Journal of Chromatography B, 877 (2009) 3231–3236

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short communication

A comprehensive method for the quantification of the non-oxidative pentose phosphate pathway intermediates in *Saccharomyces cerevisiae* by GC–IDMS

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ARTICLE INFO

Article history:
Received 27 April 2009
Accepted 15 July 2009
Available online 21 July 2009

Keywords:
Methoxime-trimethylsilyl (MOX-TMS) derivatives
GC–MS
Isotope dilution mass spectrometry
Metabolomics
Pentose phosphate pathway
Saccharomyces cerevisiae

ABSTRACT

A gas chromatography isotope dilution mass spectrometry (GC–IDMS) method was developed for the quantification of the metabolites of the non-oxidative part of pentose phosphate pathway (PPP). A mid-polar GC column (Zebron ZB-AAA, 10 m, film composition 50% phenyl 50% dimethyl polysiloxane) was used for the chromatographic separation of the intermediates. The optimized GC–MS procedure resulted in improved separation performances and higher sensitivities compared to previous methods. Furthermore, the use of ^{13}C-labeled cell extracts as internal standards improved the data quality and eliminated the need to perform a recovery check for each metabolite. The applicability of the new method was demonstrated by analyzing intracellular metabolite levels in samples derived from aerobic glucose-limited chemostat cultures of *Saccharomyces cerevisiae* at steady state as well as following a short-term glucose pulse. The major achievements of the proposed quantitative method are the independent quantification of the epimers ribulose-5-phosphate and xylulose-5-posphate and the measurement of compounds present at very low concentrations in biological samples such as erythrose-4-phosphate and glyceraldehyde-3-phosphate.

1. Introduction

The pentose phosphate pathway (PPP) is required for NADPH generation and nucleotide biosynthesis. Besides this anabolic role, the PPP is a primary catabolic route for the fermentation of pentose sugars, such as xylose and arabinose. Lately, the growing interest in ethanol production from lignocellulosic material, which is very rich in pentose sugars, has given a significant input for extensive metabolic engineering aimed at improving the efficiency of pentose fermentation in many different microorganisms [1]. The microorganism of choice has so far been the yeast *Saccharomyces cerevisiae*, which can efficiently ferment hexose sugars to ethanol, but is not able to metabolize pentose sugars. Many successful examples have recently been reported describing *S. cerevisiae* strains engineered to efficiently convert xylose and arabinose to ethanol [2–5]. During growth on pentose sugars, the non-oxidative part of PPP becomes the main catabolic pathway for sugar consumption, leading to a complete rearrangement of the reversible fluxes, possibly due to changes in intracellular concentrations of its intermediates, compared to growth on other substrates [5]. In this respect, the measurement of the intracellular levels of non-oxidative PPP intermediates would allow a better understanding of the metabolic state of the cells, help identifying metabolic limitations and provide relevant information for the development of kinetic models.

The flourishing of reports describing analytical procedures for the comprehensive quantification of non-oxidative PPP intermediates clearly shows the great interest for this issue [3,5–8]. Recently, GC–MS procedures have been reported describing the use of non-polar long capillary columns (30–60 m) for the measurement of organic acids, sugars and sugar phosphate [9–11]. Besides GC–MS-based methods, alternative approaches have been developed based on LC-MS/MS [3,6,7], high performance anion exchange chromatography coupled to pulsed amperometric detection system [5] and reversed-phase liquid chromatography–mass spectrometry (RPLC–MS) [8]. Despite the great effort, none of the published works succeeded in the comprehensive quantification of the non-oxidative PPP intermediates. The measurement of metabolites present at low concentration in biological samples, such as erythrose-4-phosphate (E4P) and glyceraldehyde-3-phospate (GAP), as well as the separation of the isomer couples GAP/dihydroxyacetone phosphate (DHAP) and ribulose-5-phosphate (RBU5P)/xylulose-5-phosphate (X5P) still

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doi:10.1016/j.jchromb.2009.07.019

remain an analytical challenge [6–10]. In particular, at the best of our knowledge, the independent quantification of the epimers ribulose-5-phosphate and xylulose-5-phosphate has not yet been reported.

Here we describe a gas chromatography isotope dilution mass spectrometry (GC–IDMS) method for the quantification of all the non-oxidative PPP intermediates, including RBU5P, X5P, E4P, and GAP. The method was applied to the quantification of intracellular metabolite levels in *S. cerevisiae* cells growing in continuous cultures, both at steady state and during the short-term response to a sudden addition of extracellular glucose. The use of ^{13}C-labeled cell extracts as internal standards according to isotope dilution mass spectrometry (IDMS) improved measurement reproducibility and eliminated the need to perform a recovery check of extraction for each individual metabolite [12,13].

2. Experimental

2.1. Chemicals

The following chemicals were purchased from Sigma–Aldrich: erythritol (≥99%), ribose-5-phosphate (R5P, ≥99%), ribulose-5-phosphate (RBU5P, ≥96%), xylulose-5-phosphate (X5P, ≥90%), erythrose-4-phosphate (E4P, ≥61%), glucose-6-phosphate (G6P, 100%), dihydroxyacetone phosphate (DHAP, ≥95%) and glyceraldehyde-3-phosphate (GAP, 260–320 mM). Sedoheptulose-7-phosphate (S7P, ≥98%) was purchased from Glycoteam-GMbH. Methoxyamine·HCl and N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) were purchased from Pierce, Rockford, IL, USA.

2.2. Cultivation conditions and sampling for intracellular metabolites

The *S. cerevisiae* strain used in this study was CEN.PK 113-7A pYX222:mtlD (W303-1a pYX222: mtlD trp1-1 ura3-1 ade2-1) [14]. Cells were grown aerobically on defined mineral medium [15] in a 7 l fermentor (Applikon, The Netherlands) with a working volume of 4 l. The glucose-limited chemostat culture was carried out at a dilution rate of 0.1 h^{-1}, 30 °C, pH 5.0, as previously described [14]. The steady-state biomass concentration was 3.6 g_{DW} l^{-1}. Samples for intracellular metabolite analysis (1 ml) were withdrawn from the bioreactor (<0.8 s) via a custom-made low dead-volume rapid sampling setup [16]. Cold methanol quenching, washing, boiling ethanol extraction and sample concentration by vacuum evaporation were carried out as described in Canelas et al. [14]. Uniformly labeled ^{13}C-cell extract was added to the cell pellets before extraction, as internal standard [13,14,17]. Dried samples were resuspended in 0.5 ml distilled water, resulting in a concentration factor of two.

For the glucose pulse experiment, 13 ml of a sterile concentrated glucose solution (3.1 M) was injected into the bioreactor, bringing the extracellular glucose concentration from residual levels (<0.2 mM) to approximately 10 mM. Time zero (steady-state) samples were collected just before the pulse. After the pulse, samples were collected at the indicated timepoints.

2.3. Sample preparation and derivatization

Erythritol was added as standard check for GC–MS performances to 100 μl of cell extract at a final concentration of 40 μM [18]. Samples were then lyophilized, resuspended in 50 μl of 240 mM methoxyamine·HCl in pyridine solution and incubated 50 min at 60 °C. The extracts were then silylated for 50 min at 60 °C by adding 80 μl of MSTFA. The resulting methoxime-trimethylsilyl (MOX-TMS) derivatives were injected for GC–MS analysis.

2.4. Analysis of metabolites by GC–MS

GC–MS measurements were carried out on a Trace GC Ultra (Thermo Finnigan, Boston, MA, USA) coupled to a Trace DSQ single quadrupole mass spectrometer (Thermo Finnigan, Boston, MA, USA). The optimized conditions for the measurement of the metabolites of interest in cell extracts were as following: 1 μl of sample was injected on a Zebron ZB-AAA column (10 m × 250 μm internal diameter, 0.25 μm film thickness; Varian Inc, Palo Alto, CA) using programmed temperature vaporization (PTV) injection in splitless mode. Straight glass liners with CarboFrit were used (Restek). The PTV temperature was set to 70 °C with a splitless time of 0.7 min. After the injection the PTV temperature was raised to 220 °C with 600 °C min^{-1} and held for 5 min. Subsequently the temperature was raised with 870 °C min^{-1} to 300 °C for cleaning purposes. Carrier gas (helium) flow during the analysis was set at 1.5 ml min^{-1}. The GC temperature gradient for the separation of complex sugar phosphate mixtures was 70 °C for 1 min, 1 °C min^{-1} up to 76 °C, 10 °C min^{-1} to 150 °C, 30 °C min^{-1} up to 320 °C. The temperature of the transfer line to the MS was set at 250 °C and the ion source was set at 280 °C. Electron ionization was operated with 70 eV. For quantitative measurements the MS was used in selected ion monitoring (SIM) mode. The mass resolution was 1 mass unit throughout the mass range 1–1050 amu.

3. Results and discussion

3.1. Use of internal standard and chromatography

Uniformly ^{13}C-labeled yeast cell extract was used as internal standard both in the samples and in the standard calibration mixtures according to the IDMS procedure [13]. The application of ^{13}C-labeled cell extract as internal standard has been described for quantitative measurements of glycolytic and tricarboxylic acid intermediates in *S. cerevisiae* using anion exchange liquid chromatography [13,14,17]. The co-extraction of the labeled internal standards with intracellular metabolites eliminated the need to perform a recovery check for each individual metabolite and improved data quality. The specific fragments used for the quantification of each metabolite and their ^{13}C-labeled analogs are reported in Table 1. The contribution of each target compound to its ^{13}C-internal standard due to the natural abundance of C, H, O, Si and P isotopes was calculated according to van Winden et al. [19] and is reported in Table 1, expressed as percentage of the target compound. The correct areas of ^{13}C-internal standards were derived by calculating such a contribution for each metabolite and subtracting the obtained value from the measured area of its ^{13}C-internal standard.

All the metabolites of interest were successfully separated, with the only exception of RBU5P and X5P (Fig. 1). Typically, methoxime-derivatives of sugar phosphate give two peaks in the chromatogram due to the presence of two isomers (syn and anti) and the ratio between the two peaks is constant for each compound and stable for a given reaction condition [20,21]. For quantification purposes we always considered the area of the highest peak. The linearity range of calibration lines spanned about two to three orders of magnitudes, with coefficient of determination values (R^2) above 0.99 and standard error below 3.0% for all metabolites. The limit of detection (LOD), calculated as three times the standard deviation of the intercept divided by the slope of the calibration curve, was in the fmol range (Table 1) resulting in overall higher sensitivity compared to previous reports [6,9].

Table 1
GC–MS parameters of methoxime-TMS derivatives of the metabolites analyzed in the present study.

Metabolite	RT[a] (min)	Specific ions, *m/z* [^{12}C, ^{13}C]	% target compound in IS[b]	Linearity range (μM)	R^2	SE%	LOD (fmol)
Erythritol	9.01	217, NA	NA	NA	NA	NA	NA
GAP[c]	13.73	328, 331	NA	0.02–2.5	0.9947	0.28	15.6
DHAP	14.19	400, 403	3.1%	0.08–20	0.9994	2.63	28.9
E4P[c]	15.03	357, 359	NA	0.02–1.25	0.9984	0.82	7.2
R5P	15.72	217, 220	1.2%	0.08–20	0.9990	1.76	9.4
Rbu5P	15.83	357, 359	14.4%	0.2–10	0.9994	3.03	41.3
X5P	15.83	357, 359	14.4%	0.05–36	0.9996	0.87	0.6
F6P	16.29	217, 220	1.2%	0.08–30	0.9992	1.49	194
G6P	16.32	357, 359	14.4%	0.16–80	0.9997	0.20	49.3
S7P	16.85	357, 359	14.4%	0.2–40	0.9995	1.06	22.3

RT, retention time; *m/z* [^{12}C, ^{13}C], specific fragments used for quantification and their ^{13}C-labeled analogs; IS, internal standard; SE%, standard error expressed as percentage; LOD, limit of detection; NA, not applicable.

[a] In case of double peaks the retention time refers to the highest peak.

[b] Contribution of the target compound to the ^{13}C-internal standard, due to the natural abundance of C, H, O, Si and P isotopes, expressed as percentage.

[c] ^{13}C-R5P was used as Internal Standard for E4P and GAP since their respective ^{13}C-isotopologues were below detection limit in the used ^{13}C-extract.

3.2. Measurement of X5P and RBU5P

RBU5P and X5P showed same retention time (Figs. 1 and 2) and same mass spectrum (data not shown). However, the peak area ratio between the second and the first eluting peak typical of MOX-TMS derivatives differed significantly between the two compounds. As shown in Fig. 2, this ratio was 26.9 for a pure solution of X5P and 1.8 for a pure RBU5P solution (independent of the concentration), while for a mixture of these two the ratio changed based on the contribution of each compound (Fig. 2C). This feature was used to separately quantify these two metabolites. By knowing the peak area ratio of the pure compounds and by measuring the areas of the two peaks in every mixture ($\sum$Pk1 and $\sum$Pk2, intended as the areas of the first and second eluting peak divided by the area of the second eluting peak of the ^{13}C-labeled internal standard, according to the IDMS procedure), it was possible to calculate the peak areas relative to the single compounds as follows:

1. X5P_Pk1 (area of X5P first eluting peak) $= (\sum Pk2 - 1.8 \cdot \sum Pk1)/(26.9 - 1.8)$.
2. X5P_Pk2 (area of X5P second eluting peak) = X5P_Pk1·26.9.

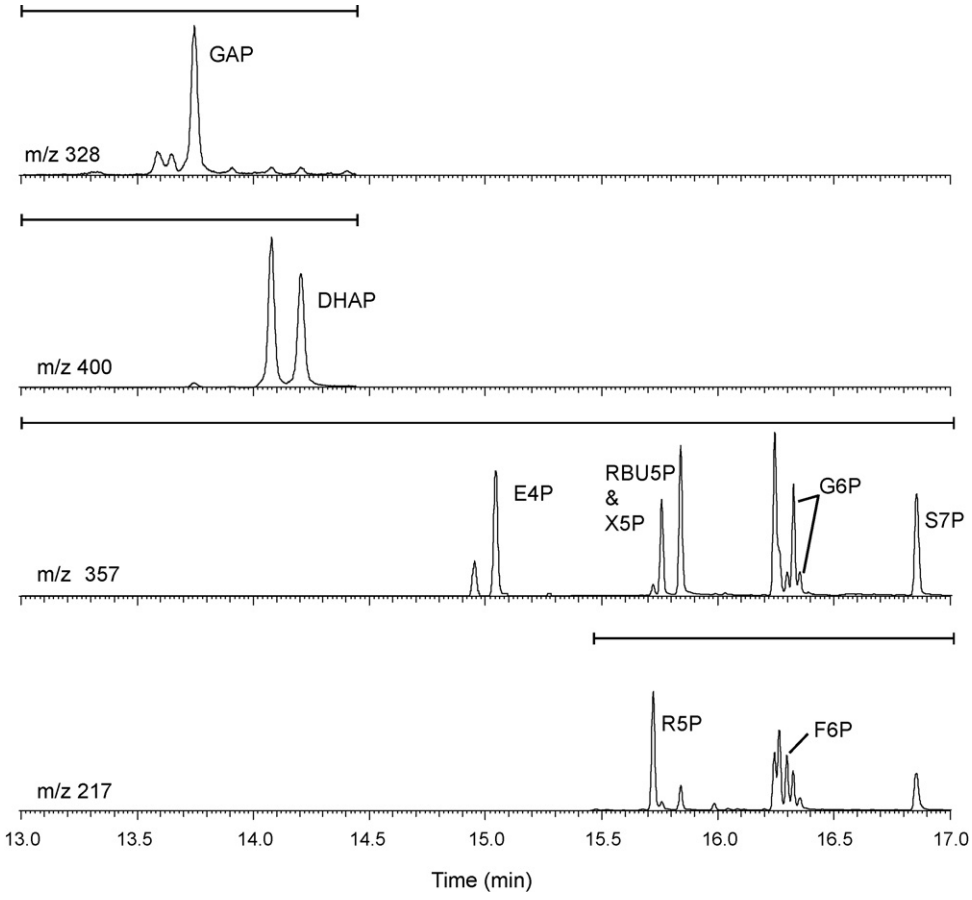

Fig. 1. Chromatographic profiles of MOX-TMS derivatives of the metabolites analyzed in the present study. The specific *m/z* values used for quantification are given for each compound. Horizontal bars indicate the time window in which the scan for the indicated *m/z* value was performed. For better visibility the *y*-axis for some of the peaks has been changed.

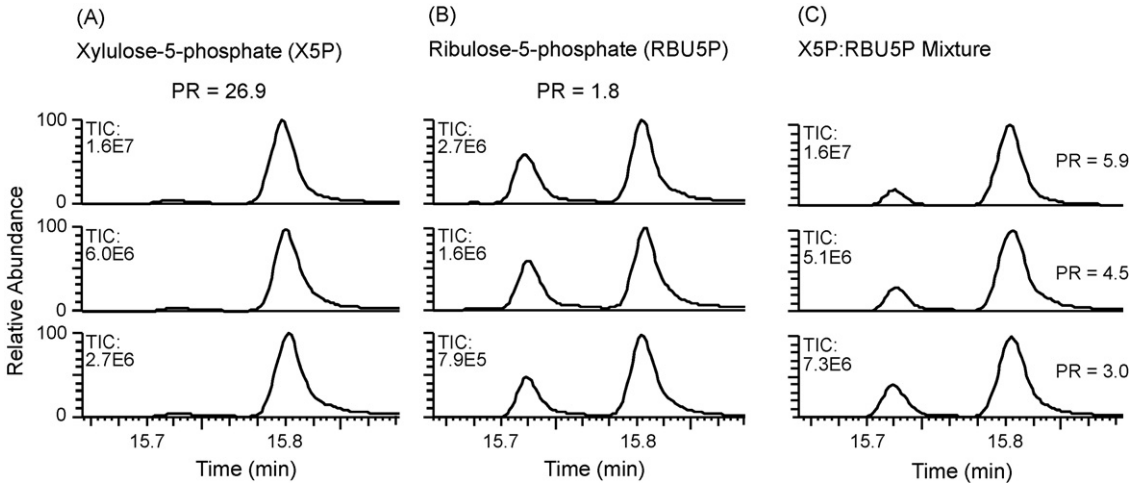

Fig. 2. Chromatographic profiles of pure solutions of MOX-TMS derivatives of xylulose-5-phospate (A) and ribulose-5-phosphate (B) at increasing concentrations (from bottom to top: 2 μM, 5 μM, and 10 μM) and of different X5P–RBU5P mixtures (from bottom to top: X5P:RBU5P = 1:4 (5 μM:20 μM), 1:2 (5 μM:10 μM) and 1:1 (30 μM:30 μM) (C)). PR (peak ratio) indicates the area ratio between the second and the first eluting peak; TIC, total ion current.

3. RBU5P_Pk2 (area of RBU5P second eluting peak) = $\sum$Pk2 − X5P_Pk2.

The calculated concentrations of RBU5P and X5P in known mixtures were in good agreement with the expected values (deviation below 15%) confirming the validity of this approach.

3.3. Quantification of non-oxidative PPP intermediates in S. cerevisiae cell extracts

For the measurement of intracellular metabolites in *S. cerevisiae* during glucose-limited growth at steady state, samples were collected from two chemostat cultures (three samples from each steady-state) and measured in duplicate. The results are reported in Table 2. For identification purposes retention times (RT) as well as mass spectra (derived from analysis performed in full scan mode) were compared for each metabolite between standard solutions and yeast samples. For quantification purposes, the analysis was run in selected ion monitoring (SIM) mode by selecting specific diagnostic fragments for the analytes in yeast samples and in the ^{13}C-labeled internal standard, as reported in Table 1. Peak areas of each analyte were integrated and the obtained values were divided by the area of their respective ^{13}C-isotopologues present in the internal standard according to the IDMS procedure [17]. Concentrations were calculated by using calibration lines prepared by mixing increasing concentrations of standard metabolites with a fix amount of ^{13}C-labeled internal standard identical to the one added in the samples at the time of extraction. The reproducibility was good, with relative standard deviations (RSD) around or below 10% for most of the analyzed compounds (Table 2). DHAP showed a RSD of 24%, while E4P and GAP showed the highest RSDs, around 35%. The analytical error (defined as the error between duplicate measurements) was around or below 2% for most metabolites indicating that most of the variation in biological samples derived from sample processing and biological variability rather than measurement errors. In the case of E4P and GAP the high RSD values were most probably due to the low levels of these metabolites and to the fact that ^{13}C-R5P was used as internal standard for these compounds instead of their respective ^{13}C-isotopologues which were below detection limit in the used ^{13}C-extract. To reduce this error, an alternative ^{13}C-internal standard may be used in which these metabolites are present at quantifiable concentrations. In addition, the levels of the measured metabolites can be further increased as needed by changing the concentration factor during the lyophilization step.

Table 2
Intracellular levels and analytical error of the metabolites analyzed in the present study in S. *cerevisiae* cells.

Metabolite	Intracellular levels[a] (μmol/g_{DW})	Workflow and biological error (n = 6)[b] %RSD	Analytical error[c] (n = 19)
GAP	0.017 ± 0.007	38.9	10.4
DHAP	0.28 ± 0.067	24.0	6.01
E4P	0.002 ± 0.0007	33.0	6.20
R5P	0.38 ± 0.020	5.27	1.50
RBU5P	0.14 ± 0.017	12.3	2.24
X5P	0.43 ± 0.042	9.75	2.10
F6P	0.98 ± 0.056	5.72	1.58
G6P	3.38 ± 0.183	5.42	0.74
S7P	1.77 ± 0.119	6.76	1.00

%RSD, relative standard deviation expressed as percentage.

[a] The intracellular levels represent average and standard deviation of six independent samples (measured in duplicate) derived from two steady state glucose-limited chemostats.

[b] Errors due to sample processing and biological variation. The values derived from the samples collected at steady state (six samples measured in duplicate).

[c] It was calculated by averaging the standard deviation of duplicate measurements for 19 samples (including steady state and glucose pulse experiment).

To further demonstrate the applicability of the method, a short-term response experiment was performed by applying a glucose pulse to a glucose-limited culture of *S. cerevisiae* and sampling for intracellular metabolite analysis within a time window of 0–335 s. As shown in Fig. 3, robust and consistent metabolic profiles with very low standard deviations were obtained for all metabolites. The trends of G6P and F6P closely matched previously reported LC-MS/MS data [22] and the ratio F6P/G6P remained stable around 0.27 both at steady state and throughout the transitory state following the pulse, consistent with the fast equilibrium reaction catalyzed by the phosphoglucose isomerase (K_{eq} = 0.28 [23]). Similarly, R5P, RBU5P and X5P showed parallel trends reflecting the fast equilibrium of the reactions catalyzed by the enzymes ribulose-5-phosphate epimerase and ribulose-5-phosphate isomerase [24].

Besides non-oxidative PPP intermediates, dihydroxyacetone phosphate (DHAP) was measured to provide a consistency check based on thermodynamic principles. Triose-phosphate isomerase (TPI), being the most active enzyme in glycolysis, is thought to maintain equilibrium between the pools of DHAP and GAP, in which case the ratio of their concentrations should be approximately 22:1 (K_{eq}, TPI = 0.045 [25]). The measured ratio of DHAP vs GAP was

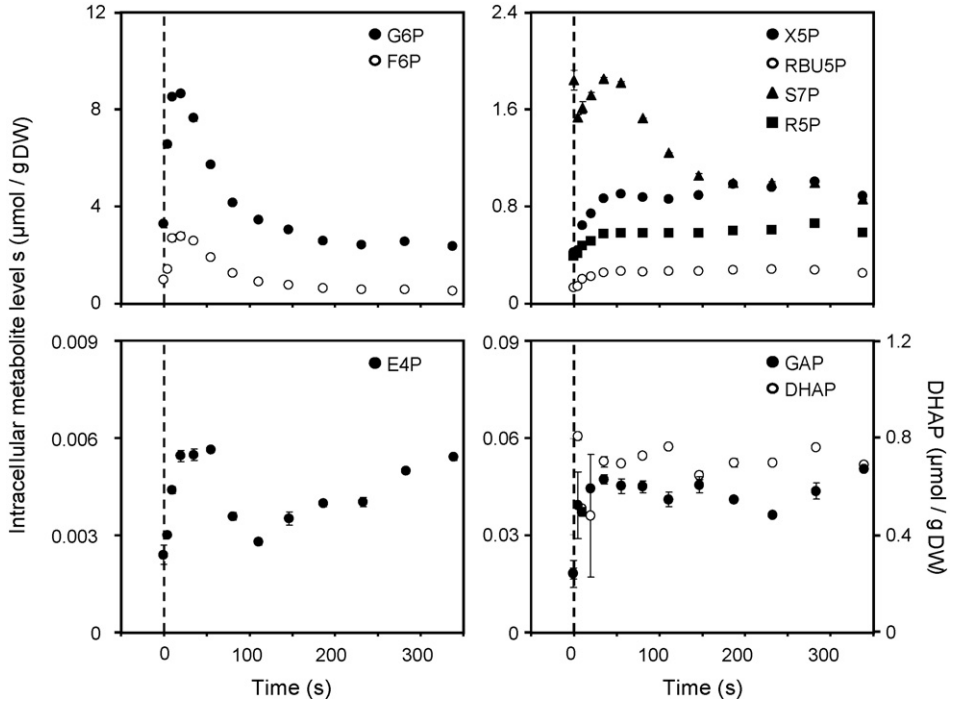

Fig. 3. Short-term dynamic profiles of intracellular levels of non-oxidative PPP intermediates in *S. cerevisiae* cell extracts following a glucose pulse. Legends are included within each panel. The reported error bars derive from duplicate measurements.

indeed close to the TPI equilibrium constant both at steady state and throughout the pulse further supporting the validity of the results (Table 2 and Fig. 3).

3.4. Comparison of results with data obtained using LC-MS/MS

Three of the metabolites measured with the present GC–IDMS method, G6P, S7P and F6P, were also measurable with our routine LC-MS/MS method [26]. As a cross-validation, these metabolites were measured in 30 independent yeast samples using both platforms. The difference between the two methods averaged 2.8% for G6P, 5.4% for S7P and 16.8% for F6P.

4. Conclusions

A GC–IDMS procedure has been developed for the comprehensive quantitative measurement of the non-oxidative PPP metabolites. This method presented superior chromatographic performances and higher sensitivity for the analysis of non-oxidative PPP intermediates compared to current available procedures [6–10]. The subset of analyzed metabolites presented significant analytical challenges due to the low abundance and the presence of isomers and epimers (the latter giving identical fragmentation pattern) which are difficult to separate by chromatography. Furthermore, the high interconversion rates typical of the non-oxidative PPP reactions make the sampling/processing procedure a critical point affecting the overall quality of the data when analyzing biological samples. At the best of our knowledge, this is the first report in which all these issues have been successfully addressed. The choice of a short (10 m) mid-polar capillary column, as reported in the present work, resulted in improved chromatographic performances compared to previous works in which a long (30 m) non-polar capillary column was used [9,10]. Here we showed perfect separation between the isomers GAP and DHAP, perfect separation between the first and second eluting peak of RBU5P and X5P and co-elution between RBU5P and X5P which allowed the calculation of the concentration of the two metabolites. The quality of the data was not affected when switching from standard mixtures to biological samples. This was achieved through the use of well-defined culture conditions and sample treatment procedures. In particular, the application of ^{13}C-labeled internal standards according to the IDMS procedure allowed to correct for matrix effects (which strongly affect derivatization and separation of metabolites), reduce errors due to sample preparation and discrimination of less volatile derivatives during injection. As such, the IDMS procedure eliminated the need to perform recovery check and controls for derivatization efficiency and resulted in improved reproducibility compared to previous works [9,10]. The high sensitivity allowed us to successfully measure intracellular metabolites usually present at very low concentration in biological samples, such as E4P and GAP. In the case of three metabolites which could also be analyzed with an LC-MS/MS-based method, G6P, S7P and F6P, excellent agreement was found between the two techniques indicating that the absolute concentration values obtained with this new method are reliable. Finally, preliminary data suggest that the method could be further developed to include an extended range of metabolites, such as sugars and organic acids.

Acknowledgements

This project is financially supported by the Netherlands Ministry of Economic Affairs and the B-Basic partner organizations (www.b-basic.nl) through B-Basic, a public–private NWO-ACTS programme (ACTS = Advanced Chemical Technologies for Sustainability).

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