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Research Article

Sample preparation workflow for the liquid chromatography tandem mass spectrometry based analysis of nicotinamide adenine dinucleotide phosphate cofactors in yeast†

The accurate quantification of the highly unstable intracellular cofactor nicotinamide adenine dinucleotide phosphate in its oxidized and reduced forms demands a thorough evaluation of the analytical workflow and dedicated methods reflecting their solution chemistry as well as the biological importance of their ratio. In this work, we present a workflow for the analysis of intracellular levels of oxidized and reduced nicotinamide adenine dinucleotide phosphate in the yeast *Pichia pastoris*, including hot aqueous extraction, chromatographic separation in reversed-phase conditions employing a 100% wettable stationary phase, and subsequent tandem mass spectrometric analysis. A thorough evaluation and optimization of the sample preparation procedure resulted in excellent biological repeatabilities (on average $<10\%$, $N = 3$) without employing an internal standardization approach. As a consequence, the methodology proved to be appropriate for the relative assessment of intracellular levels of oxidized and reduced nicotinamide adenine dinucleotide phosphate in different *P. pastoris* strains. The ratio of reduced versus oxidized nicotinamide adenine dinucleotide phosphate was significantly higher in an engineered strain overexpressing glucose-6-phosphate dehydrogenase than in the corresponding wildtype strain. Interestingly, a difference was also observed in the nicotinamide adenine dinucleotide phosphate pool size, which was significantly higher in the wildtype than in the modified strain.

Keywords: Metabolite extraction / Nicotinamide adenine dinucleotide phosphate / Reduction charge / Reversed-phase chromatography
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1 Introduction

Nicotinamide adenine dinucleotide phosphate (NADP) is an important intracellular cofactor. The oxidized form $NADP^+$ acts as a precursor for messenger molecules in some organisms [1], whereas the reduced form NADPH provides reducing power to intracellular antioxidant systems [1–4] and transfers reduction equivalents from substrates to anabolism [5,6]. Moreover, there is evidence that NADPH plays a role in processes associated with the metabolic burden that decreases biomass yield and thus the overall product yield in high-level production of heterologous protein [7]. Despite the biological importance of NADP, its accurate determination suffers from the sensitivity of NADPH in a variety of conditions that are usually applied in sample preparation for metabolomics [8,9]. The development of a suitable analytical workflow must therefore include an elaborate study of analyte stability with a major focus on the sample preparation procedure, which was shown to be the major contributor to overall measurement uncertainty [10]. In particular, low pH and elevated temperatures must be avoided when the reduced NADPH is to be extracted from biological samples [8, 9]. The oxidized form $NADP^+$ on the other hand is said to be more stable than NADPH, even at low pH [11]. Another factor that further limits the available options for metabolite extraction is the susceptibility of the organism of interest to cell lysis. Depending on the cell wall characteristics of an organism, more or less harsh conditions have to be applied in order to release the cellular content into the extraction solvent. Among the most widely used extraction procedures for a number of organisms including yeast is boiling in ethanol [12, 13]. Alternatives are methods using cold methanol and/or chloroform in combination with cycles of freeze–thawing or vigorous shaking for metabolite extraction [12, 14, 15]. According to Canelas

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Abbreviations: **AMP**, adenosine monophosphate; **G6PDH**, glucose-6-Phosphate dehydrogenase; **MRM**, multiple reaction monitoring; **$NADP^+$, NADPox**, nicotinamide adenine dinucleotide phosphate, oxidized; **NADPH, NADPred**, nicotinamide adenine dinucleotide phosphate, reduced; **PGC**, porous graphitic carbon chromatography; ***P. pastoris***, *Pichia pastoris*

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et al., near-complete extraction of a number of metabolites with good reproducibility was possible using boiling ethanol, hot water, and cold methanol extraction [12]. Maharjan and Ferenci did a similar study on extraction techniques for *Escherichia coli*, which found extraction with cold methanol at −40°C most promising for the extraction and analysis of a wide range of metabolites [16].

The solution chemistry of NADP suggests the use of an alkaline extraction solvent for NADPH and an acidic solvent for $NADP^+$. Although this approach is used in some protocols [2, 17, 18], Maharjan and Ferenci found that nucleotides may adsorb to the precipitating salts that are formed during the neutralization step after extraction [16] and would therefore be lost during the subsequent sample preparation steps. Ogasawara et al. proposed that a heating step is necessary in an extraction protocol for NADPH, as some of it is protein-bound and is only released in this step [4]. Gonzalez et al. assessed that NADH, which is similarly unstable as NADPH, is stable in boiling ethanol extracts as long as the extraction solvent is buffered to pH 7.5 [13]. The fact that there are large discrepancies between reported intracellular $NADP^+$ and NADPH levels [2, 3, 6, 17, 19–22] indicates that there is still a need for further investigation and discussion of this analytical problem.

The redox couple $NADP^+$ and NADPH is generally considered a challenging target for quantitative analysis in literature. Although various enzymatic assays are commercially available as ready-to-use kits, these are reportedly sensitive to changes in the sample matrix and exhibit poor reproducibility [4] since usually they do not offer simultaneous analysis of both $NADP^+$ and NADPH within one assay. Several LC–MS methods have been published in the context of comprehensive metabolomics studies where $NADP^+$ and NADPH were among the target analytes. These methods employed ion-pairing chromatography [3, 19, 21–24], HILIC [8] and porous graphitic carbon chromatography [20]. However, such typical analytical methods for metabolomics represent a trade-off between broad coverage and optimum conditions for a given metabolite. Only recently, a dedicated LC–MS/MS method focusing on a number of intracellular coenzymes, including $NADP^+$ and NADPH, was published [22]. These highly unstable cofactors require a thorough evaluation and dedicated methods that reflect their solution chemistry and the biological importance of their ratio, where small artefactual shifts lead to a very different biological interpretation. To date, a systematic study on metabolite extraction, sample preparation, and LC–MS analysis strictly focusing on $NADP^+$ and NADPH has not been performed.

In this work, we present an analytical workflow for the quantification of $NADP^+$ and NADPH in yeast cell extracts. The chromatographic method development included different stationary phases and was closely linked to the evaluation of potential sample preparation procedures that avoid critical conditions for the two redox cofactors. The fact that isotopically enriched $NADP^+$ and NADPH standards were not available, and hence state-of-the-art isotope dilution strategies could not be implemented, was challenging. Therefore, the major aim was to develop an analytical workflow where all sample preparations steps could be carried out with highest possible precision and recovery while preserving the redox state of both analytes. In this way, a robust and reproducible determination of these important intracellular cofactors from a complex biological matrix was enabled.

2 Materials and methods

2.1 Standards and materials

$NADP^+$ disodium salt, NADPH tetrasodium salt hydrate, digitonin, LC–MS grade acetonitrile, water, formic acid, and ammonium acetate were purchased from Sigma–Aldrich. LC–MS grade methanol and ethanol absolute were purchased from Fisher Scientific.

2.2 *P. pastoris* fed batch culture

Pichia pastoris wildtype X-33 was cultivated in minimal medium using a 1 L benchtop bioreactor (DASGIP Parallel Bioreactor System, Germany). The system was operated in fed batch at 25°C, pH 5, and 20% pO_2. After inoculation at an optical density (OD, at 600 nm) of 0.2, the system was operated in batch with a working volume of 320 mL for 35 h. Samples were drawn after 2 h of exponential growth in fed batch. The cultivation broth was sampled directly into a fourfold volume of cold quenching solvent (60% v/v methanol). The quenched cell suspension was kept at -30 ± 3°C, aliquoted, and centrifuged (4000 *g*, −20°C, 10 min in a Sorvall RC 6+ centrifuge, Thermo Scientific). The supernatant was discarded and the pellet washed twice by resuspension in fresh quenching solution and centrifugation. Finally, the cell pellets were stored at −80°C until extraction.

2.3 *P. pastoris* batch culture

P. pastoris wildtype X-33 and a modified *P. pastoris* strain ZWF1 overexpressing glucose-6-phosphate dehydrogenase were grown in parallel batch cultures in shake flasks at 25°C, pH 5.7, and 170 rpm. Precultures of both strains were inoculated from YPD agar plates, grown for 24 h in 5 mL YPD medium, and used for inoculation of the main culture at OD 0.2 in 10 mL minimal medium. After 24 h, 1 mL aliquots were collected by pipetting, immediately pelleted for 2 min at 13 000 *g* in a precooled table-top centrifuge (Heraeus Biofuge Pico), put on dry ice, and immediately used for metabolite extraction. Simultaneously, OD as a measure of the biomass content was determined using a WPA CO8000 Cell Densitymeter.

2.4 Metabolite extraction

$^{15}N_5$-5′-Adenosine monophosphate (AMP) was added to each sample prior to extraction and used as a monitoring tool.

2.4.1 Freeze–thawing extraction

This protocol was modified from Ref.[25]. The cell pellet was resuspended in 2.5 mL of pre-cooled extraction solvent (60% v/v methanol, 40% v/v H_2O with 0.1% w/v digitonin), frozen in liquid nitrogen for 3 min, and thawed in a nonthermostatted ultrasonic bath for 15 min. After centrifugation (10 min at 4000 *g*), the supernatant was dried under reduced pressure in a Thermo Savant SPD121P SpeedVac Concentrator. The dried extract was reconstituted in 1 mL of 5 mM ammonium acetate pH 8.0 prior to analysis.

2.4.2 Boiling ethanol extraction

This protocol was adapted from Ref. [26]. The pellet was resuspended in 4 mL of preheated 75% v/v ethanol, incubated at 85°C for 3 min with intermediate mixing, cooled on dry ice, and centrifuged for 10 min at 4000 *g*. The supernatant was dried under reduced pressure in a Thermo Savant SPD121P SpeedVac Concentrator. The dried extract was reconstituted in 1 mL of 5 mM ammonium acetate pH 8.0 prior to analysis.

2.4.3 Hot aqueous extraction

This protocol was adapted from Ref.[12]. The cell pellet was resuspended in 900 μL of preheated 5 mM ammonium acetate pH 8.0, incubated at 85°C for 3 min with intermediate mixing, cooled on dry ice, and centrifuged for 10 min at 4000 *g*. The supernatant was transferred to HPLC vials for analysis.

2.4.4 Mechanical cell lysis with glass beads

The cell pellet was resuspended in 2 mL of 5 mM ammonium acetate buffer pH 8.0. The sample was split and transferred into two tubes containing 500 mg glass beads and subjected to two homogenizing cycles of 20 s at 6.5 m/s in a FastPrep-24 from MP Biomedicals. In between, the samples were cooled on ice. After centrifugation (10 min, 4000 *g*), the supernatant was transferred to HPLC vials for analysis.

2.5 Standard recovery study

The standard recovery was determined for all intermediate steps of the tested extraction procedures. The standards were prepared as mixtures in the respective extraction solvent ($N = 3$) containing 5 μM of $NADP^+$ and NADPH, respectively. All samples were analyzed by flow injection (85% v/v 5 mM ammonium acetate pH 6.0, 15% v/v methanol) in a 2 min run at 250 $\mu L/min^{-1}$ in negative MRM mode on a Thermo TSQ Vantage triple quadrupole mass spectrometer.

2.6 LC–MS analysis

An Agilent 6220 LC-TOFMS system was used for initial method development and the standard stability study. All measurements were performed using ESI in negative ion mode, with 4000 V capillary voltage, 350°C gas temperature, 10 L/min^{-1} drying gas flow, 25 psig nebulizer pressure, 180 V fragmentor voltage, and 60 V skimmer voltage. Spectra were acquired in the mass range of 100–1000 m/z. Extracted ion chromatograms (EIC) were obtained from total ion chromatograms (TIC) by extraction of the exact masses (742.0682 m/z for $NADP^+$, 744.0838 m/z for NADPH, and 351.0418 m/z for $^{15}N_5$–5′-AMP) with an extraction window of ± 0.005 m/z.

A Thermo TSQ Vantage triple quadrupole mass spectrometer with an Accela 1250 HPLC system was used for LC–MS/MS analysis. ESI was performed in negative ion mode with 300°C capillary temperature, 3300 V spray voltage, 40 psig sheath gas pressure, and 10 psig auxiliary gas pressure. The transitions monitored in MRM mode (collision energies in brackets) were 742.0 m/z > 408.0 m/z (32 V) and 620.0 m/z (18 V) for $NADP^+$, 744.0 m/z > 159.0 m/z (41 V) and 426.0 m/z (32 V) for NADPH, and 351.0 m/z > 79.1 m/z (41 V) and 139.1 m/z (32 V) for $^{15}N_5$-5′-AMP (used as monitoring tool).

2.6.1 Hydrophilic interaction liquid chromatography

A bridged ethylene hybrid particle (BEH) based HILIC column (Xbridge Amide, 2.1 × 150 mM, 3.5 μM, Waters) was tested with 5 mM ammonium acetate pH 6.0 (A) and acetonitrile (B). The column was kept at 40°C and run with a flow rate of 250 μL/min. The injection volume was 5 μL. The gradient start conditions were 85% B, followed by a decrease to 55% in 4 min and reequilibration within a total run time of 15 min.

2.6.2 Porous graphitic carbon chromatography

A PGC-packed column (Hypercarb, 3 × 50 mm, 5 μm, Thermo Scientific) was tested in several conditions. The mobile phase was mixed from either 50 mM ammonium formate pH 9.0, 20 mM ammonium formate pH 6.0, or 20 mM ammonium acetate pH 9.0 (A) and either acetonitrile or methanol (B). The flow rate was set to 500 μL/min and the column heated to 40°C. The injection volume was 5 μL. Gradient conditions were either from 90 to 50% B or from 2 to 15% B followed by column reequilibration.

2.6.3 Reversed-phase chromatography

A silica-based column with C_{18} ligands (Atlantis T3, 2.1 × 150 mm, 3 μm, Waters) was employed for reversed-phase separation. The flow rate was set to 250 μL/min and the column thermostatted at 40°C. The injection volume was 5 μL. The chromatographic run started at 100% A (5 mM ammonium acetate pH 6.0), maintained for 2 min, followed by a gradient from 0 to 17% B (100% methanol) in 3.5 min. With a washing step at 90% B and column equilibration time, the overall run time was 12 min.

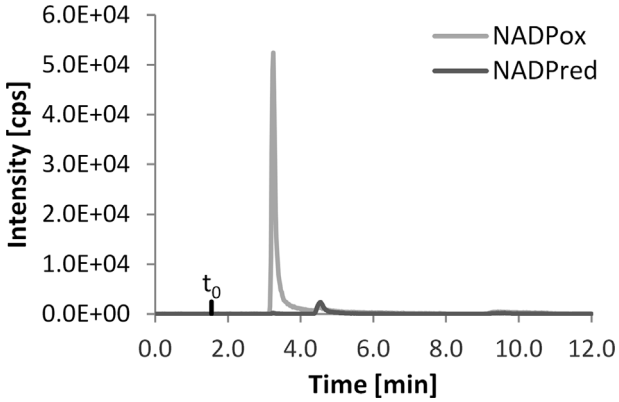

Figure 1. Optimized LC–MS/MS analysis of a mixture of $NADP^+$ and NADPH (5 μM each) using a Thermo TSQ Vantage system in negative MRM mode, precursor ions: 742.0 *m/z* ($NADP^+$) and 744.0 (NADPH), product ions (collision energy in brackets): 408.0 *m/z* (32 V) and 620.0 *m/z* (18 V) for $NADP^+$; 159.0 *m/z* (41 V) and 426 *m/z* (32 V) for NADPH.

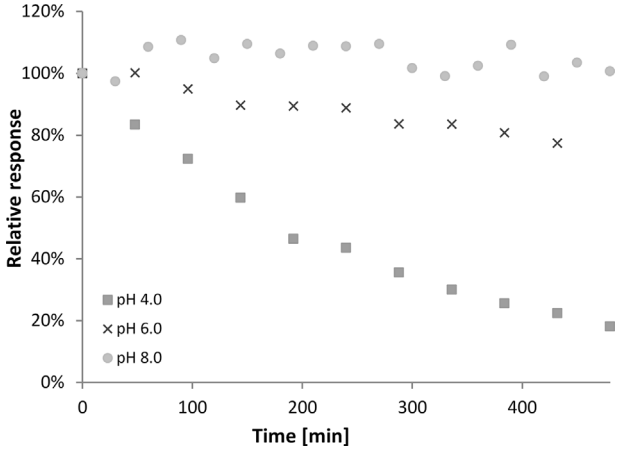

Figure 2. Stability of NADPH (5 μM) in 5 mM ammonium acetate pH 4.0, 6.0, or 8.0 during 8 h at 6°C. Low pH negatively affects NADPH stability, whereas NADPH is stable at pH 8.0.

3 Results and discussion

3.1 Chromatographic separation

The chromatographic separation of $NADP^+$ and NADPH was successfully achieved on a 100% wettable reversed-phase stationary phase (Atlantis T3, Waters) within a total run time of 12 min. As can be seen in Fig. 1, an excellent chromatographic resolution (calculated based on the full peak width at half-maximal height, FWHM) of 4.9 was obtained. LC–TOF-MS was used for initial method development and the standard stability study, whereas LC–MS/MS was used for the detection and quantification of $NADP^+$ and NADPH in yeast samples. The corresponding analytical figures of merit are given in Table 1. As a drawback, it was observed that even less than 10% of organic solvent in the sample led to a near-complete loss of retention on the reversed-phase stationary phase, which was a limitation for the selection of the extraction procedure. HILIC was tested as an alternative chromatographic separation, being highly compatible with organic solvents. In fact, the tested BEH-based stationary phase (Xbridge Amide, Waters) allowed a separation of $NADP^+$ and NADPH, even though the sample solvent had to match the gradient start conditions to avoid peak splitting. However, using this stationary phase it was observed that NADPH was oxidized to a large extent (approximately 80%). Hence, this method was not further considered. Other HILIC phases (silica-based phases) showed poor retention and peak broadening. Finally, porous graphitic carbon (PGC) was tested. However, in accordance with previous reports [20], high ionic strength (at least 50 mM ammonium formate pH 9.0) in the mobile phase was necessary to reduce the strong retention of these very polar compounds, thus severely compromising the robustness of this LC–MS approach. Accordingly, the PGC was excluded from further evaluation.

Table 1. Analytical figures of merit for the RPLC-MS analysis of $NADP^+$ and NADPH in a 5 μM standard mixture on two different LC–MS instruments, an Agilent 6220 LC-TOFMS system and a Thermo TSQ Vantage LC–MS/MS system.

	LC–TOF-MS		LC–MS/MS	
	$NADP^+$	NADPH	$NADP^+$	NADPH
Retention time [min]	5.2	5.7	3.2	4.5
FWHM [s]	9	5	7	12
Peak area RSD ($N = 5$ inj.)	6.0%	7.0%	3.4%	4.5%
LOD [pmol]	0.80	1.2	0.04	0.15
Resolution (FWHM concept)	2.5		4.9	

3.2 Standard stability

As NADPH is known to be unstable in solution in a variety of conditions, a separate standard stability study was included in this work. Repeated LC–TOF-MS measurement of standard solutions of $NADP^+$ and NADPH in 5 mM ammonium acetate adjusted to pH 4.0, 6.0, or 8.0 kept at 6°C confirmed literature reports [8, 9, 11], i.e. the NADPH signal decreased during storage at pH 4.0 and 6.0, with less than 20% of the original signal still observable after 8 h at pH 4.0 (Fig. 2). At pH 8.0 on the other hand, NADPH degradation was insignificant with a recovery of 104 (± 4)% within 17 injections during 8 h. $NADP^+$ was stable in all conditions with recoveries of 97 (± 5)% at pH 4.0, 103 (± 4)% at pH 6.0, and 95 (± 6)% in 17 injections during 8 h. Thus, at pH 8.0 both $NADP^+$ and NADPH exhibited RSDs below the typically observed measurement repeatability ($N = 5$, Table 1) during this measurement, therefore this solvent was used for all further standard and sample preparation.

3.3 Metabolite extraction

The dominant challenge in the development of a suitable sample preparation workflow for $NADP^+$ and NADPH was the lack of isotopically enriched internal standards. Although $U^{13}C$ cell extracts are widely used as internal standards in metabolomics since they contain the whole metabolome fully ^{13}C-labeled, we found only low levels of NADPH in such a cell extract. The major criterion for a suitable sample preparation workflow in this work was therefore that it ensures the stability of both analytes and preserves their redox state.

Representatives of all types of extraction procedures typically used in metabolomics were evaluated in terms of their applicability for the extraction of $NADP^+$ and NADPH from yeast cells and subsequent LC–MS analysis. Separate studies were performed with the aim of (1) assessing the recovery of $NADP^+$ and NADPH during all steps of the sample preparation protocol, and (2) evaluating the extraction efficiency, i.e. the ability of a certain protocol to give access to the entire intracellular NADP pool. For the standard recovery study, standard solutions of $NADP^+$ and NADPH were prepared for each step of the procedure separately, treated accordingly and analyzed by flow injection on a Thermo TSQ Vantage LC–MS/MS system. The obtained signal responses were normalized to the response in the untreated sample for each protocol. The extraction efficiency was evaluated using a homogenous set of cell pellets with identical biomass content from a *P. pastoris* fed batch culture. Subsets of this sample pool were extracted employing the different extraction methods ($N = 3$) and analyzed via RPLC–MS/MS. The NADP pool size, i.e. the sum of $NADP^+$ and NADPH, was calculated for each sample and normalized to the biomass content. In order to account for instrumental drifts and volume losses, isotopically enriched $^{15}N_5$-5′-AMP was added to all samples prior to extraction and used as monitoring tool. Only LC–MS/MS measurements showing a RSD <10% for the AMP signal were considered valid.

As an example for cold extraction, a freeze–thawing protocol in buffered 60% methanol with digitonin was tested. In the standard recovery study (Figs. 3 and 4), only minor losses of $NADP^+$ were observed after freezing and thawing. However, extensive losses were observed for both $NADP^+$ and NADPH after drying the extracts under reduced pressure, resulting in recoveries of 65.2 (± 0.2)% for $NADP^+$ and 49.8 (± 1.3)% for NADPH. Moreover, the extraction efficiency was very low for this procedure. Even though digitonin, a mild nonionic detergent that permeabilizes the cell membrane, was added to the extraction solvent, it seems that the strong *Pichia* cell wall was left intact during this treatment. In fact, freeze–thawing is more frequently applied for organisms where cell lysis is more readily achieved than for yeast cells. In this work, freeze–thawing was therefore not further considered.

Quite divergent behavior of $NADP^+$ and NADPH was observed during glass bead-assisted mechanical cell lysis in aqueous solution. After the first homogenization cycle, 80.8 (± 4.4)% of $NADP^+$ but only 49.1 (± 3.4)% of NADPH could be recovered (Figs. 3 and 4). Critical factors affecting metabolite stability are the friction of the glass beads and the introduction of large amounts of air during mixing, increasing the risk for unwanted oxidation. In general, this procedure does not allow a tight control of the sample temperature, which is especially important for unstable metabolites like $NADP^+$ and NADPH. These critical factors were also evident in the extraction efficiency study, where the overall extraction yield was low despite the very efficient cell lysis. Moreover, this procedure does not ensure the complete inactivation of all

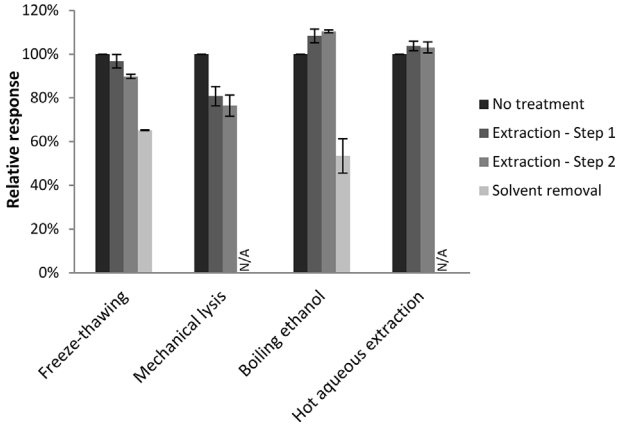

Figure 3. Standard recovery of $NADP^+$ during different extraction procedures. Each bar corresponds to the recovery of a 5 μM standard solution relative to an untreated 5 μM standard solution ($N = 3$). Steps 1 and 2 refer to the actual extraction steps, i.e. freezing and subsequent thawing at 21°C or by ultrasonication for the freeze–thawing extraction, 20 and 40 s homogenization for mechanical lysis and incubation at 85°C for 1.5 and 3 min for the hot extraction procedures. Solvent removal by drying under reduced pressure was not necessary for all procedures.

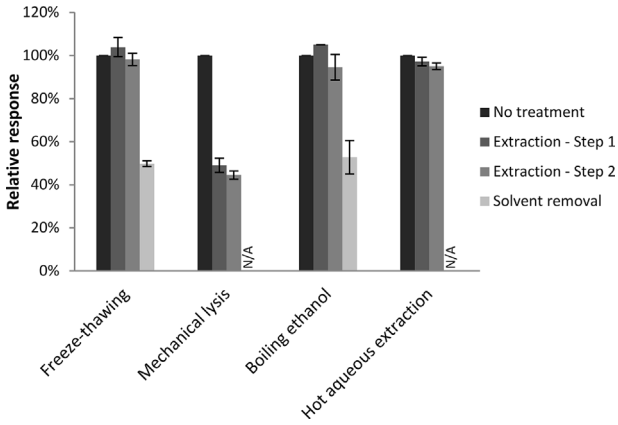

Figure 4. Standard recovery of NADPH during different extraction procedures. Each bar corresponds to the recovery of a 5 μM standard solution relative to an untreated 5 μM standard solution ($N = 3$). Steps 1 and 2 refer to the actual extraction steps, i.e. freezing and subsequent thawing at 21°C or by ultrasonication for the freeze–thawing extraction, 20 s and 40 s homogenization for mechanical lysis and incubation at 85°C for 1.5 and 3 min for the hot extraction procedures. Solvent removal by drying under reduced pressure was not necessary for all procedures.

Table 2. Intracellular $NADP^+$ and NADPH levels in two strains of *P. pastoris* (wildtype and G6PDH overexpressing). Three independent cultures of each strain were analyzed, the results were normalized to the biomass content.

		P. pastoris wildtype X-33					modified *P. pastoris* ZWF1				
		1	2	3	Average	SD	1	2	3	Average	SD
Extraction yield [nmol]	$NADP^+$	0.78	0.79	0.79	0.79	<0.01	0.52	0.32	0.43	0.40	0.11
	NADPH	0.14	0.14	0.14	0.14	<0.01	0.29	0.25	0.26	0.27	0.02
NADP pool [nmol]		0.92	0.92	0.93	0.92	<0.01	0.81	0.57	0.60	0.66	0.13
Reduction charge		0.15	0.15	0.15	0.15	<0.01	0.36	0.43	0.43	0.41	0.04

enzymes and was therefore not considered a suitable procedure for the aim of this work.

The two protocols employing hot extraction were very similar in their recovery pattern, i.e. both $NADP^+$ and NADPH were stable during the extraction at 85°C (Figs. 3 and 4). Solvent removal was necessary to make ethanolic cell extracts compatible with the presented chromatographic system, but introduced losses of approximately 50% of $NADP^+$ and NADPH, respectively. Moreover, this step also prolongs sample preparation by several hours for a typical extraction volume of 2 mL. In terms of extraction efficiency, hot extraction yielded the highest extractable NADP levels within this study while exhibiting excellent repeatability <10%. Remarkably, the overall yield of the boiling ethanol procedure was higher than for the otherwise studied methods, even though a solvent removal step had to be included in this specific protocol. Boiling ethanol extraction is indeed well established and widely used for the extraction of intracellular metabolites from yeast and other organisms. The benefits of this method are effective cell lysis within a relatively fast and simple procedure as well as the inactivation of enzymes and a low protein content in the extracts. When 5 mM ammonium acetate pH 8.0 is used as extraction solvent, the extracts can be used for RPLC–MS/MS analysis directly after extraction. The resulting hot aqueous extraction procedure is simple, fast, and reproducible and was therefore the method of choice for the purpose of this work.

3.4 Proof of principle—measuring relative differences between yeast strains

In order to test the ability of the presented method to reflect intracellular NADP levels, a modified *P. pastoris* ZWF1 strain overexpressing glucose-6-phosphate dehydrogenase (G6PDH) was compared with *P. pastoris* wildtype. G6PDH catalyzes the reduction of $NADP^+$ to NADPH in pentose phosphate pathway, so that its overexpression should lead to increased intracellular NADPH levels. In fact, the ZWF1 strain showed a significantly higher ratio of NADPH over $NADP^+$ than the wildtype, indicating a shift of the NADP pool toward the reduced form (Table 2). As a result, the anabolic reduction charge, i.e. the fraction of NADPH in the NADP pool, was 0.15 (± <0.01) for the wildtype and 0.41 (± 0.04) for the overexpressing strain. Interestingly, also the NADP pool size itself was different between the two strains, with the wildtype showing 1.4-fold higher total extractable NADP levels (Table 2). As the two strains also showed differences in growth rate, the difference in NADP pool size is most probably an effect of the metabolic burden associated with the increased production of G6PDH. Although excellent repeatability precisions ($N = 3$) of 0.7% for NADPH and 0.4% for $NADP^+$ were observed for the wildtype strain, the variability was higher for the modified strain, where repeatability precisions of 8.4% for NADPH and 27% for $NADP^+$ were observed. At this point, it should be mentioned that OD was used to compensate for the apparently different growth rates of the two investigated strains in this study. Indeed, for the wild type strain the excellent biological repeatability of cell growth resulted in repeatability precisions of 0.7 and 0.4% for $NADP^+$ and NADPH determinations, respectively. However, in the case of the genetically modified strain, this approach showed shortcomings resulting in poor repeatability of NADP determinations. In this case, an accurate biomass determination would significantly improve the biological repeatability of the determined NADP levels. Given these considerations and the inherently high variability of complex biological samples, the observed repeatability precisions are excellent, especially since internal standardization could not be applied to correct for fluctuations and errors introduced during sample preparation.

4 Concluding remarks

Typical LC–MS methods in metabolomics target not only one metabolite but at least several intermediates of certain metabolic pathways simultaneously. Although it would be desirable to include $NADP^+$ and NADPH into such methods, to our experience this is hardly possible because these two analytes require special attention in all steps of the sample preparation and analysis workflow. The specific solution chemistry of $NADP^+$ and NADPH and the biological importance of their ratio must be considered in the development of a dedicated method since small artefactual shifts in the intracellular levels lead to a very different biological interpretation. Although this workflow has been evaluated here thoroughly and was shown to be appropriate for relative quantification of

intracellular $NADP^+$ and NADPH, future work will have to focus on the implementation of an isotope dilution strategy before absolute quantification of these important intracellular metabolites can be attempted.

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The authors have declared no conflict of interest.

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