analyses. After ionization, each precursor ion was isolated in the first quadrupole (Q1), collided with gas in Q2, and then fragment ions were detected in Q3 (MS/MS experiments). Capillary voltage and collision energy values were optimized for each standard, wherein the capillary voltages were similar for all metabolites (3500 V). The highest and/or most selective fragment ion was selected for MRM analyses (Table 3). No fragmentation ion was detected for acetaldehyde (Acald), probably due to its low molecular weight (44 Da), and as such it was excluded from this study.

After the optimization of mass spectrometer parameters, the next step involved developing a chromatographic method capable of separating all metabolites. Liquid chromatography is crucial for the separation of isomeric compounds, especially for metabolites with same MRM channels (Q1 and Q3).
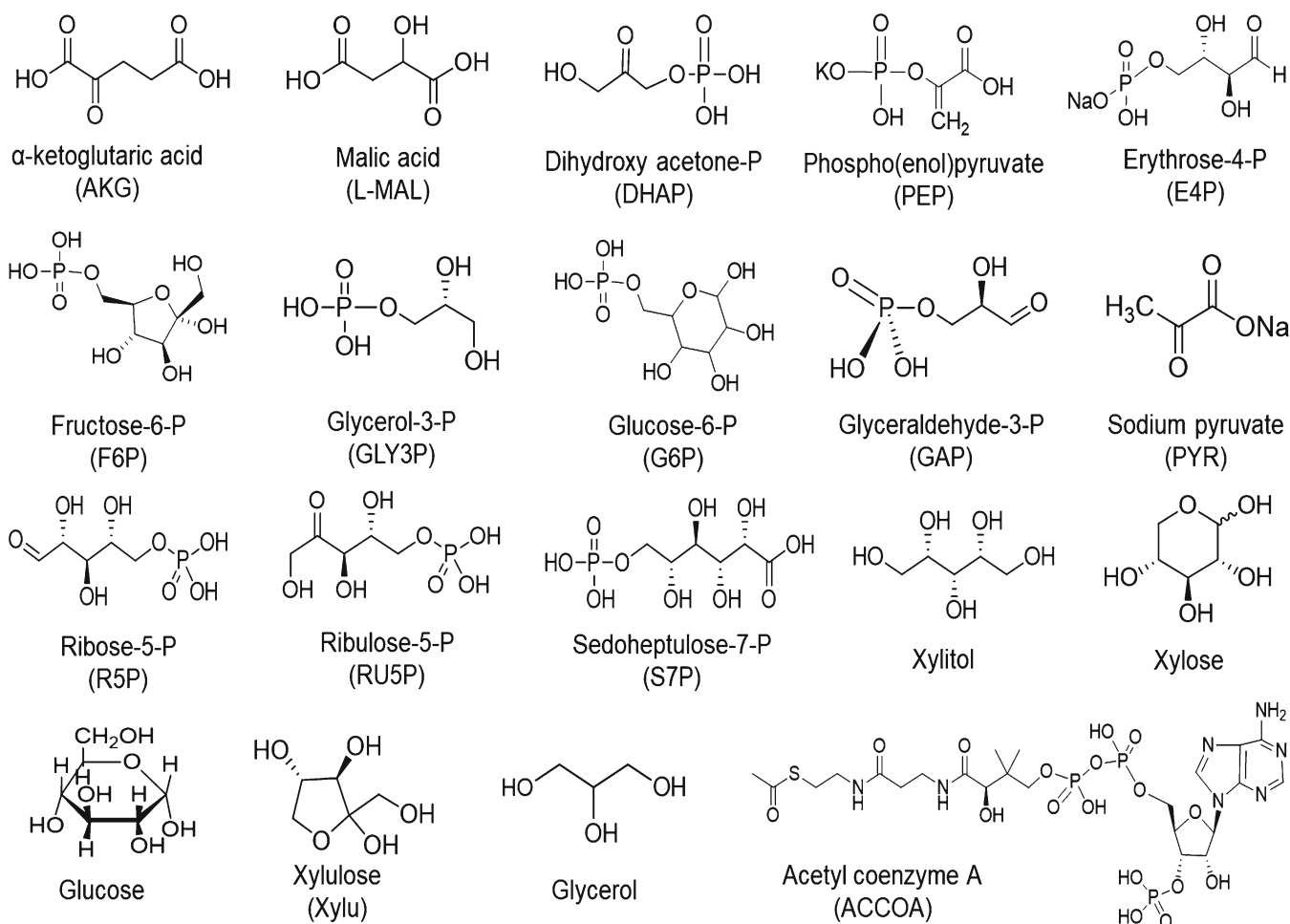
Figure 1. Chemical structures of metabolites from glycolysis, pentose phosphate pathway and tricarboxylic acid cycle analyzed by UHPLC-MS/MS. Metabolites: alpha ketoglutaric acid (AKG), D-malic acid (L-MAL), dihydroxy acetone phosphate (DHAP), phospho(enol)pyruvate (PEP), erythrose-4-phosphate (E4P), fructose-6-phosphate (F6P), glycerol-3-phosphate (GLY3P), glucose-6-phosphate (G6P), glyceraldehyde-3-phosphate (GAP), sodium pyruvate (PYR), ribose-5-phosphate (R5P), ribulose-5-phosphate (RU5P), sedoheptulose-7-phosphate (S7P), xylitol, xylose, D-(+)-glucose, xylulose (Xylu), glycerol and acetyl coenzyme A (ACCOA)

Table 3. The List of Metabolites Quantified by the Developed UHPLC-MS/MS Methods

Method	Metabolite	Cone voltage (V)	Q1 (*m/z*)	Q3 (*m/z*)	Collision energy (eV)	Retention time (min)
IPC	ACCOA	60	808.1	408.0	50	18.50
IPC	AKG	18	144.6	56.8	15	15.39
IPC	DHAP	25	169.0	97.0	18	8.37
IPC	E4P	20	198.8	78.8	20	7.52
IPC	F6P	32	258.6	96.8	18	6.66
IPC	G6P	32	258.7	96.8	20	6.16
IPC	GAP	20	168.8	96.8	18	7.27
IPC	GLY3P	30	170.5	78.7	25	6.91
IPC	L-MAL	18	132.6	114.8	10	14.89
IPC	PEP	20	166.6	78.8	10	16.35
IPC	PYR	20	86.8	42.8	8	9.03
IPC	R5P	30	228.8	96.8	20	6.56
IPC	RU5P	30	228.8	79.0	20	7.47
IPC	S7P	32	288.4	138.9	25	6.64
HILIC	Glycerol	18	91.0	59.1	18	3.36
HILIC	Glucose	15	179.1	58.9	18	5.12
HILIC	Xylitol	25	151.0	58.9	20	4.75
HILIC	Xylose	10	149.0	59.0	14	4.57
HILIC	Xylu	10	149.0	59.0	14	3.83

Abbreviations: Ion pair chromatography (IPC), hydrophilic interaction liquid chromatograpy (HILIC), acetyl Co-enzyme A (ACCOA), alpha ketoglutaric acid (AKG), di-hydroxyacetone phosphate (DHAP), erythrose-4-phosphate (E4P), fructose-6-phosphate (F6P), glucose-6-phosphate (G6P), glyceraldehyde-3-phosphate (GAP), glycerol-3-phosphate (GLY3P), malic acid (L-MAL), phospho(enol)piruvate (PEP), sodium pyruvate (PYR), ribose-5-phosphate (R5P), ribulose-5-phosphate (RU5P), sedoheptulose-7-phosphate (S7P), and xylulose, (Xylu).

Ultra-High Performance Liquid Chromatography (UHPLC) Chromatographic parameters such as mobile phase composition, column temperature, and elution modes (gradient and isocratic) were tested to improve peak separation for the 19 metabolites. However, no single set of conditions was able to provide a complete peak separation with good resolution for all metabolites, due to their distinct chemical structures. In metabolomics analyses, a single approach is desirable for measuring all metabolites, but this is a challenge for complex matrix samples with a wide range of chemical classes of metabolites at different concentrations. As such, two main groups were established based on their chemical characteristics: (1) a group
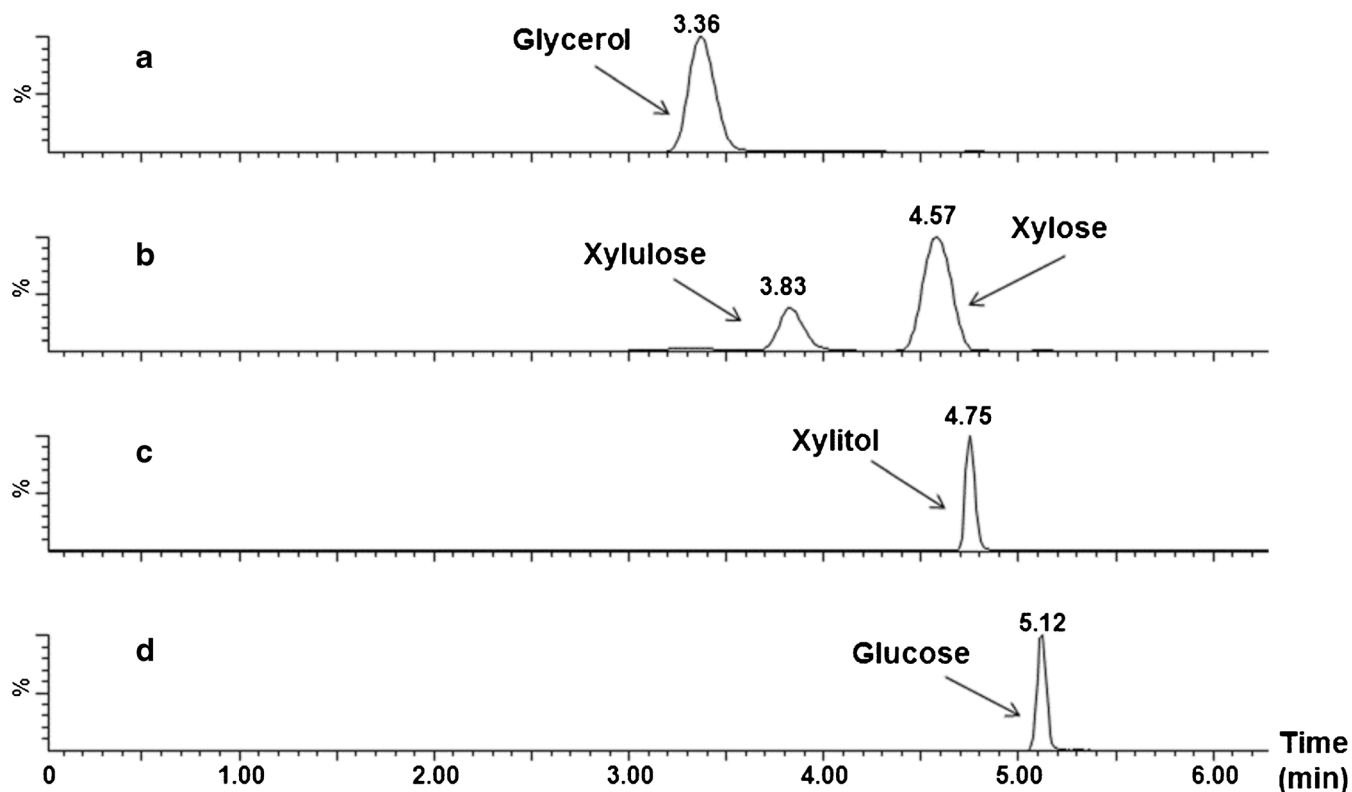

Figure 2. Multiple reaction monitoring (MRM) experiments using HILIC-ESI(-)-MS/MS for channels (Q1 > Q3): a) *m/z* 91 > 59 (glycerol); b) *m/z* 149 > 59 (xylulose and xylose); c) *m/z* 151 > 59 (xylitol) and d) *m/z* 179 > 59 (glucose)
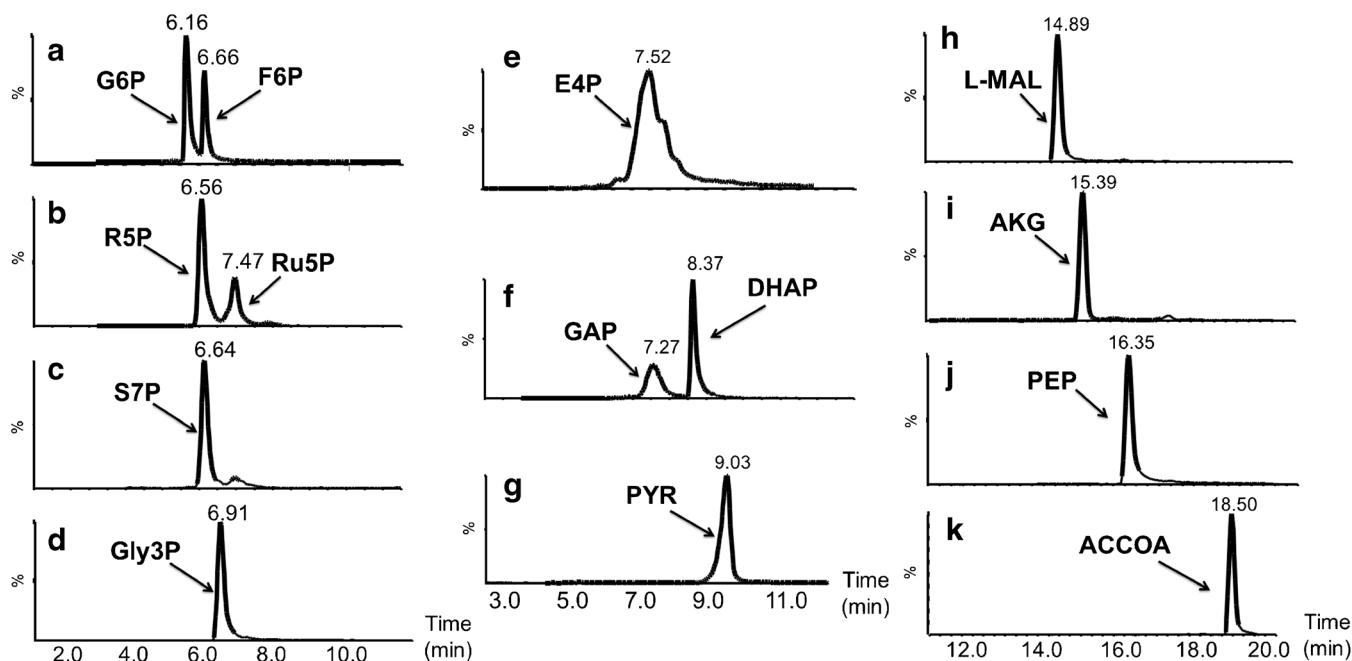
Figure 3. Multiple Reaction Monitoring experiments using IPC-ESI(-)-MS/MS: (**a**) *m/z* 259 > 97 (glucose-6-phosphate and fructose-6-phosphate); (**b**) *m/z* 229 > 97 (ribose-5-phosphate) and *m/z* 229 > 79 (ribulose-5-phosphate); (**c**) *m/z* 288 > 139 (sedoheptulose-7-phosphate); (**d**) *m/z* 170 > 79 (glycerol-3-phosphate); (**e**) *m/z* 199 > 79 (erytrose-4-phosphate); (**f**) *m/z* 169 > 97 (glyceraldehyde-3-phosphate and dihydroxyacetone phosphate); (**g**) *m/z* 87 > 43 (pyruvic acid); (**h**) *m/z* 133 > 115 (malic acid); (**i**) *m/z* 145 > 57 (alpha ketoglutaric acid); (**j**) *m/z* 167 > 79 (phospho(enol)pyruvate); (**k**) *m/z* 808 > 408 (acetyl Co-enzyme-A)

of sugars (monosaccharides) and alcohols; and (2) a group of organic acids and sugar phosphate compounds.

Hydrophilic Interaction Liquid Chromatography (HILIC)
Polar stationary phases, such as chemically modified silica, linked to organic groups, such as amine, amide, diol, cyano, and others, are often used in HILIC. The mechanism of compound separation by HILIC is still poorly understood. Tang and co-workers [35] proposed that water molecules are attracted by polar groups of the stationary phase, resulting in an aqueous layer on the surface. Partition of the analyte between the mobile phase (hydrophobic) and immobilized aqueous layer best explains the HILIC mechanism [29]. In HILIC, the eluting solvent is usually a mixture of water and acetonitrile with a modifier, such as ammonium salts [36]. Initially, the potential of the amide column was tested for separation of 14 metabolites, including phosphate sugars, organic acids, sugars (monosaccharides), and alcohols. Ammonium formate, ammonium acetate, and ammonium hydroxide were tested in different solutions of acetonitrile and water (Supplementary Table S1). The use of ammonium formate as a modifier in isocratic elution mode retained only seven metabolites (Supplementary Figure S1), and the gradient elution mode using ammonium acetate (Supplementary Table S2) provided poor separation, particularly for phosphate compounds (Supplementary Figure S2). The use of ammonium hydroxide, in gradient elution mode (Supplementary Table S3), showed good separation only for the neutral compounds (glycerol, xylulose, xylose, xylitol, and glucose). However, the phosphate compounds and

acids such as malic acid and alpha ketoglutaric were not separated (Supplementary Figure S3).

From these results, metabolites in the study were divided into two groups according to their chemical characteristics and interaction with the chromatography column. The first group consists of sugars (monosaccharides), xylitol, and glycerol, and the second consists of phosphate sugars and organic acids. For this first group the ideal chromatographic condition was observed using the mobile phase: A (0.1% ammonium hydroxide) and B (acetonitrile with 0.1% ammonium hydroxide) at 50 °C (column temperature) in a gradient mode (Table 1). A baseline peak separation of five metabolites: glycerol (91 > 59), xylulose (149 > 59), xylose (149 > 59), xylitol (151 > 59), and glucose (179 > 59) was performed in a 12 min analysis (Figure 2). To our knowledge, this is the first time that an ultrafast chromatography method coupled to mass spectrometry has been reported to detect these compound classes.

The isomers xylose and xylulose have the same values of Q1 (*m/z* 149) and Q3 (*m/z* 59) in the MRM experiment; therefore, chromatography was essential for separation and quantitation (Figure 2b). After the injection of each standard solution, the peaks were assigned to xylulose (3.83 min) and xylose (4.57 min) (data not shown).

Ion-Pair Chromatography (IPC) Ionic compounds are often separated by IPC, which usually uses a hydrophobic stationary phase and an ion pair (IP) reagent as the mobile phase. In general, volatile alkylamines and organic acids are used as IP reagents, and a polar compound, such as methanol or propan-2-
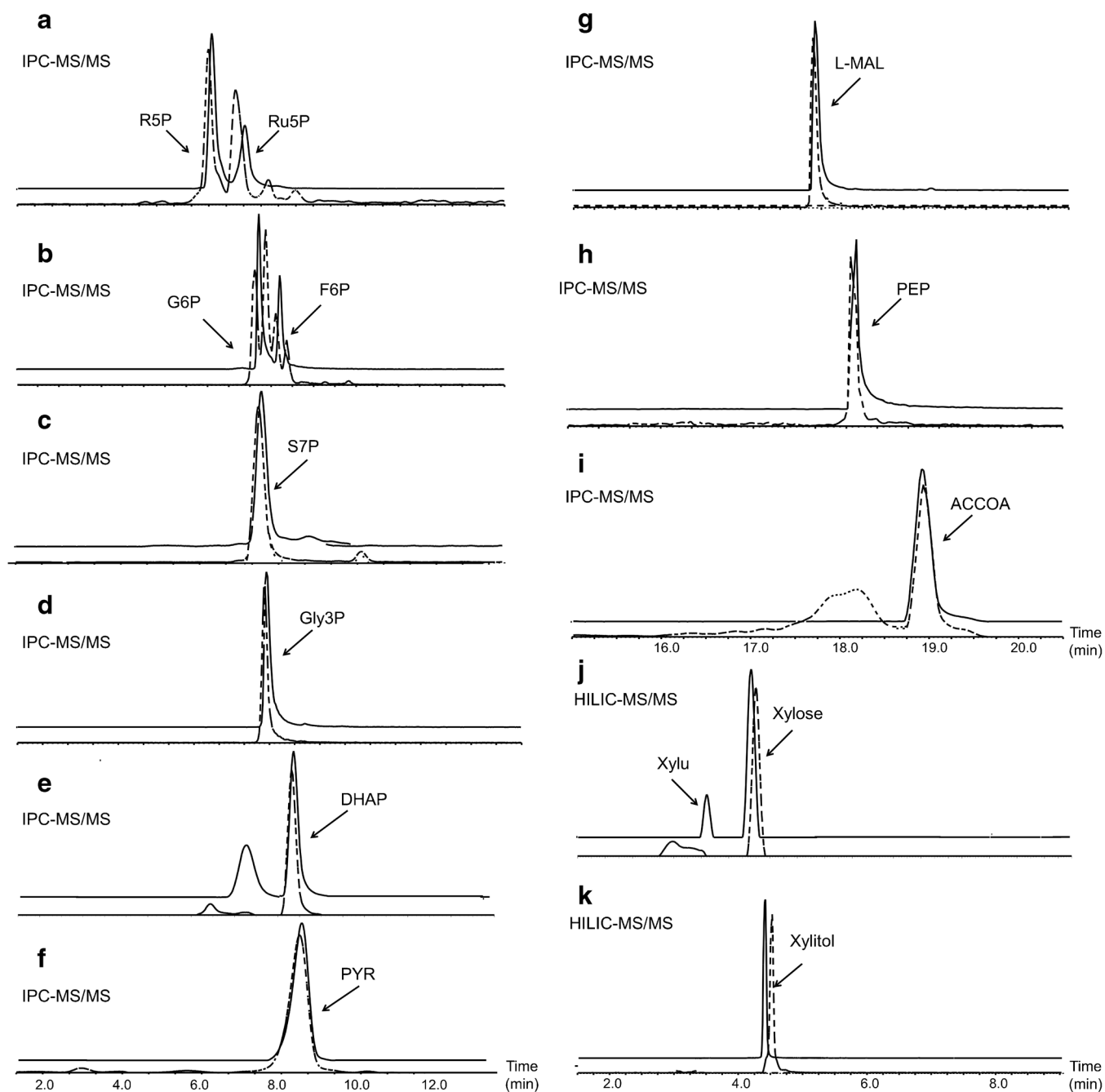
Figure 4. MRM chromatograms of 13 metabolites detected using IPC and HILIC, X axis is retention time in minutes. The upper solid line correspond to the standard mixture of metabolites and lower dashed lines correspond to the metabolites detected in *S. arborariae* cell extracts: (**a**) ribose-5-phosphate (R5P) and ribulose-5-phosphate (Ru5P), (**b**) glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P), (**c**) sedoheptulose-7-phosphate (S7P), (**d**) glycerol-3-phosphate (Gly3P), (**e**) dihydroxy acetone phosphate (DHAP), (**f**) pyruvic acid (PYR), (**g**) malic acid (L-MAL), (**h**) phospho(enol)pyruvate (PEP), (**i**) acetyl Co-enzyme A, (**j**) xylose, (**k**) xylitol

ol, as an organic modifier. IP reagent interacts with the stationary phase, creating an opposite charge to the analyte, thus increasing retention time and selectiveness of the reverse phase column. In the current study, a reverse phase column (HSS-T3- Waters), was tested using two IP reagents, triethylamine (TEA) and tributylamine (TBA), at different concentrations, pH values, temperatures, and elution modes (gradient and isocratic)

(Supplementary Table S6). Acceptable separation of phosphorylated compounds was not seen using TEA (Supplementary Figure S4). However, improved results were seen using TBA, with better peak separation, resolution, and sensitivity, as the alkyl chain size of the IP reagent was directly related to the analyte elution time [27]. Two organic eluents were tested, and a better separation of AKG, L-MAL, DHAP, and GAP was

Table 4. Regression coefficients (R^2), range of calibration curve, limits of detection (LOD), limits of quantification (LOQ), and metabolite concentrations in samples of yeast *Spathaspora arborariae* under aerobic and oxygen- limited condition

#	Metabolite	Range (µg/mL)	R^2	LOD (µg/mL)	LOQ (µg/mL)	Aerobic		Oxygen-limited	
						(µg/mL)	RSD (%)	(µg/mL)	RSD (%)
1	ACCOA	7.4–80	0.9975	2.35	7.12	17.04	7.68	6.61	5.46
2	AKG	3.5–50	0.9958	0.92	3.08	< LOQ	–	< LOQ	–
3	L-MAL	0.2–50	0.9984	0.03	0.11	64.95	18.97	14.03	8.37
4	Gly3P	3.5–50	0.9994	0.09	0.29	7.58	5.77	2.61	15.77
5	G6P	1.5–50	0.9990	0.43	1.43	5.43	10.02	4.45	12.09
6	F6P	3.5–50	0.9980	0.96	3.19	7.77	14.19	6.65	15.38
7	DHAP	2.0–50	0.9992	0.59	1.99	1.90	6.98	< LOQ	–
8	GAP	3.5–50	0.9979	0.92	3.06	ND	–	ND	–
9	R5P	2.0–50	0.9992	0.65	1.99	2.87	13.08	< LOQ	–
10	Ru5P	2.5–50	0.9991	0.61	2.04	21.92	2.72	5.79	15.87
11	E4P	1.0–50	0.9996	0.29	0.98	ND	–	ND	–
12	S7P	1.4–30	0.9983	0.38	1.27	37.33	19.46	20.55	17.23
13	PEP	2.5–50	0.9919	0.66	2.21	18.50	9.91	4.26	13.11
14	PYR	0.5–50	0.9998	0.14	0.43	4.63	9.90	1.65	16.86
15	Xylu	0.5–75	0.9971	0.20	0.50	ND	–	ND	–
16	Xylose	0.5–50	0.9990	0.20	0.50	3275.00	11.26	4896.00	6.10
17	Glucose	0.5–50	0.9991	0.20	0.50	ND	–	ND	–
18	Glycerol	0.5–50	0.9993	0.20	0.50	< LOQ	–	< LOQ	–
19	Xylitol	0.5–75	0.9988	0.20	0.50	1140.00	5.92	401.52	12.16

achieved using methanol instead of acetonitrile, in a gradient mode (data not shown). Variation in pH values (2.8, 4.8, 5.1, and 6.2) (Supplementary Figure S5A–D) showed a greater co-elution at the lower pH value in the separation of phosphates sugars (Supplementary Figure S5B). The separation of ribose-5-phosphate was achieved at pH 4.8, and this pH value improved separation of compounds with carboxylic groups such as malic acid, alpha ketoglutaric acid, and phospho(enol)pyruvate.

Three concentrations of TBA, (2, 5, and 10 mM - Supplementary Table S5), were investigated, and the chromatographic profile was similar using 5 and 10 mM (Supplementary Figure S6). In general, studies in the literature have been performed using 10 mM TBA [26, 27, 37]; however, the use of TBA has the disadvantage of contaminating the mass spectrometer, and it is difficult to remove and requires constant cleaning. As such, 5 mM TBA in the mobile phase was chosen for this study. The best chromatographic condition was achieved using the mobile phases: A (5 mM tributylamine, 10 mM acetic acid, and 5% (v/v) methanol); B (methanol) at 45 °C (column temperature) in a gradient mode (Table 2). A 26 min chromatographic run is described here for the first time, for separation of 14 compounds, including the isomers: G6P and F6P; and R5P and Ru5P (Figure 3). OAA did not show good stability and reproducibility and it was excluded from further study.

A summary table describing the metabolites and most significant UHPLC-MS/MS optimized parameters such as the separation mode, retention time, cone voltage, MRM transitions and collision energy, is presented (Table 3).

Yeast Metabolomics

Quantitative Analysis of Spathaspora arborariae Metabolites

Metabolomics analysis of a naturally xylose-fermenting yeast,

S. arborariae [38], was performed using the UHPLC-MS/MS methods developed here, after the yeast was cultivated in a fermenter under aerobic and oxygen-limited conditions. Optimization of sample preparation was carried out to extract the highest possible quantity of intracellular metabolites using fewer steps in order to avoid loss and chemical degradation, and to increase the sensitivity of analysis. Yeast media volumes of 1-5 mL were collected, processed, and analyzed, and a volume of 2 mL was chosen for further analysis.

An important consideration in metabolomics analysis is the wide range of concentrations for different metabolites in a biological sample, ranging from pmol/L to mol/L. UHPLC-MS/MS injections of *S. arborariae* samples have detected high concentrations of xylose and xylitol, and so samples were diluted 100-fold before HILIC-MS/MS analysis. Furthermore, malic acid and sedohptulose-7-phosphate reached the upper limit of the detection curve, and so a 5-fold dilution was required before IPC-MS/MS analysis.

A total of 13 metabolites (xylitol, xylose, pyruvic acid, sedoheptulose-7-phosphate, glucose-6-phosphate, fructose-6-phosphate, glycerol-3-phosphate, malic acid, phospho(enol)-pyruvate, acetyl co-enzyme-A, ribose-5-phosphate, ribulose-5-phosphate, and dihydroxy acetone phosphate) were quantified in *S. arborariae* samples under aerobic conditions. Figure 4 shows a significant overlap of MRM chromatograms for some of these metabolites identified in yeast samples and standard solutions using IPC and HILIC-MS/MS. The matrix effect exists but was not significant for data interpretation as shown in Figure 4, since the retention times for standards and yeast intracellular metabolites were similar. Furthermore, other isomers, different from the standards, were detected in some MRM channels (Figure 4a and b).

The limit of detection (LOD), limit of quantitation (LOQ), and regression coefficients (R^2) for each metabolite were
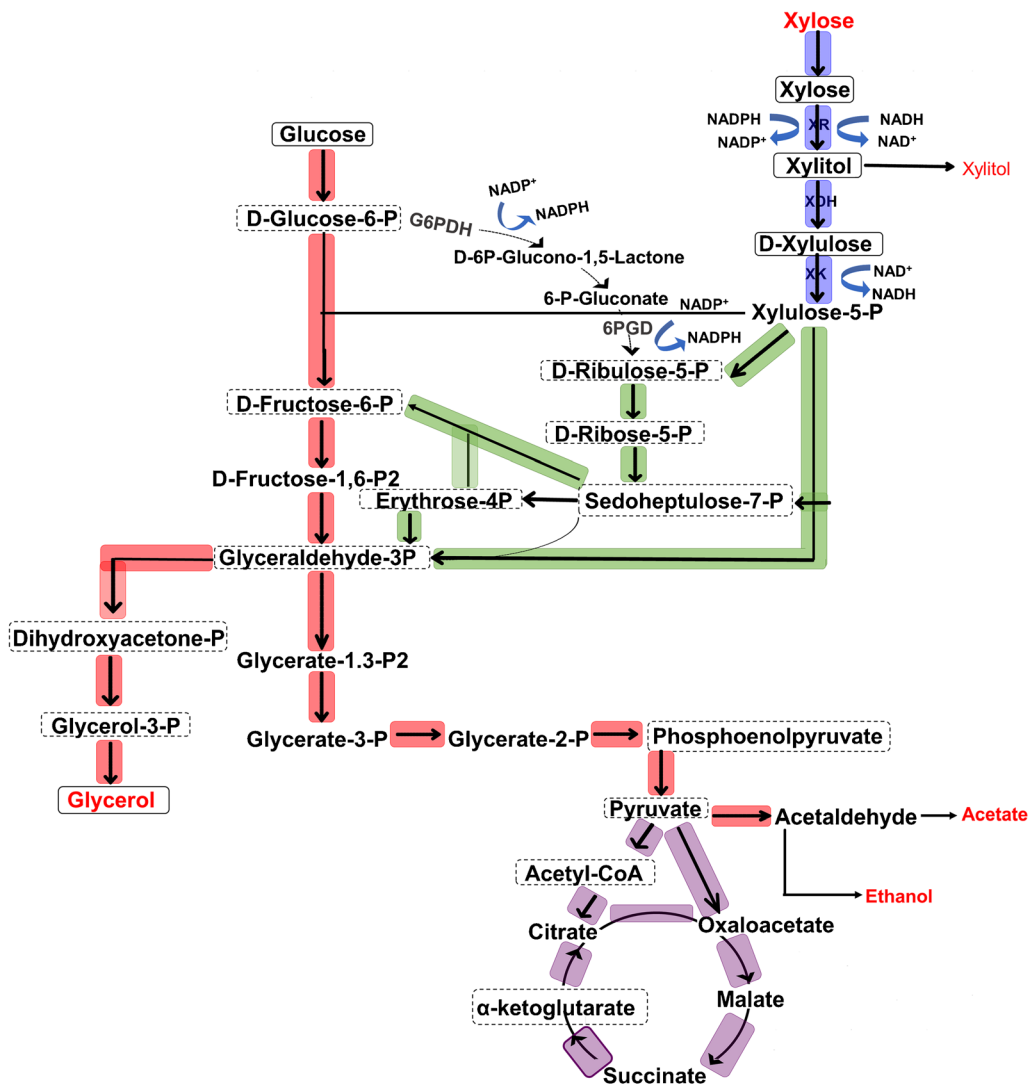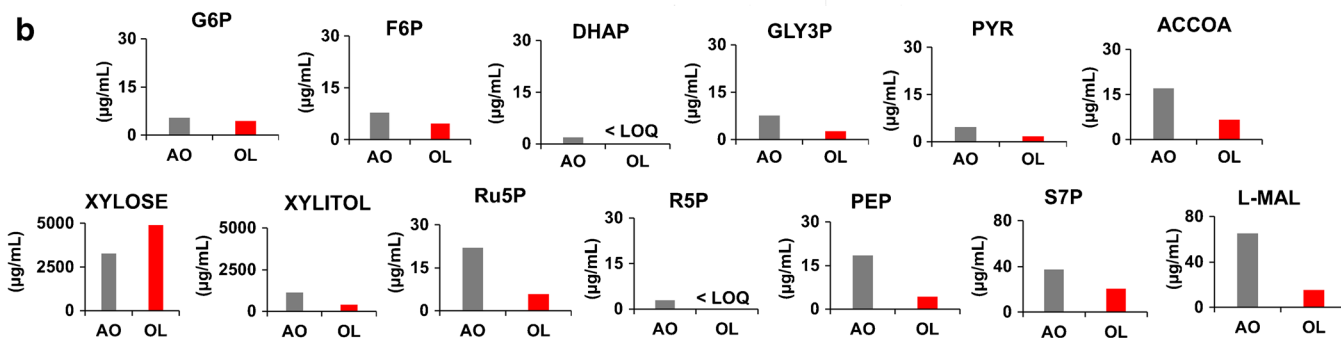
Figure 5. (**a**) Simplified schematic of central carbon metabolism from xylose to ethanol by yeast. Metabolic pathways assigned: oxidoreductive xylose assimilation (blue), pentose phosphate pathway (green); glycolysis (red); and tricarboxylic acid cycle (purple). The dotted boxes indicate which of these metabolites are detected by the HILIC method and the solid boxes indicate those detected by the IPC method. (**b**) Concentrations of 13 metabolites under aerobic (AO) and oxygen-limited (OL) conditions.

established for quantitative analysis (Table 4). Calibration curves ranged from 0.5 to 80 µg/mL (Supplementary Figures S7 and S8); LOD values ranged from 0.03 (L-MAL) to 3.19 µg/mL (ACCOA), and LOQ from 0.11 (L-MAL) to 7.12 µg/mL (ACCOA). Using these parameters, the metabolites detected during exponential growth of *S. arborariae* in

xylose medium were quantified. The concentrations were shown as an average of triplicates (µg/mL), and relative standard deviation (RSD) presented values below 20% as shown in the Table 4.

Concentrations of intracellular metabolites associated with central carbon metabolism in *S. arborariae* were, to

the best of our knowledge, determined for the first time here during xylose fermentation under aerobic and oxygen-limited conditions (Figure 5). Xylose was the most concentrated metabolite, followed by xylitol under both aerobic (3275.00 µg/mL; 1140.00 µg/mL), and oxygen-limited (4896.00 µg/mL; 401.52 µg/mL) conditions (Table 4). Although high concentration of xylose was expected due to this sugar being the substrate for the experiment and cells not being washed before metabolite extraction, the excess of xylitol indicates that the available oxygen was not sufficient for redox balance. Therefore, the accumulation of xylitol can be explained by an imbalance in cofactors required by the enzymes XR and XDH for their respective activities. This is in accordance with data previously reported in the literature, which demonstrated xylitol production, especially when the yeast is cultivated under oxygen-limited conditions [8]. These observations may be explained by the fact that XR from *S. arborariae* uses mainly NADPH as a cofactor in xylose reduction, whereas XDH is strictly NAD^+-dependent. Furthermore, the presence of glucose-6-P (G6P) and fructose-6-P (F6P) (Figure 5) indicates the need for regeneration of the cofactor NADPH in the non-oxidative PPP [39].

In addition, three metabolites of PPP were detected (ribose-5-phosphate (R5P), ribulose-5-phosphate (RU5P), and sedoptulose-7-phosphaste (S7P)), with S7P the most abundant under aerobic and oxygen-limited conditions (Table 4). The excess of S7P in *S. arborariae* can be attributed to insufficient activity of PPP enzymes, as occurs in native *S. cerevisiae* [39]. Overexpression of non-oxidative PPP genes in *S. cerevisiae* was shown to improve xylose conversion to ethanol [9].

As expected, the yeast produced two times more biomass under aerobic (0.31 g g^{-1}) than oxygen-limited (0.15 g g^{-1}) conditions (data not shown). Aerobic metabolism allows carbon flux through the TCA cycle, which in turn results in greater ATP formation [40]. Indeed, higher activity of the TCA cycle under aerobic compared with oxygen-limited conditions was confirmed by the approximately 4.5 and 3.0 times higher concentrations of L-MAL and ACCOA, respectively, (Table 4). In general, all metabolites quantified under oxygen-limited conditions are found at lower concentrations compared with aerobic conditions, and this is possibly related to biomass yield.

Conclusions

Metabolomics studies, although challenging, can contribute to the understanding of xylose metabolism in yeast. Here, we first developed two complementary methods based on ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) to quantify 19 metabolites involved in the glycolysis, PPP, and TCA pathways in

yeast. A faster run method based on IPC-MS/MS (26 min of total chromatographic run time), using 5 mM TBA concentration (lower than previously described) was capable of separating 14 metabolites with good peak resolution. Furthermore, a new HILIC-MS/MS method was developed to quantify xylose, glucose, glycerol, xylitol, and xylulose in a 12 min chromatographic run. Previous methods described are slower and do not separate all of these metabolites. Analytical parameters such as LOD, LOQ, and calibration curves were established for quantitative analysis of targeted metabolites.

To our knowledge, this is the first time that intracellular metabolites from *S. arborariae* have been successfully quantified using the metabolomics protocol developed in this work, characterizing the metabolic flux after xylose consumption. We showed that the yeast growth under aerobic conditions leads to respiratory metabolism. Analysis under oxygen-limited conditions showed fermentation metabolism, but with imbalance of cofactor regeneration, resulting in xylitol accumulation. These results indicate that fine control of oxygen levels during fermentation is necessary to optimize ethanol production with *S. arborariae*.

The protocol presented here may be further applied to other yeast species, and data generated may be used to identify limiting steps in xylose metabolism, and consequently, support yeast genetic engineering to improve second-generation ethanol production.

Compliance with Ethical Standards

Conflict of Interest Author Christiane Gonçalves Campos declares that she has no conflict of interest.

Author José Antônio de Aquino Ribeiro declares that he has no conflict of interest.

Author Patrícia Pinto Kalil Gonçalves Costa declares that she has no conflict of interest.

Author Katiúscia Pereira Araújo declares that he has no conflict of interest.

Author Henrique César Teixeira Veras declares that he has no conflict of interest.

Author João Ricardo Moreira de Almeida declares that he has no conflict of interest.

Author Clenilson Martins Rodrigues declares that he has no conflict of interest.

Author Patrícia Verardi Abdelnur declares that she has no conflict of interest.

Ethical Approval   This article does not contain any studies with human subject or animal participation.

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