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Master thesis

Quantitative Analysis of $NADP^+$ and NADPH in Yeast

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Abstract

Nicotinamide adenine dinucleotide phosphate (NADP) is a coenzyme and redox agent involved in many anabolic reactions in all cell types and organisms. The ratio of the reduced form (NADPH) and the oxidized form ($NADP^+$) contributes to the overall redox state of the cell. There are currently only estimates of the intracellular levels of these compounds since the accurate determination is hindered by methodological problems. The instability of NADPH under various conditions restricts the available options for metabolite extraction from the cell. In this work, a variety of cold, hot and mechanical extraction protocols was evaluated concerning their ability to give access to the intracellular NADP pool of the target organism of this work, the yeast *Pichia pastoris* as well as the stability of $NADP^+$ and NADPH in the extraction conditions. Different separation mechanisms and columns were tested to develop an analytical method for the chromatographic separation of NADPH and $NADP^+$ from cell extracts. Time-of-flight and triple quadrupole mass spectrometry were used for detection. The overall analytical workflow established in this work includes sample preparation using aqueous hot extraction at pH 8.0, chromatographic separation in reversed-phase conditions at pH 6.0 within a total run time of 12 min with mass spectrometric detection using electrospray ionization and a triple quadrupole mass analyzer. It was shown that this procedure delivers data fit for the evaluation of intracellular NADP levels in yeast.

Kurzfassung

Nicotinamid-adenin-dinukleotid-phosphat (NADP) ist ein wichtiger Redoxkofaktor im Anabolismus aller Zellen. Das Verhältnis der reduzierten Form (NADPH) und der oxidierten Form ($NADP^+$) trägt wesentlich zum Redoxstatus der Zelle bei. Die genaue Bestimmung der intrazellulären Konzentration von $NADP^+$ und NADPH ist allerdings aufgrund von methodischen Komplikationen schwierig. Insbesondere die Instabilität von NADPH stellt vor allem bei der Extraktion der Analyten aus dem Zielorganismus Pichia pastoris ein großes Hindernis dar. Im Rahmen dieser Arbeit wurden mehrere kalte, heiße und mechanische Extraktionsmethoden bezüglich der Ausbeute und der Stabilität der Analyten in den Extraktionsbedingungen getestet. Zur chromatographischen Trennung der beiden Substanzen wurden mehrere Trennmechanismen mit massenspektrometrischer Detektion getestet. Der im Rahmen dieser Arbeit etablierte analytische Prozess gliedert sich in die Schritte (1) Probenvorbereitung mittels heißer wässriger Extraktion bei pH 8,0, (2) chromatographische Trennung in Umkehrphasenbedingungen bei pH 6.0 innerhalb einer Gesamtlaufzeit von 12 min sowie (3) Elektrospray-Ionisierung und Detektion mittels Triple-Quadrupol-Massenspektrometrie. Die Daten aus dieser Analyse sind dafür geeignet, Aussagen über den intrazellulären NADP-Gehalt von Hefen zu treffen.

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LIST OF ABBREVIATIONS

ADP … adenosine diphosphate

AMP … adenosine monophosphate

ATP … adenosine triphosphate

cps … counts per second

EDTA … ethylene diamine tetraacetic acid

ESI … electrospray ionzation

GSH … glutathione

GSSG … glutathione disulfide, formed by two oxidized glutathione molecules

HILIC … hydrophilic interaction liquid chromatography

HPLC … high-pressure liquid chromatography

LC … liquid chromatography

LC-MS … liquid chromatography coupled to mass spectrometry

LC-MS/MS … liquid chromatography coupled to tandem MS analysis with 2 mass analyzers

LOD … limit of detection

LOQ … limit of quantification

MS … mass spectrometry

NAD … nicotinamide adenine dinucleotide

NAD^+ … oxidized form of nicotinamide adenine dinucleotide

NADH … reduced form of nicotinamide adenine dinucleotide

NADP … Nicotinamide adenine dinucleotide phoshphate

$NADP^+$ … oxidized form of nicotinamide adenine dinucleotide phosphate

NADPH … reduced form of nicotinamide adenine dinucleotide phosphate

OD … optical density

PGC … porous graphitic carbon chromatography

QQQ … triple quadrupole mass analyzer

RPC … reversed-phase chromatography

RSD … relative standard deviation

SRM … selected reaction monitoring

TOF …time-of-flight mass analyzer

$U^{13}C$- … prefix for uniformely ^{13}C labeled compounds

UV … ultraviolet light

1 OBJECTIVE

The yeast *Pichia pastoris* is a popular host for recombinant protein production. It is more easily cultivated and genetically modified than mammalian cells and can be grown to high cell densities. Prokaryotic organisms share these general features, but *Pichia pastoris* as eukaryotic organism has the additional potential to produce high concentrations of soluble, correctly folded and posttranslationally modified proteins. A thorough understanding of the protein biosynthesis and related pathways in the cell is a prerequisite for the optimization efforts that are still ongoing for this cell factory.

The pentose phosphate pathway is among the most important pathways in anabolism. It provides reducing equivalents in the form of NADPH, that is required for reductive biosynthesis and is crucial in the prevention of oxidative stress in the cell. NADPH and its oxidized form $NADP^+$ are an important redox couple in the cell and are involved in many intracellular reactions. There is evidence that such redox processes contribute to the metabolic burden, that decreases the biomass yield and thus the overall yield in high-level production of heterologous protein. Knowledge about the redox state of a cell and its regulation could provide valuable information for the optimization of recombinant protein production in yeast.

The aim of this work is to establish an analytical workflow for the quantitative determination of $NADP^+$ and NADPH in yeast. This includes a strategy for metabolite extraction and sample preparation as well as the development of a method for chromatographic separation with mass spectrometric detection and analysis of $NADP^+$ and NADPH from cell extracts of *Pichia pastoris*.

2 INTRODUCTION

2.1 Physiological role of NADP

Nicotinamide adenine dinucleotide phosphate (NADP) is a coenzyme and redox agent involved in many anabolic reactions in the cell. It occurs in all cell types both in its oxidised ($NADP^+$, see Figure 1a) and its reduced form (NADPH, see Figure 1b). Except for an additional 2'-phosphate group attached to the adenosine ribose moiety, NADP is structurally identical to NAD (nicotinamide adendine dinucleotide). Both compounds are fully deprotonized in physiological conditions [24] [25].

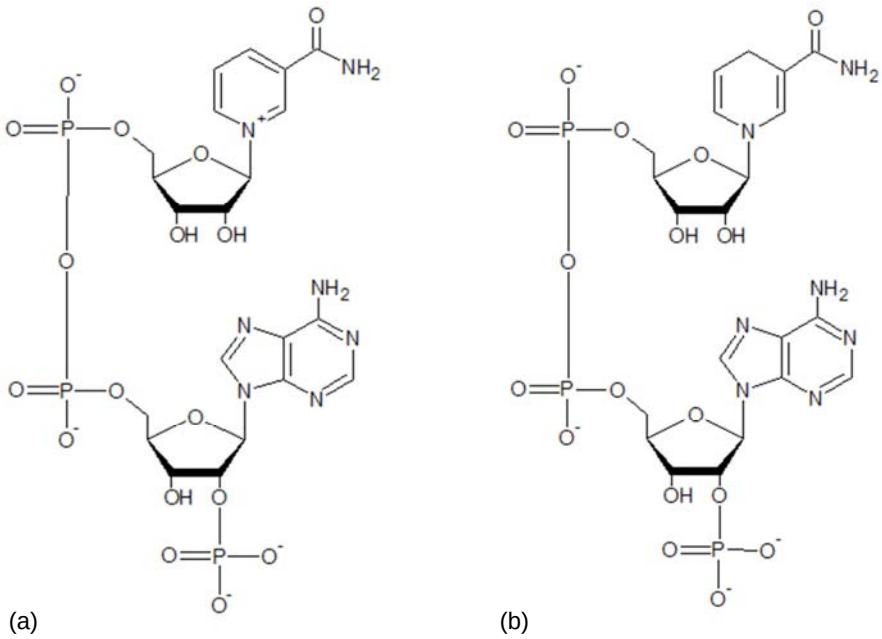

Figure 1: Chemical structures of Nicotinamide adenine dinucleotide phosphate (NADP). (a) The oxidized form, $NADP^+$, (b) the reduced form, NADPH.

Physiologically, the two coenzymes have different functions. While NADP is mainly involved in substrate reduction, NAD is mostly used by enzymes catalyzing substrate oxidation [1]. Moreover, the cell's NAD pool (including NAD^+ and NADH) is much bigger than the NADP pool (including $NADP^+$ and NADPH) in physiological conditions [1]. In contrast to the NAD pool, it is estimated that the reduced form, NADPH, is more abundant than the oxidized $NADP^+$ in the cell [1] [2] [3]. The exact ratio of the two forms and the NADPH consumption of a cell depend on the organism's growth stage and substrate [4] [5]. However, there are currently only estimates of the overall concentration, the resulting redox state of the cell and

whether NADP is predominantly free or protein-bound, since the accurate determination of these parameters is hindered by methodological problems due to the instability of NADP under various conditions [2] [6] [7].

NADPH is an important reducing agent that is involved in many cellular functions, such as the reductive biosynthesis of amino acids, lipids and nucleotides [8]. Another very important function is to provide redox power to antioxidant systems which mediate the cell's response to oxidative stress [2] [9] [10] [3]. In addition, it has been revealed that NADP also plays an important role in cell death [1]. $NADP^+$ on the other hand is involved in many signalling reactions, where it acts as precursor for messenger molecules [2]. For example, the $NADP^+$ derivative NAADP (nicotinic acid adenine dinucleotide phosphate) is currently known as the most potent intracellular Ca^{2+}-mobilizing messenger [2] [11]. However, yeasts do not seem to use these $NADP^+$ conversions in signaling [2].

De novo synthesis of the two forms of NADP is mediated by many different enzymes. For $NADP^+$ generation, there are multiple NADPH-dependent enzymes which catalyze the oxidation of NADPH to $NADP^+$, but also NAD^+ kinases that form $NADP^+$ from NAD^+ [1]. NADPH on the other hand can be formed from $NADP^+$ by multiple different enzymes such as glucose-6-phosphate dehydrogenase and 6-gluconate phosphate dehydrogenase that are involved in the pentose phosphate pathway. Additional sources are reactions involving isocitrate dehydrogenases, malic enzyme and mitochondrial transhydrogenase, which uses NADH and $NADP^+$ for NADPH generation [1] [2]. In addition, there are reports of NADH kinases that catalyze NADH phosphorylation to form NADPH in *Saccharomyces cerevisiae* [11] [12].

Studies suggest that NADP's major role in metabolism is important for biotechnological applications. Alteration of the $NADP^+$/NADPH balance was shown to have an effect on the xylose metabolism in yeasts [13]. This furtherly boosts modeling efforts [14] and targeted strain engineering [15]. However, even more knowledge is required in order to effectively use these assumptions in the optimisation of cell factories. A very useful tool in this task is LC-MS analysis, a modern technique that is widely used in such metabolomic problems [16]. It is very selective, accurate and sensitive, but care must be taken that the sample reflects the true relations as found in the cells and is not falsified during sample preparation [17]. In fact, the choice of protocol for metabolite extraction from the cell is crucial for accurate and reliable metabolite analysis [17] [18] [19] [20]. This is especially critical in the analysis of NADP due to its instability under most of the commonly applied extraction conditions [2] [6] [7].

2.2 Solution chemistry

Perhaps the most challenging aspect of NADP analysis is the stability of the two analytes. In fact, the stability is among the most frequently named sources of error in analysis of NADP [21]. Especially the reduced form is sensitive to a variety of conditions that are usually applied in sample preparation and analysis [6] [7]. In correlation with its biological function as reductive agent, NADPH is sensitive to oxidizing agents such as oxygen, ferricyanide, riboflavin, phenazine and peroxide [21], but other factors have been identified that have a much greater influence on the stability of this compound. Less data is available for the solution chemistry of $NADP^+$, which is in general considered to be much more stable than its reduced form. The general estimation in literature is that $NADP^+$ is, in contrast to NADPH, unstable at high pH and degrades during thermal treatment at 60°C for 30 min [21] [22] [23].

Hofmann *et al.* assessed the thermal stability of NADPH in the solid state and in aqueous solution [7]. This study concluded that NADPH in solution is subject to significant degradation, while it is relatively stable in the dry state. Even incubation at 95°C for 16 h did not induce degradation of solid NADPH and it was even observed that it remains stable for several days at 40-50°C [7]. Aqueous solutions of NADPH however were shown to be much more sensitive to thermal treatment. The aim of this study was to identify the products of the degradation process, which were intensively present already at 50°C. Among them were products of bond cleavage like nicotinamide, ATP, ADP, AMP and their respective dehydration products [7]. However, this study did not give any information about the stability at lower temperatures or the degradation kinetics.

Wu *et al.* did an extensive study on the influence of various factors on the kinetics of NADPH degradation [6]. The findings from this investigation clearly indicate that the pH has the greatest effect on the stability of NADPH in solution. At low pH values, the degradation of NADPH occurs very fast, resulting in half-life times of 2.7 min at pH 3 and 30°C [6]. Between pH 3 and 7.5, the rate constants of the degradation increased according to a linear function. Above pH 7.5, the degradation rate of NADPH was found to be much smaller, indicating a significantly higher stability. In fact, Wu *et al.* observed a half-life time of 517 min for NADPH at pH 7 and 30°C [6]. Moreover, elevated temperatures and high ionic strength of the solution were also found to increase the rate of NADPH degradation. At 41°C, half of the NADPH would be degraded within 1 h at pH 6, while the same process at the same pH takes more than 8 h at 19°C [6]. Additionally, it was shown that acetate and phosphate accelerate the degradation of NADPH. This effect was found to be concentration-dependent and was rather insignificant at concentrations of less than 100 mM of the respective anion [6].

All these stability issues represent a challenge for the sample preparation and analysis of NADPH, including metabolite extraction which has to avoid these conditions but must still be effective. Solutions of NADPH can be stabilized in alkaline conditions, but even the protective effect of pH does not allow long-time storage, as Lowry *et al.* stated [22]. In fact, it was shown in this study that the storage temperature plays a vital role in storage stability. Alkaline solutions of NADPH were stable for one week at 4℃ and -85℃, respectively, but showed a 13% loss when stored at -20℃ for the same time [22]. This may be explained with the different availability of liquid phase at these temperatures. Considering that some impurities are always present and that they mostly catalyze oxidation, they must be more diluted at 4℃ than at -20℃, where the amount of residual liquid phase is small [22]. A higher concentration of these oxidants means an increased rate of degradation and a decreased storage stability. At -85℃, almost no liquid phase is left and the catalysts are therefore not available in solution, which results in a higher stability [22]. For short-term stability however, pH seems to be the determining parameter. Reports say that no loss of NADPH was observed in a 1 h thermal treatment at 100℃ in a 0.1 N NaOH solution [22] [23].

2.3 Analytical challenge

$NADP^+$ and NADPH are both polar compounds with three phosphate groups that are deprotonated in physiological conditions, providing four negative charges. $NADP^+$ and NADPH differ only in the nicotinamide moiety, where the pyridine ring of the nicotinamide can be in the oxidised ($NADP^+$) or reduced (NADPH) state, leading to an additional charge in the $NADP^+$ molecule (see Figure 1). This positive charge compensates one of the negative charges issuing from the deprotonated phosphate groups, so that effectively, $NADP^+$ is three times negatively charged in physiological conditions [24]. In contrast, NADPH is four times negatively charged in physiological conditions [25].

The reduction reaction from NADP+ to NADPH implicates a change in the electron configuration of the nicotinamide moiety that allows both spectrophotometric and spectrofluorimetric detection of NADPH [3] [20]. In the reduced state, the pyridine ring shows absorbance at 340 nm [20] [21] and emission at 460 nm [3] [15] [21], which is not the case for the oxidized state. Several well-established methods exist for the determination of NADP, which combine enzymatic assays with the measurement of UV absorption or fluorescence. Basically any enzyme that converts NADP can be used in such an enzymatic assay, although substrate specificity and the optimum conditions for the enzymatic reaction must be

considered and matched with the conditions in which NADP is stable. Examples for enzymes used in direct enzymatic assays are glucose-6-phosphate dehydrogenase and isocitrate dehydrogenase for $NADP^+$ and glutamate dehydrogenase or glutathione reductase for NADPH [3] [20] [21]. Depending on the sample concentration and matrix, coupling with another reaction with a more selectively and/or sensitively detectable product may be necessary. NADP's reactive nature can be used in this context to encourage the formation of adducts with characteristic UV absorption and fluorescence by reaction with various compounds such as cyanide, bisulphite, carbonyl compounds, hydroxylamine and hydrazine [21] [26]. An alternative luminometric method for NADPH determination was reported by Wulff, that involves oxidation of NADPH by flavinmononucleotide (FMN) reductase and conversion of the thus reduced $FMNH_2$ in a luciferase reaction under emission of light [27]. In optimized conditions, the intensity of the emitted light is proportional to the concentration of NADPH.

The mentioned enzymatic approaches to the determination of NADP suffer from a few problems and disadvantages. First of all, the accurate determination of $NADP^+$ and NADPH levels in biological samples, i.e. cell extracts, may be hindered by unspecific enzyme reactions. Some of the enzymes that use NADP as a cofactor in their usual biological role may also react with NAD^+ or NADH. Since cell extracts contain both cofactors, a close examination of the kinetics of the enzymatic conversion is necessary. If possible at all, elimination of this interference may require the introduction of another enzymatic reaction prior to the real enzymatic assay that consumes NAD but not NADP. For example, the glutamate dehydrogenase in the assay proposed by Klingenberg [21] reacts with NADH and NADPH at roughly the same rate, so that NADH must first be oxidized with glycerol phosphate dehydrogenase in order that it is no longer available as a substrate for glutamate dehydrogenase. The luminometric method mentioned above also works both with NADH and NADPH, so that discrimination between the two pyridine dinucleotides is not possible [27]. Another source of interference is the prescence of pigments or other compounds that absorb strongly in the same range as NADP and are present in complex biological samples from some sources [20]. Besides all these interference problems, the stability of NADP in the samples was identified as a major source of error [21] [27].

Several ready-to-use kits are available for the measurement of $NADP^+$ and NADPH in biological samples. The quantitation is based on the enzymatic conversion of NADP by specific enzymes in a cycling assay [22], followed by colorimetric detection. The possible interference exhibited by simultaneously present NAD in biological samples is mostly eliminated in commercially available kits by using engineered highly specific enzymes that only recognize NADP. These kits conveniently also include buffers and reagents for

metabolite extraction. However, most of them were developed for mammalian cells, for which relatively mild conditions suffice to lyse the cells and extract intracellular metabolites such as NADP. Most manufacturers state that they cannot guarantee the applicability of the kit to any other biological material than mammalian cells. Considering the differences in cell wall structure and strength, by far not all organisms relevant in metabolomic studies, e.g. yeasts, are accessible with these enzymatic kits. Cycling assays are in general considered to be very specific and due to the amplification provided by the enzymatic cycling procedure also very sensitive [22]. However, reproducibility seems to be low and automatisation as well as the handling of a large number of samples is complicated [3].

Enzymatic assays for the analysis of NADP often allow the determination of NADPH and total NADP, but not of the oxidized $NADP^+$ individually, or require the total absence of the reduced NADPH if $NADP^+$ is to be measured. In order to achieve that, the protocols apply conditions in which one of the forms is most unstable or completely converted into the respective other form. In the first case, this means acidic treatment to remove NADPH and alkaline treatment or incubation at 60°C to degrade $NADP^+$ and allows the individual determination of either one of the two forms [22]. In the second case, this is usually performed in a preliminary enzymatic step that involves the complete oxidation or reduction of NADP and allows determination of the total amount of NADP present in the sample. Thus, simultaneous determination of $NADP^+$ and NADPH from the same sample is at best complicated, if not unfeasible. In fact, many procedures that involve the determination of both $NADP^+$ and NADPH apply two different extraction procedures, alkaline extraction for NADPH and acidic extraction for $NADP^+$ [26]. Klaidman *et al.* proposed a fluorimetric HPLC method which allows the determination of $NADP^+$ and NADPH after a simple extraction procedure from the same sample in a single reversed-phase chromatographic run [26]. The reduced form is detected simply as NADPH which exhibits fluorescence naturally, while $NADP^+$ is detected as stable addition product NADP-CN after treatment with cyanide. Only this addition product and not the oxidized form alone is fluorimetrically detectable. However, this method is rather time-consuming and the treatment with cyanide may not be suitable for all biological matrices [3]. Ogasawara *et al.* developed another faster HPLC method that involves an enzymatic assay combined with subsequent fluorimentric determination of NADPH and total NADP [3]. The level of total NADP in the sample is determined after the reduction of $NADP^+$ to NADPH by glucose-6-phosphate dehydrogenase, while NADPH is determined in a separate sample aliquot without enzymatic conversion. One advantage noted in this report is that since the cell extracts are alkalized after enzymatic conversion, NADPH should be sufficiently stable so that an autosampler can be used for the handling of large sample numbers. On the other hand, the sample needs to be split into two aliquots that are treated separately for the determination of NADPH and total NADP, respectively [3].

In the recent years, a growing interest in the determination of a cell's metabolic state revealed new questions, summarized in the term "metabolomics". Especially scientists in the field of biotechnology are interested in the elucidation of the complex network of intracellular metabolites and the influence of gene expression on the level of metabolites and vice versa [16]. Detailed knowledge about the metabolism of a cell is a prerequisite for effective strain engineering in the design of cell factories for recombinant protein production. Answering these questions in metabolomics requires analytical methods that are sensitive, fast and allow the identification of known or unknown compounds from complex samples [16]. Mass spectrometry (MS) meets all these requirements. MS spectra may provide a lot of information about the amount as well as the structure of the analyte, but are still relatively easy to handle [16]. Additionally, several software solutions are available that facilitate interpretation and data handling. MS can be combined with separation techniques such as HPLC, resulting in LC-MS, a powerful tool for the analysis of multiple analytes from a complex biological sample [16]. While the direct infusion of the sample into the mass spectrometer is possible, high ionic strength of the sample leads to ion suppression, thus the signal appears lower than it actually is. Additionally, not all solvents used in sample preparation are suitable for electrospray ionization, which is widely used in LC-MS [16]. Chromatographic separation acts as a purifying step and delivers the analytes in separate, concentrated pulses in an appropriate solvent to the mass analyzer. In an optimized LC-MS system, this technique allows sensitive high-throughput analysis that combines the simultaneous identification and detection of multiple compounds in complex samples [16] [28]. Luo *et al.* for example proposed an LC-MS/MS method using tributylamine as volatile ion pairing reagent that allows separation and simultaneous quantification of 29 metabolites from central carbon metabolism [28].

One major advantage of MS analysis is that it allows the use of isotopically enriched internal standards [29]. Internal standardization is used to correct for any losses of the target analyte that may occur during sample preparation and/or measurement. This includes the possible degradation of the analyte. It is of outmost importance that the substance used as internal standard has the same characteristics and will be subject to losses to the same degree as the target analyte. Especially for analytes with a very specific pattern of instability such as NADP, it is hard to find a suitable internal standard according to the criteria mentioned above. The fact that MS allows the discrimination between molecules of slightly different mass is very convenient in this context. When, for example, all the ^{12}C atoms in a compound are replaced by isotopes, most molecules will not change their behaviour in chromatographic separation, but have a different mass than the original molecule and can therefore be distinguished in MS analysis. This technique using such isotopical analogs as internal standards is very frequently used in MS analysis in general and metabolomics in particular

[16]. In metabolic profiling, sometimes even fully labelled extracts of cells grown on $^{13}C_6$-glucose as carbon source are added to biological samples prior to sample preparation [30].

The basic characteristics of NADP require an elaborate approach to the development of a chromatographic separation method for the two molecules. Reversed phase chromatography for polar or ionic analytes often depends on mobile phase additives, such as ion pairing agents [7] [28] [31] [32] or stationary phases that allow the use of highly aqueous solvents in order to achieve sufficient retention on the stationary phase. Alternatives are ion chromatography (IC), hydrophilic interaction liquid chromatography (HILIC) [33] or the use of stationary phases that are designed to separate highly polar compounds, such as porous graphitic carbon (PGC) [34] [35] [36] [37]. However, since LC-MS analysis uses electrospray ionization, some of these options have to be excluded or applied very carefully due to the high ionic strength or mobile phase additives involved that interfere with the analyte signal or cause ion source polution [7] [34]. For example, salts with one of the widely used reagents in ion pairing chromatography, tetrabutylammonium, are not volatile and therefore not compatible with subsequent MS analysis [28]. On the other hand, there are special stationary phases for reversed phase chromatography that retain polar analytes and allow the use of solvents that are perfectly compatible with electrospray ionization. An overview on published analytical methods using LC-MS for NADP analysis is given in Table 1.

Table 1: Overview on published LC-MS methods for NADP analysis.

Reference	Analyte(s)	Separation mechanism	Column	Mass analyzer
Buescher *et al.* [31]	$NADP^+$ NADPH	Ion-pairing RPC[1]	Waters Aquity T3	QQQ[2]
Hofmann *et al.* [7]	NADPH	HILIC[3]	SeQuant ZIC®-pHILIC	Ion Trap
Luo *et al.* [28]	$NADP^+$ NADPH	Ion-pairing RPC	Phenomenex Synergi Hydro	Ion Trap
Mozzicafreddo *et al.* [38]	$NADP^+$ NADPH	Ion-pairing RPC	Phenomenex Luna	QQQ
Pabst *et al.* [34]	$NADP^+$	PGC[4]	Thermo Hypercarb	Q-TOF[5]
Ralser *et al.* [10]	$NADP^+$ NADPH	Ion-pairing RPC	Waters Symmetry C18	QQQ
Yang *et al.* [29]	$NADP^+$ NADPH	Ion-pairing RPC	Agilent Zorbax C18	TOF[6]

[1] RPC … reversed-phase chromatography
[2] QQQ … triple quadrupole mass analyzer
[3] HILIC … hydrophilic interaction liquid chromatography
[4] PGC … porous graphitic carbon chromatography
[5] Q-TOF …quadrupole time-of-flight mass analyzer
[6] TOF …time-of-flight mass analyzer

2.4 Metabolite extraction

Metabolite extraction is probably the most important part of sample preparation and metabolome analysis. Not only is it usually very time-consuming, but it also has high potential to introduce losses that falsify the analytical result. An elaborate suitability study aiming at the minimization of losses and optimization towards the chemical features of the target analyte(s) is indispensable during the development of a sample preparation procedure. A good extraction protocol introduces minimal loss of the target metabolite, prevents any alterations of the metabolite and uses a solvent that is compatible with the analyte as well as the subsequent analytical procedure [16] [39]. In addition, extraction should be fast and in very closely controlled conditions.

Another important aspect of metabolite extraction is the inactivation of enzymes and other biochemical processes during sampling, which is necessary in order to avoid a change of the metabolite levels in the samples during sample preparation or storage [16]. Since the metabolic turnover for some components is very high, this has to be achieved rapidly [40] [16] [41]. This step is commonly referred to as “quenching” and is most frequently carried out by sampling the cells directly into methanol at ≤ -40℃ [16] [19] [29] [40], but also by exposure to liquid nitrogen, acid or alkali [16] [42]. However, the dwelling time of the cells in this quenching solution must be optimized to minimize the leakage of metabolites from the cells [43] [16].

Due to the chemical variability of the compounds involved in cellular metabolism, it is virtually impossible to design one extraction protocol that is suitable for extraction and subsequent analysis of the whole metabolome [16] [19] [43] [41]. Metabolites may be polar or nonpolar, acid- or alkalistable, temperature-sensitive or have other specific characteristics or requirements. The choice of extraction solvent is therefore not trivial, especially since also the analytical platform used must be considered. Certain solvents may be incompatible with the desired analytical technique [16] [39]. This is especially important for MS analysis, which is widely used in metabolomics [16] and is not compatible with all solvents. As a result, organic solvents or mixtures of polar and non-polar organic solvents are most frequently used for metabolite extraction [16].

Metabolite extraction can be achieved in many ways. For extracellular metabolites, a simple separation of the cells from the culture broth with subsequent purification and isolation steps is sufficient. On the other hand there are many intracellular metabolites of interest, such as $NADP^+$ and NADPH, which may either be localized in the cell’s cytoplasm or in cell compartments. For analysis of these compounds, the cell must be disrupted during the

extraction process. When a protocol is chosen, not only the cell type and species [17] but also the nature of the analyte must be considered [16] [39] [43]. In other words, the conditions applied must be harsh enough to open the cell and make all analyte pools completely available, while the metabolite(s) of interest must not be damaged or destroyed during sample preparation [16] [17] [39] [43].

An extraction protocol for metabolite analysis must be optimized for a specific target organism [41]. The success of a certain protocol for cell lysis will largely depend on the cell wall characteristics of the organism, which may be very different even within groups of related organisms. As a consequence, protocols optimized for microbial cells have to be adapted to be suitable also for mammalian cells due to the different structure of the cell envelope [43]. For instance, some yeasts have very thick cell walls, while mammalian cells only have a cell membrane, which is easier to overcome. *Pichia pastoris* is usually disupted by rather harsh protocols using mechanical cell lysis [44] or boiling [30], indicating that the cell wall is very robust and care must be taken that the cell actually breaks in the extraction conditions. Available extraction protocols for yeasts include exposure of the cell to hot acid, boiling ethanol or high hydroxide concentrations [39] [41]. Alternatives to the common protocols mentioned above are methods that use cold methanol and/or chloroform in combination with cycles of freeze-thawing or vigorous shaking for metabolite extraction [17] [39] [41]. Canelas *et al.* did an extensive study of various techniques for the extraction of intracellular metabolites from yeast, including hot extraction with water (HW) or ethanol (BE), cold extraction with chloroform and methanol (CM) or acidified methanol (AANM) as well as a freeze-thawing method with methanol (FTM) [17]. These procedures represent the standard approaches to metabolite extraction. Canelas *et al.* found BE, HW and CM to reproducibly yield near-complete extraction and good reproducibility, while the results from FTM and AANM showed that some metabolites are especially well recovered, while the extraction efficacy was very low for others [17]. 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 [45].

NADP, especially the reduced form, is sensitive to a variety of conditions that are usually applied in sample preparation for metabolomics [6] [7]. The development of a suitable extraction protocol must therefore include an elaborate study of analyte stability. In particular, low pH must be avoided when the reduced NADPH is to be extracted [6]. Moreover, high ionic strength of the extraction solvent may induce signal suppression in MS analysis [39]. 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 released in this step [3]. Buescher *et al.* measured NADP from extracts from boiling ethanol extraction [31], which is one of the most

frequently used extraction protocols for various organisms including microbes [17] [19]. Gonzalez *et al.* assessed that NADH, which is similarly unstable as NADPH, is stable in such boiling ethanol extracts as long as the extraction solvent is buffered to pH 7.5 [19]. The stability requirements of NADP suggest the use of an alkaline extraction solvent for NADPH and an acidic solvent for $NADP^+$. While this approach is used in some protocols [9] [13] [21], Maharjan and Ferenci found that nucleotides may absorb to the precipitating salts that are formed during the neutralization step after extraction [45]. However, this step is necessary in order to increase metabolite stability in the extracts [45] and to decrease the ionic strength of the sample that may otherwise cause signal suppression. An overview on published protocols for the extraction of NADP from biological samples is given in Table 2 along with the ratios of $NADPH/NADP^+$ given in literature. The fact that the reported values for the ratio $NADPH/NADP^+$ are very divergent indicates that the determination is not straight-forward.

Table 2: Overview on published methods for the extraction of $NADP^+$ and NADPH and measurment of the ratio $NADPH/NADP^+$ in biological samples.

Reference	Organism(s)	Extraction method / solvent	Analytical method	ratio $NADPH/NADP^+$
Buescher *et al.* [31]	Microbial cells, mammalian cells	Boiling in buffered ethanol pH 7.2	LC-MS/MS	-
Hou *et al.* [8]	*S. cerevisiae*	2-phase extraction using methanol and chloroform	LC-MS/MS	0.04[1] 0.2[2]
Klingenberg [21]	Mammalian tissue	Mechanical cell lysis, separate extraction with perchloric acid ($NADP^+$) and potassium hydroxide (NADPH)	Enzymatic assay, UV detection	-
Luo *et al.* [28]	*E. coli*	Alkaline extraction with potassium hydroxide, neutralization prior to analysis	LC-MS/MS	0.06
Pabst *et al.* [34]	Chinese Hamster Ovary (CHO)	Ice-cold sodium fluoride	LC-MS/MS	0.15
Ralser *et al.* [10]	*C. elegans*	Mechanical cell lysis combined with 2-phase extraction with phenol, chloroform, isoamyl-alcohol and EDTA	LC-MS/MS	0.15[3] 0.3[4]
Tan *et al.* [9]	*S. cerevisiae*	Separate extraction with hydrochloric acid ($NADP^+$) and potassium hydroxide (NADPH)	Enzymatic assay, UV detection	1.3
Yang *et al.* [29]	*S. cerevisiae*	Boiling in ethanol	LC-MS	4.23
Watanabe *et al.* [13]	*S. cerevisae*	Separate extraction with potassium phosphate pH 5 ($NADP^+$) and Tris/HCl pH 9 (NADPH)	Enzymatic assay, UV detection	4.92

[1] observed in a wild-type strain
[2] observed in a recombinant strain overexpressing cytosolic NADH kinase
[3] observed in wild-type cells
[4] observed in recombinant cells expressing a mutant triosephosphate isomerase

3 EXPERIMENTAL

3.1 Solvents and Chemicals

For cultivation media, the following substances were purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany): $MgSO_4.7H_2O$ 99%, KCl 99.5%, K_2HPO_4 99%, KH_2PO_4 98%, glycerol 99%. Glucose monohydrate for microbiology and citric acid 99.5%. $(NH_4)_2HPO_4$ p.a. were purchased from AppliChem (Gatersleben, Germany), $CaCl_2.2H_2O$ p.a, ammonia (25% v/v), KOH pellets pure and hydrochloric acid (30% ultrapure) from Merck KGaA (Darmstadt, Germany) and H_3PO_4 85% v/v and biotin 99% from Sigma-Aldrich (Vienna, Austria) were also used for cultivation media. The components of the trace salts stock solution were purchased from Merck, with the exception of 5.0 g $FeSO_4.7H_2O$ 99.5% that was purchased from Carl Roth.

For liquid chromatography, analytical-grade standards of NADPH and $NADP^+$, LC-MS grade acetonitrile, water, methanol and formic acid as well as ammonium acetate were purchased from Sigma Aldrich. Isotopically labeled adenosine-monophosphate ($^{15}N_5$-5'AMP) was purchased from Isotec (Sigma Aldrich).

For metabolite extraction, potassium hydroxide (KOH), ammonium acetate and digitonin were purchased from Sigma Aldrich, LC-MS grade methanol was purchased from Fisher Scientific.

3.2 Organism, growth conditions, sampling and quenching

3.2.1 Fed batch culture for homogenous cell samples

A set of homogenous cell pellets, i.e. from the same culture and sampled at the same time using the same sampling procedure, was to be created for the evaluation of the different extraction protocols. The wild type strain of *Pichia pastoris* was cultivated in fed batch using a 1 L benchtop bioreactor (DASGIP Parallel Bioreactor System, Germany). One liter of the batch and fedbatch medium contained the following components: 0.5 g $MgSO_4 \cdot 7H_2O$, 0.9 g

KCl, 0.022 g $CaCl_2 \cdot 2H_2O$, 10.98 mL 85% v/v H_3PO_4, 4.6 mL trace salt stock solution, 2 mL biotin solution (c = 0.2 g*L^{-1}) and 10 g D-glucose. Further cultuvation conditions were a temperature of 25℃, an oxygen partial pressure (pO_2) of 20% and pH 5.0, continuously adjusted using 25% NH_3. After inoculation at an optical density (OD) of 0.2, the system was operated in batch with a working volume of 320 mL for 35 h, followed by the fed batch phase. The first samples were then taken after 2 h of exponential growth, followed by another 2 h of exponential growth and another round of sampling. The cultivation broth was sampled directly into the 4-fold volume of cold quenching solvent (60% v/v methanol) at 5 mL·s^{-1} using a peristaltic pump and 5 mm silicone tubes. A cooling mixture (70:30 ethylene glycol:ethanol with dry ice pellets) was used to temper the quenched cell suspension to -30℃ ± 3℃.

After aliquotation of the cell suspension into 15 mL sample tubes (Greiner), the biomass was pelleted by centrifugation at 4000 *g* and -20℃ for 10 min in a Sorvall RC 6+ centrifuge from Thermo Scientific. To wash the cell pellet, the supernatant was discarded, 5 mL of quenching solution added and centrifuged again. After another washing step, the supernatant was discarded carefully and the samples stored at -80℃ until extraction.

3.2.2 Batch culture for strain comparison

Batch cultures of two strains of *Pichia pastoris* were performed to get biomass samples for the suitability evaluation, i.e. the comparison of the NADP levels found in these strains. The strains of choice were the *Pichia pastoris* wild type strain X33 and a strain overexpressing glucose-6-P dehydrogenase (ZWF1) under control of the constitutive pGAP1 promoter, constructed on X33 background. Four 24 h precultures of each strain in shake flasks with 5 mL minimal medium were inoculated from cryostocks, followed by the main culture in 10 mL minimal medium inoculated at an OD of 0.2. Further cultivation conditions were 25℃ and 170 rpm on an orbital shaker. The pH was set to 5.7 with 5 M KOH solution. After 24 h of main culture, three 1 mL aliquots out of the four independent shake flask cultures of the two strains were sampled into separate tubes by pipetting, immediately pelleted in a microcentrifuge (Heraeus Biofuge Pico) and the supernatant discarded. Simultaneously, the optical density as a measure of the biomass content was determined using a WPA CO8000 Cell Densitymeter. The samples were put on dry ice and immediately used for metabolite extraction.

The minimal medium contained the following components (per liter): 20 g of glucose, 20 g of citric acid, 3.15 g of $(NH_4)_2HPO_4$, 0.03 g of $CaCl_2 \cdot 2H_2O$, 0.8 g of KCl, 0.5 g of $MgSO_4 \cdot 7H_2O$, 2 mL of biotin (0.2 g·L^{-1}) and 1.5 mL of trace salts stock solution.

The trace salts stock solution contained per liter: 6.0 g of $CuSO_4 \cdot 5H_2O$, 0.08 g of NaI, 3.0 g of $MnSO_4 \cdot H_2O$, 0.2 g of $Na_2MoO_4 \cdot 2H_2O$, 0.02 g of H_3BO_3, 0.5 g of $CoCl_2$, 20.0 g of $ZnCl_2$, 5.0 g of $FeSO_4 \cdot 7H_2O$ and 5.0 mL of H_2SO_4 (95–98% w/w).

3.3 Metabolite extraction and sample preparation

Several different extraction protocols were tested for their suitability for the extraction of $NADP^+$ and NADPH from *Pichia pastoris*. Selected extraction protocols were furtherly investigated in a stepwise recovery experiment. The workflow for all protocols is described below in further detail.

3.3.1 Extraction of *Pichia pastoris* cell pellets

A set of homogenous cell pellets of *Pichia pastoris*, prepared according to the procedure described in 3.2.1, was used for the evaluation of the extraction efficiency. The extraction procedures tested in this experiment are listed and described below. Each extraction protocol was tested in three replicates. $^{15}N_5$-5'-AMP was added to each sample as monitoring tool. The extracted biomass was separated from the liquid extract and examined in a light microscope for the evaluation of cell lysis.

3.3.1.1 Alkaline cold methanol extraction

This protocol was modified from Canelas *et al.*, 2009 [17]. The cell pellet was resuspended in 100 µL of a 50 µM solution of $^{15}N_5$-5'-AMP and 2.5 mL of pre-cooled extraction solvent (60% v/v methanol, 40% H_2O with 50 mM KOH). The sample was then shaken vigorously for 45 min at ≤-37℃ and subsequently centrifuged for 10 min at 4000 *g*. The supernatant was collected by decanting and dried under reduced pressure in a Thermo Savant SPD121P SpeedVac Concentrator. The dried extract was reconstituted in 1 mL of LC-MS grade H_2O prior to analysis.

3.3.1.2 Alkaline cold methanol freeze-thawing extraction

This protocol was modified from Tredwell *et al.*, 2011 [46]. The cell pellet was resuspended in 100 µL of a 50 µM solution of $^{15}N_5$-5'-AMP and 2.5 mL of pre-cooled extraction solvent (60% v/v methanol, 40% v/v H_2O with 50 mM KOH). The sample was then frozen in liquid nitrogen for 3 min and subsequently thawed in a non-thermostated ultasonic bath for 15 min. After centrifugation (10 min at 4000 *g*), the supernatant was collected by decanting and dried under reduced pressure in a Thermo Savant SPD121P SpeedVac Concentrator. The dried extract was reconstituted in 1 mL of LC-MS grade H_2O prior to analysis.

3.3.1.3 Cold methanol freeze-thawing extraction with Digitonin

This protocol was modified from Tredwell *et al.*, 2011 [46]. The cell pellet was resuspended in 100 µL of a 50 µM solution of $^{15}N_5$-5'-AMP, 200 µL of a fully ^{13}C-labeled cell extract and 2.5 mL of pre-cooled extraction solvent (60% v/v methanol, 40% v/v H_2O with 0.1% w/v Digitonin). The sample was then frozen in liquid nitrogen for 3 min and subsequently thawed in a non-thermostated ultrasonic bath for 15 min. After centrifugation (10 min at 4000 *g*), the supernatant was collected by decanting and 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.

3.3.1.4 Boiling ethanol extraction

This protocol was adapted from Tweeddale *et al.*, 1998 [47]. After adding 100 µL of a 50 µM solution of $^{15}N_5$-5'-AMP, the pellet was resuspended in 4 mL of pre-heated 75% v/v ethanol and incubated at 85℃. The sample was vortexed thor oughly for 10 s after 1.5 min of heating and again after 3 min of heating. The tubes were cooled rapidly on dry ice and centrifuged for 10 min at 4000 *g*. The supernatant was collected by decanting and dried under reduced pressure in a Thermo Savant SPD121P SpeedVac Concentrator. The dried extract was reconstituted in 1 mL of LC-MS grade H_2O prior to analysis.

3.3.1.5 Hot water / buffer extraction

This protocol was adapted from Canelas *et al.*, 2009 [17]. After adding 100 µL of a 50 µM solution of $^{15}N_5$-5'-AMP, the cell pellet was resuspended in 900 µL of either cold or pre-heated LC-MS grade H_2O or 5 mM ammonium acetate buffer at pH 8.0. The sample was then incubated for 3 min at 85℃ and subsequently c ooled on dry ice. After centrifugation (10 min, 4000 *g*), the supernatant was collected by pipetting.

3.3.1.6 Buffered mechanical cell lysis with glass beads

The cell pellet was resuspended in 2 mL of 5 mM ammonium acetate buffer adjusted to pH 8.0 with ammonia. The sample was split and transferred into two tubes containing 500 mg glass beads and subjected to two homogenizing cycles of 30 s at a speed of 6.5 m/s in a FastPrep-24 from MP Biomedicals. Between and after the two cycles, the samples were cooled on ice. After centrifugation (10 min, 4000 *g*), the supernatant was collected by pipetting.

3.3.1.7 Redox-buffered mechanical cell lysis with glass beads

The cell pellet was resuspended in 1 mL of a 5 mM ammonium acetate buffer adjusted to pH 8.0 with ammonia containing 0.5 µM GSSG and 50 µM GSH. After adding 500 mg glass beads, the sample was homogenized in two cycles of 30 s at a speed of 6.5 m/s in a FastPrep-24 from MP Biomedicals. Between and after the two cycles, the samples were cooled on ice. After centrifugation (10 min, 4000 *g*), the supernatant was collected by pipetting.

3.3.2 Recovery study

The protocols chosen for the recovery experiment are given in Table 3. The standard recovery was determined for the listed steps of each protocol in three replicates each. The standards were prepared as mixtures in the respective extraction solvent and contained 5 µM $NADP^+$, 5 µM NADPH and 5 µM $^{15}N_5$-5'AMP. All samples were analysed by flow injection (isocratic, 85% v/v 5 mM ammonium acetate at pH 6.0, 15% v/v methanol) in a 2 min run at

250 µL·min^{-1} in negative MRM mode on a Thermo TSQ Vantage Triple Quadrupole mass spectrometer.

Table 3: List of extraction protocols, solvents and steps investigated in the extraction recovery study.

Extraction protocol	Step description	Extraction solvent
Buffered mechanical cell lysis with glass beads	(0) No treatment (1) 20 s homogenization (2) 2 x 20 s homogenization	5 mM ammonium acetate, pH 8.0
Buffered cold methanol freeze-thawing extraction with Digitonin	(0) No treatment (1) Freezing, thawing at 20°C (2) Freezing, ultrasonication (3) Solvent evaporation, reconstitution	60% v/v methanol 40% v/v 5 mM ammonium acetate, pH 8.0 with 0.1% w/v digitonin
Boiling ethanol extraction	(0) No treatment (1) 1.5 min incubation at 85°C (2) 3 min incubation at 85°C (3) Solvent evaporation, reconstitution	75% v/v ethanol, 25% v/v H_2O
Hot water / buffer extraction	(0) No treatment (1) 3 min incubation at 85°C	100% H_2O or 5 mM ammonium acetate, pH 8.0

3.4 LC-MS systems

All LC-MS analysis of NADPH and NADP$^+$ was performed either on an Agilent 6210 Series Time-of-Flight (TOF) mass spectrometer coupled with an Agilent 1200 series HPLC or on a Thermo TSQ Vantage triple quadrupole mass spectrometer coupled with an Accela 1250 HPLC system. In all cases, ionization was achieved by electrospray ionization (ESI) in negative mode.

3.4.1 Time-of-Flight Mass Spectrometer (TOF)

The LC-MS system used for LC-TOF-MS was an Agilent 6210 Series Time-of-Flight (TOF) mass spectrometer coupled with an Agilent 1200 series HPLC and a CTC autosampler. The optimized parameters for mass spectrometric detection of NADP$^+$ and NADPH on this instrument are given in Table 4.

Table 4: Optimized detection parameters for $NADP^+$, NADPH and $^{15}N_5$-5'-AMP in ESI-TOF-MS analysis in negative ionization mode.

	$NADP^+$	**NADPH**	**$^{15}N_5$-5'AMP**
Molecular formula	$C_{21}H_{29}N_7O_{17}P_3$	$C_{21}H_{30}N_7O_{17}P_3$	$C_{10}H_{12}{}^{15}N_5O_7P$
Observed mass	742.0678 m/z	744.0829 m/z	351.0418 m/z
Capillary voltage	4000 V	4000 V	4000 V
Gas temperature	350℃	350℃	350℃
Drying gas flow	10 $L \cdot min^{-1}$	10 $L \cdot min^{-1}$	10 $L \cdot min^{-1}$
Nebulizer pressure	25 psig	25 psig	25 psig
Fragmentor voltage	180 V	180 V	180 V
Skimmer voltage	60 V	60 V	60 V

3.4.2 Triple Quadrupole Mass Spectrometer (QQQ)

The LC-MS system used for LC-QQQ-MS was a Thermo TSQ Vantage triple quadrupole mass spectrometer in connection with an Accela 1250 HPLC system and a CTC autosampler. The optimized parameters for mass spectrometric detection on this instrument are given in Table 5.

Table 5: Optimized detection parameters for $NADP^+$, NADPH and $^{15}N_5$-5'-AMP in ESI-QQQ-MS analysis in negative ionization and MRM mode on a Thermo TSQ Vantage MS.

	$NADP^+$	**NADPH**	**$^{15}N_5$-5'AMP**
Molecular formula	$C_{21}H_{29}N_7O_{17}P_3$	$C_{21}H_{30}N_7O_{17}P_3$	$C_{10}H_{12}{}^{15}N_5O_7P$
Parent mass	742.0 m/z	744.0 m/z	351.0 m/z
Product ion mass / collision energy	408.0 m/z / 32 V 620.0 m/z / 18 V	159.0 m/z / 50 V 426.0 m/z / 29 V	79.1 m/z / 41 V 139.1 m/z / 32 V
Capillary temperature	300℃	300℃	300℃
Spray voltage	3300 V	3300 V	3300 V
Sheath gas pressure	40 psig	40 psig	40 psig
Auxiliary gas pressure	10 psig	10 psig	10 psig

3.5 Chromatographic separation

In order to achieve chromatographic separation of $NADP^+$ and NADPH, different types of columns and separation mechanisms were tested. The evaluation and choice of the suitable separation system was based on the separation efficiency, the type of solvents used and the compatibility with the desired sample solvent.

3.5.1 Reversed-phase separation on a silica-based stationary phase

A silica-based column with C18 ligands (Waters Atlantis T3, 2.1 x 150 mm, 3 µm particle size) was employed for reversed-phase separation. The aqueous mobile phase used was a 5 mM ammonium acetate buffer (A) adjusted to pH 6.0. The analytes were eluted with methanol (B). The flow rate was set to 250 $\mu L \cdot min^{-1}$ and the column thermostated at 40℃. The injection volume was 5 µL. The chromatographic run started at 100% A, which was maintained for 2 min, followed by a gradient from 0 to 17% methanol in 3.5 min. With a washing step at 90% B and the appropriate column equilibration time to reinstate the starting conditions, the overall run time was 12 min. If necessary, the samples were diluted in 5 mM ammonium acetate buffer at pH 8.0.

3.5.2 Chromatographic separation on a porous graphitic carbon stationary phase

A PGC-packed Hypercarb column (Thermo Scientific, 3 x 50 mm, 5 µm particle size) was tested both in reversed-phase and in hydrophilic interaction liquid chromatography (HILIC) mode. Different aqueous mobile phases (A) were tested with the Hypercarb column. Those were 50 mM ammonium formate buffer at pH 9.0, 20 mM ammonium formate buffer at pH 6.0 and 20 mM ammonium acetate buffer at pH 9.0. The organice mobile phase (B) was acetonitrile or methanol. Each time, the flow rate was set to 500 $\mu L \cdot min^{-1}$ and the column heated to 40℃. In HILIC mode, the organic content in the mobile phase was reduced from 90% to 50%, followed by a cleaning step at 50% B and re-equilibration at starting conditions. In reversed-phase mode, the organic content in the mobile phase was increased from 2% to 15%, followed by a cleaning step at 50% B and re-equilibration at starting conditions. For method development, the standards were prepared in 5 mM ammonium acetate buffer at pH 8.0 and diluted in different percentages of methanol prior to analysis.

3.5.3 HILIC separation on a silica-based stationary phase

A silica-based Nucleodur HILIC column (Macherey-Nagel, 2 x 100 mm, 1.8 µm particle size) was tested with acetonitrile as organic mobile phase (B). As aqueous mobile phase (A), a 10 mM ammonium formate buffer was tested both at pH 3.0 and pH 7.0. In both cases, the gradient started at 95% B and decreased to 10% B in 7 min. The total runtime was 20 min with a flow rate of 300 $\mu L \cdot min^{-1}$. The column was kept at 40°C in a thermostated column oven. The samples were diluted either in 5 mM ammonium acetate buffer (pH 8.0) or 60% methanol.

Another silica-based HILIC column with a zwitterionic stationary phase (SeQuant ZIC-HILIC, 2.1 x 150 mm) was also tested. The organic mobile phase was 100% acetonitrile (B), while the aqueous mobile phase was 5 mM ammonium formate at pH 4.0 (A). The run started at 80% B, followed by a gradient from 80% to 35% in 5 min within a total run time of 15 min. The flow rate was set to 250 $\mu L \cdot min^{-1}$.

3.5.4 HILIC separation on an Ethylene Bridge Hybrid particle stationary phase

For HILIC separation, an Ethylene Bridge Hybrid particle (BEH) based Xbridge Amide column (Waters, 2.1 x 150 mm, 3.5 µm particle size) was tested. The column is packed with particles that are stable across the entire pH-range and is compatible with a wide range of mobile phases and temperatures. The mobile phases were 5 mM ammonium acetate at pH 6.0 (A) and acetonitrile (B). The column was kept at 40°C in a column oven and run with a flow rate of 250 $\mu L \cdot min^{-1}$. During the chromatographic run, the organic content of the mobile phase was decreased from 85% to 55% in 4 min, followed by a cleaning step at 90% A and an appropriate column re-equilibration time, adding up to a total run time of 15 min. All samples and standards were diluted in acetonitrile to match the starting conditions.

4 RESULTS & DISCUSSION

4.1 LC-MS method for quantitative analysis of NADP

4.1.1 Mass spectrometric detection

In accordance with nucleotide measurements [48], ionization in negative ion mode was found optimal for the detection of $NADP^+$ and NADPH. Both compounds ionize well in negative mode and provide sufficiently high signal intensity. Ionization in positive mode is unfavourable due to the instability of the two compounds in the acidic conditions required as well as the occurrence of multiply charged ions.

4.1.1.1 Time-of-Flight mass spectrometry (TOF-MS)

In this work, TOF-MS was used for the initial method development, including separation optimization and identification of the monoisotopic masses of $NADP^+$ and NADPH, as well as stability measurements. TOF-MS is especially suitable for this task because of the high mass resolution (>13,000 at 2722 m/z) and mass accuracy (< 2 ppm) that are achievable with TOF-MS instruments such as the Agilent 6210 TOF mass spectrometer. However, quantification is limited by the restricted linear dynamic range.

Figure 2 shows a schematic view of the TOF-MS instrument used in this work. The HPLC flow forms a stable spray in the ion source, producing analyte ions that are drawn through the heated transfer capillary. A voltage is applied at the end of this capillary that allows the fragmentation of the ions. The ion beam is then focused by octopole ion guides and a focus lens and enters the mass analyzer as a parallel beam. As the ions enter the ion pulser, they are deflected by high voltage pulses and are sent into the flight tube. At the end of the flight tube, the ions are reflected towards the ion pulser. This lens system focuses the ion beam to the detector, where the mass of the ion is determined based on the time it took the ion to travel through the flight tube. Ions with insufficient kinetic energy, i.e. ions that are generated after the flight tube due to metastable decay, cannot maintain the right trajectory and miss the detector. The achievable resolution is therefore high in this setup.

The accurate monoisotopic masses of $NADP^+$ and NADPH, calculated based on the molecular formulas given in Table 4 using Masshunter 3.2 software, are 742.0682 m/z for $NADP^+$ and 744.0838 m/z for NADPH. The difference of 2 m/z between the two forms of NADP can be explained by the fact that $NADP^+$ inherently carries a positive charge, so that a negatively ionized form of the molecule must have lost two hydrogens, i.e. $NADP^+$ is found as $(M-2H)^-$ while NADPH is found as $(M-H)^-$. The observed masses were 742.0678 m/z for $NADP^+$ and 744.0829 m/z for NADPH. In both cases, the deviation of the observed mass from the accurate mass, i.e. the mass accuracy, is well below 2 ppm.

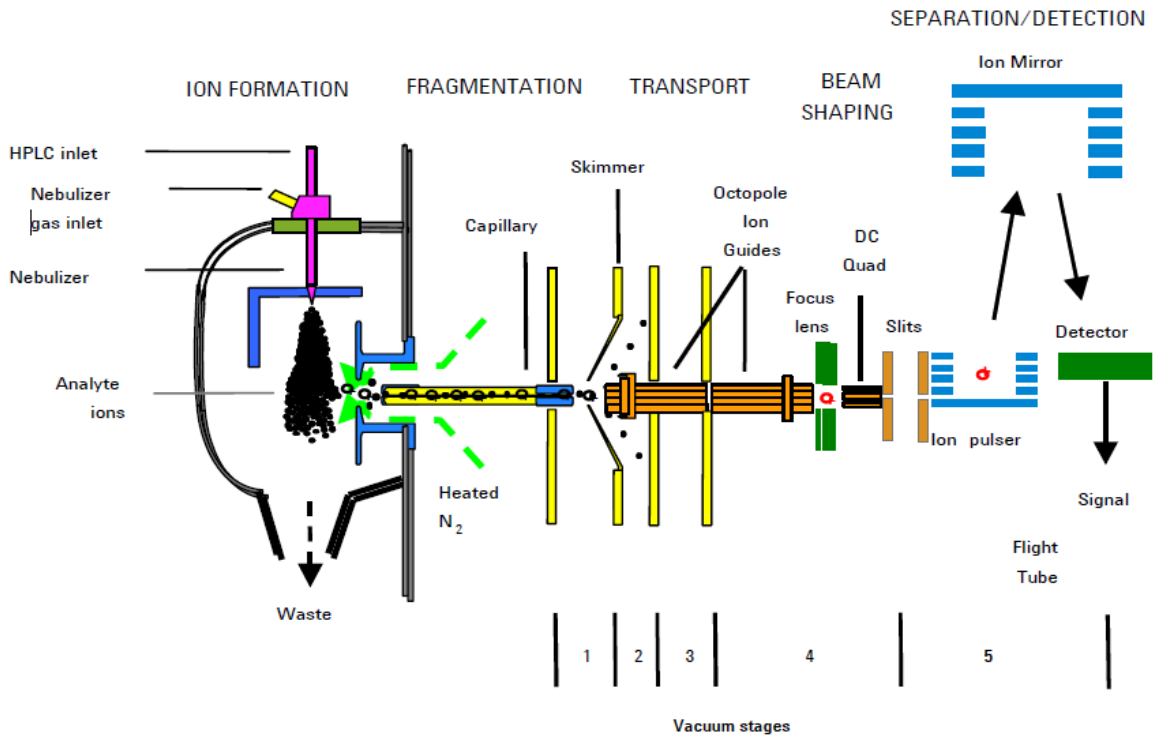

Figure 2: Schematic view of the Agilent 6210 TOF mass spectrometer including electrospray ionization (ESI), ion optics and a time-of-flight mass analyzer.

Both compounds occur as single-charged ions in negative ionization mode, which was confirmed both considering the molecular weight and the isotopic pattern. The signals corresponding to the two forms of NADP were extracted from the total ion current using the Agilent Mass Hunter 3.1 Qualitative Analysis software with an extraction window of 0.005 m/z, which resulted in an extracted ion chromatogram, from which a mass spectrum was generated for the two peaks separately. As is shown in Figure 3, the observed mass spectrum confirmed the expected isotopic pattern. Quantification was performed in the Agilent Mass Hunter Quantitative Analysis 3.2 software based on the peak area in the extracted ion current chromatogram with an extraction width of 0.005 m/z.

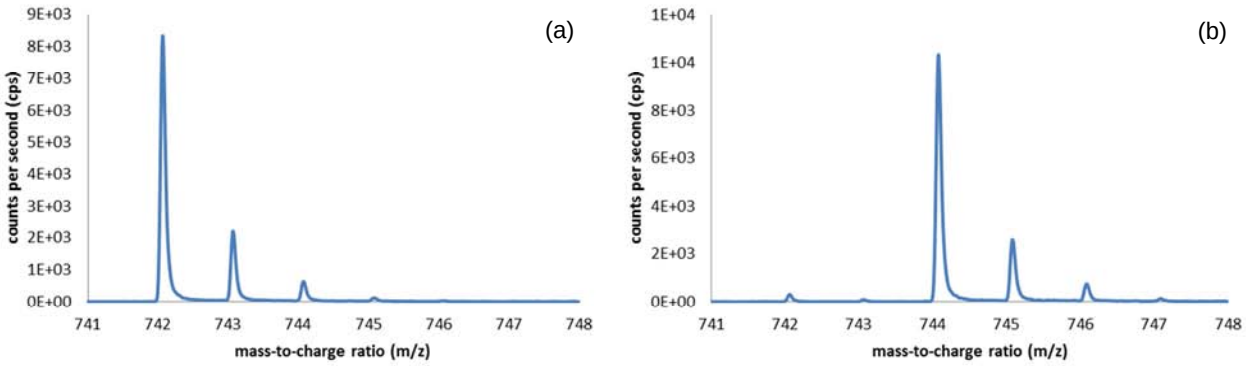

Figure 3: Observed mass spectra of the two forms of NADP from LC-TOF-MS analysis of a 5 µM standard solution on an Agilent 6210 series Time-of-Flight mass spectrometer. (a) $NADP^+$, (b) NADPH.

4.1.1.2 Triple quadrupole mass spectrometry (QQQ-MS)

In this work, the dedicated system for accurate quantification was a QQQ-MS instrument with an extended linear dynamic range and high sensitivity. Detection in this setup is based on a substance-specific fragmentation pattern that allows selective analysis, which contributes to the very high signal-to-noise ratios that are achievable with QQQ-MS instruments.

The signal detection was performed in selected reaction monitoring (SRM) mode. First, optimization of the ionization and fragmentation settings was performed using single standards in direct infusion analysis. 30 µM single standards of $NADP^+$ and NADPH respectively were injected using a syringe pump at 15 $\mu L \cdot min^{-1}$ into the mobile phase flow provided by the HPLC system at 250 $\mu L \cdot min^{-1}$. The mobile phase composition was adjusted to the anticipated elution conditions in chromatographic separation and consisted of 80% 5 mM ammonium acetate (pH 6.0) and 20% methanol. The main parameter for identification and optimization of the fragmentation pattern is the collision energy, i.e. the voltage applied in the collision cell of the QQQ-MS instrument where the fragments are created (see Figure 4). Figure 5 shows the main product ions resulting from fragmentation of $NADP^+$ at different collision energies. The product ion with the highest intensity was found already at 20 V, with a mass-to-charge ratio of 620 m/z. At higher collision energies, several product ions with a much lower signal intensity were found (408, 272 and 159 m/z). Luo *et al.* suggested that the 620 m/z fragment is a result of the loss of the nicotinamide moiety [28]. It was observed that this loss can already occur in the ion source, so that the voltage applied in the spray chamber must be optimized in order to minimize the in-source fragmentation of $NADP^+$ that would result in a loss of signal and a drastic reduction in sensitivity. A similar overview is given in Figure 6 for the fragmentation of NADPH, which resulted in multiple transitions, yielding product ions of 159, 397, 408 and 426 m/z, respectively.

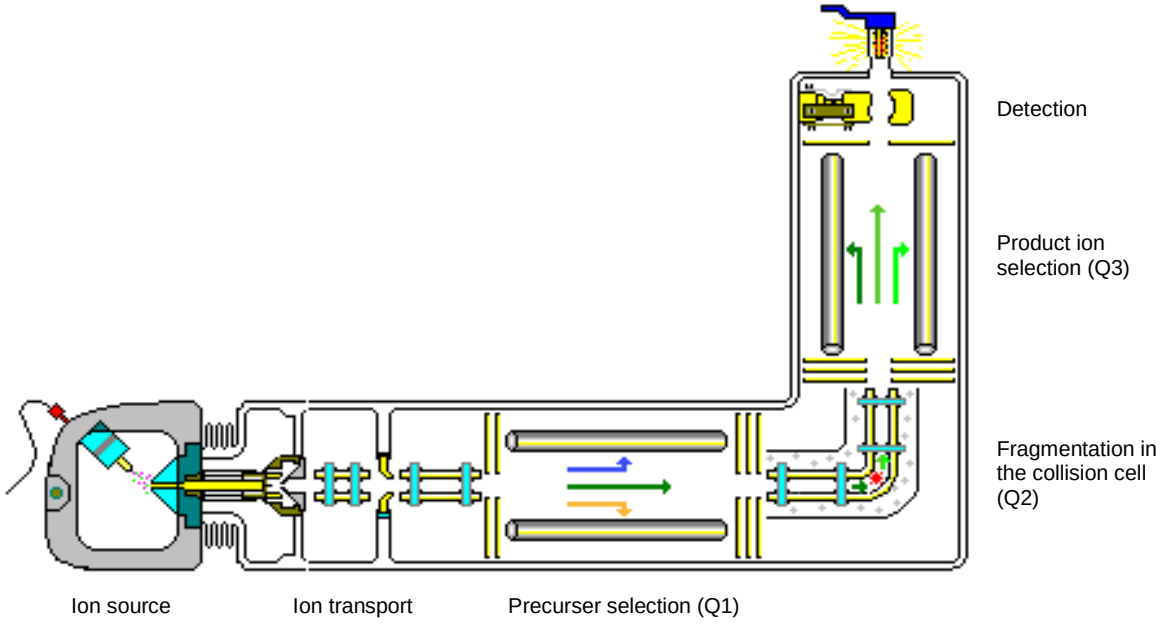

Figure 4: Schematic view of the Thermo TSQ Vantage mass spectrometer including electrospray ionization, ion optics and a triple stage quadrupole mass analyzer. This system allows the selection of a precurser ion, fragmentation of the precurser and scanning for selected product ions.

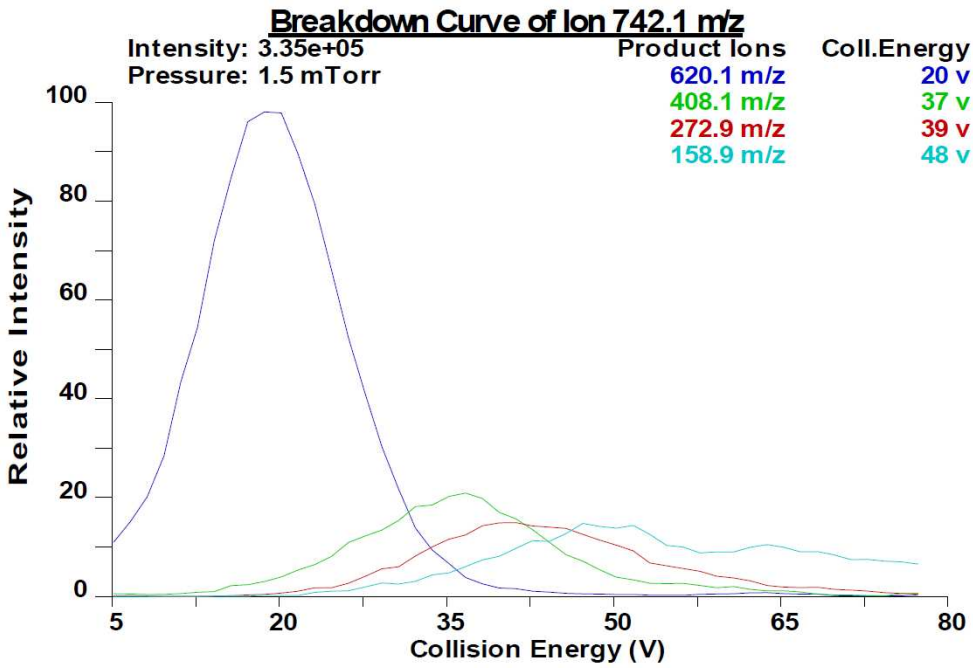

Figure 5: The main product ions resulting from fragmentation of $NADP^+$ at different collision energies between 5 and 80 V, as observed on a Thermo TSQ Vantage triple quadrupole MS. A 30 µM standard solution of $NADP^+$ was analyzed by direct infusion. The mobile phase (80% 5 mM ammonium acetate at pH 6.0, 20% methanol) was delivered by the HPLC system at a flow rate of 250 $\mu L \cdot min^{-1}$. The standard solution was injected into this flow using a syringe pump at 15 $\mu L \cdot min^{-1}$

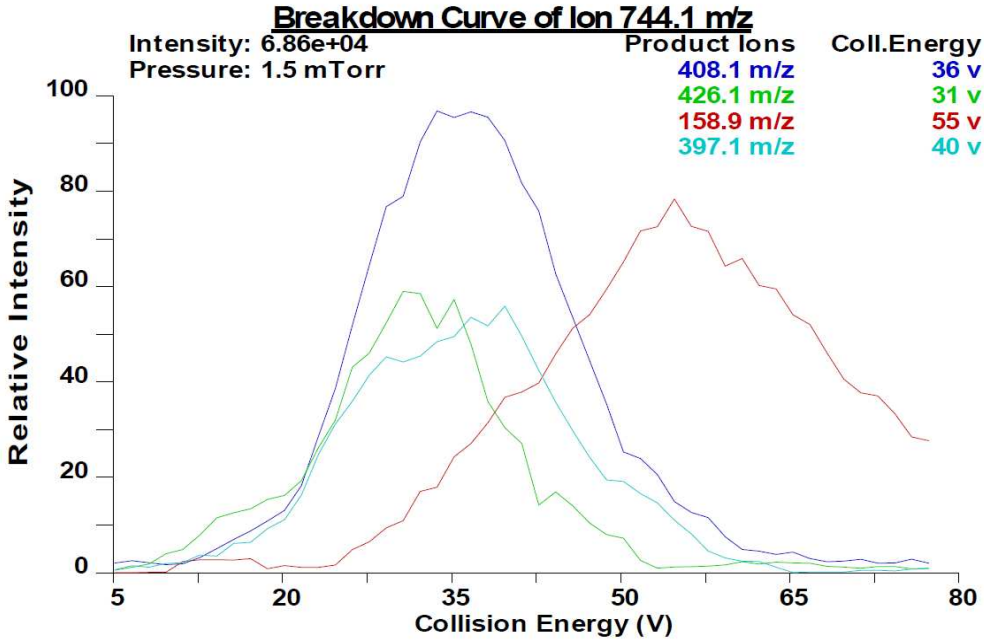

Figure 6: The main product ions resulting from fragmentation of NADPH at different collision energies between 5 and 80 V, as observed on a Thermo TSQ Vantage triple quadrupole MS. A 30 µM standard solution of NADPH was analyzed by direct infusion. The mobile phase (80% 5 mM ammonium acetate at pH 6.0, 20% methanol) was delivered by the HPLC system at a flow rate of 250 µL·min^{-1}. The standard solution was injected into this flow using a syringe pump at 15 µL·min^{-1}

For quantification, the two most intense transitions per analyte were chosen and included into the MS acquisition method. These were 742 > 620 m/z and 742 > 408 m/z for $NADP^+$ and 744 > 426 m/z and 744 > 159 m/z for NADPH. Calibration and quantification was based on the sum signal of these transitions for the respective analyte. Quantification of the signal yielded from a certain sample was then based on the peak area in the extracted ion current chromatograms. The calibration curves of $NADP^+$ and NADPH along with detection and quantitation limits are given in 4.1.3 for the overall LC-MS method.

4.1.2 Chromatographic separation of $NADP^+$ and NADPH

The state of the art in the chromatographic separation of $NADP^+$ and NADPH is presented in 2.3. As can easily be seen, ion-pairing chromatography, hydrophilic interaction liquid chromatography (HILIC) and porous graphitic carbon chromatography (PGC) are optional separation methods. In this work, reversed-phase chromatography, HILIC and PGC were tested to develop an analytical method for separation and quantification of NADPH and $NADP^+$. The reversed-phase chromatography employed a 100% wettable stationary phase. All method development was performed on the LC-TOF-MS system described in 3.4.1.

Among the HILIC columns tested, different problems occurred. The silica-based HILIC stationary phases (Macherey-Nagel Nucleodur HILIC and SeQuant ZIC-HILIC) were unable to separate the two forms of NADP in the conditions tested, which were adjusted to the desired overall analytical system. Acetonitrile was used as the non-eluting organic component of the mobile phase. Various 10 mM ammonium formate buffers adjusted to pH 3, pH 4.75, pH 6, pH 7 and pH 8 were tested as the eluting aqueous components of the mobile phase. At pH 3 and pH 4.75 both positive and negative mode ionization was tested, while at all other pH values, ionization was only performed in negative ion mode. Positive ionization mode measurement revealed the interference of multiply charged ions that complicates accurate detection, while the chromatographic performance was equally unsatisfactory in all pH conditions investigated. Gradient elution was tested on both columns, with starting conditions of 95% acetonitrile and 5% of the respective buffer and a subsequent linear gradient to 10% acetonitrile and 90% buffer. On the Nucleodur HILIC column, strong peak tailing and a lack of separation was observed, while peak broadening and insufficient retention was the main problem when using the ZIC-HILIC column. However, it must be mentioned that the standards were diluted in 60% methanol and not in the exact starting conditions.

On the third HILIC column (Waters Xbridge Amide), the separation of the two compounds was successful, even though severe peak tailing was observed for the oxidized form. This indicates that some unspecific interactions occur on this stationary phase, which could probably be eliminated by conditioning the column with e.g. phosphate buffer. However, a closer examination of the chromatographic separation revealed a more severe problem, namely that NADPH is oxidized on the packing material to a large extent. While a single standard of $NADP^+$ produced the expected single peak, a single standard of NADPH consistently resulted in a chromatogram with two peaks, one at the expected retention time of NADPH and a much larger one at the retention time of $NADP^+$. Extraction of the MS spectrum at these time points reveiled that the second peak actually corresponds to $NADP^+$ and indicates that oxidation occurs. Judging from the peak areas, more than 80% of NADPH is oxidized. Interestingly, this was only observed when the organic mobile phase was acetonitrile or isopropanol. However, even though oxidation was not observed when methanol was used as organic solvent in the mobile phase, neither one of the compounds was retained on the column in these conditions. This indicates that methanol is not a suitable organic solvent for the separation mechanism HILIC on this stationary phase. As a consequence of the findings described above, simultaneous measurement and quantification of $NADP^+$ and NADPH from the same sample would be impossible on this stationary phase, which makes this separation unsuitable for the aim of this work. Moreover, attempts to calibrate this method for the measurement of the NADP pool, i.e. the sum of $NADP^+$ and

NADPH in the sample, were marked by memory effects, causing the signal to increase significantly in long-term measurements, as well as further unspecific interactions, so that a linearity of the calibration curve could not be established.

On porous graphitic carbon as stationary phase, both $NADP^+$ and NADPH were retained. The crystalline surface of this stationary phase is lined with hexagonally arranged carbon atoms without any pores or chemically bound ligands. Retention on this stationary phase is based on dispersive and charge-induced interactions of the analyte with the polarized graphite surface. In the case of $NADP^+$ and NADPH, the interaction with the stationary phase was found to be so strong that the two compounds could not be eluted properly. Among the mobile phases tested were 10 and 20 mM ammonium formate buffers adjusted to pH 9 as well as a similar buffer of higher molarity that was prepared by adjusting a 0.3% formic acid solution to pH 9.0 with ammonia, as published by Pabst *et al.* [34]. As organic mobile phase, either acetonitrile or methanol was used. Moreover, both “reversed-phase” and “HILIC-mode” gradients as well as isocratic elution were tested. Separation or even elution of the two compounds from the Hypercarb column was not successful in any of the tested conditions. The difficulties experienced in these experiments are in accordance with the findings of Pabst *et al.*, who reported that very high ionic strength is required to elute strongly retained compounds like NADP from a PGC stationary phase [34]. While chromatography can be run in these conditions in principle, solvents of high ionic strength may cause severe problems like ion source pollution and signal suppression in most ESI-MS instruments and also the one used in this work. There are additional reports about a near-total oxidation of the reduced NADPH on this stationary phase [personal communication].

The only successful chromatographic separation in suitable conditions was achieved on a reversed-phase stationary phase (Waters Atlantis T3). The packing material of this column was designed to be compatible with highly acqueous mobile phases, i.e. it is 100% wettable and suitable for the separation of both polar and hydrophobic analytes [49]. Because of the high polarity and charge state of the two analytes, they are hardly retained on common reversed-phase stationary phases and elute at an organic content in the mobile phase of less than 20% methanol. The special characteristics of the Waters Atlantis column allow the employment of these highly aqueous conditions in which $NADP^+$ and NADPH are retained and separated. This would not be possible on most other reversed-phase stationary phases since the alkyl chains tend to collapse or aggregate due to hydrophobic interactions in a highly aqueous environment. However, it was observed that even less than 10% of organic solvent in the injection solvent lead to a near-complete loss of retention on the reversed-phase stationary phase. This requires the samples to be free of organic solvent, which has to be considered in the choice of extraction protocol (see 4.3). A more detailed description of

the reversed-phase separation in combination with MS detection is given in the following section.

4.1.3 Reversed-phase LC-MS method for quantifying NADP

Concluding the results presented in 4.1.1 and 4.1.2, an LC-MS method combining reversed-phase chromatographic separation and TOF-MS or QQQ-MS detection was established. The chromatographic separation of the two compounds on the silica-based Atlantis T3 column (Waters) allows simultaneous analysis of $NADP^+$ and NADPH from the same sample within a total run time of 12 min in MS-friendly solvents. As can be seen in the sample chromatogram shown in Figure 7, both compounds elute as separate sharp peaks at approximately half the total run time. The retention times of the two analytes were stable after sufficient pre-run equilibration of the column, but were slightly different between the LC-TOF-MS and LC-QQQ-MS system used (see Table 6). This is an instrument-specific effect caused by the different void volumes of the two HPLC systems rather than a method-specific problem. Table 6 gives an overview on the basic analytical characteristics of the reversed-phase LC-MS method.

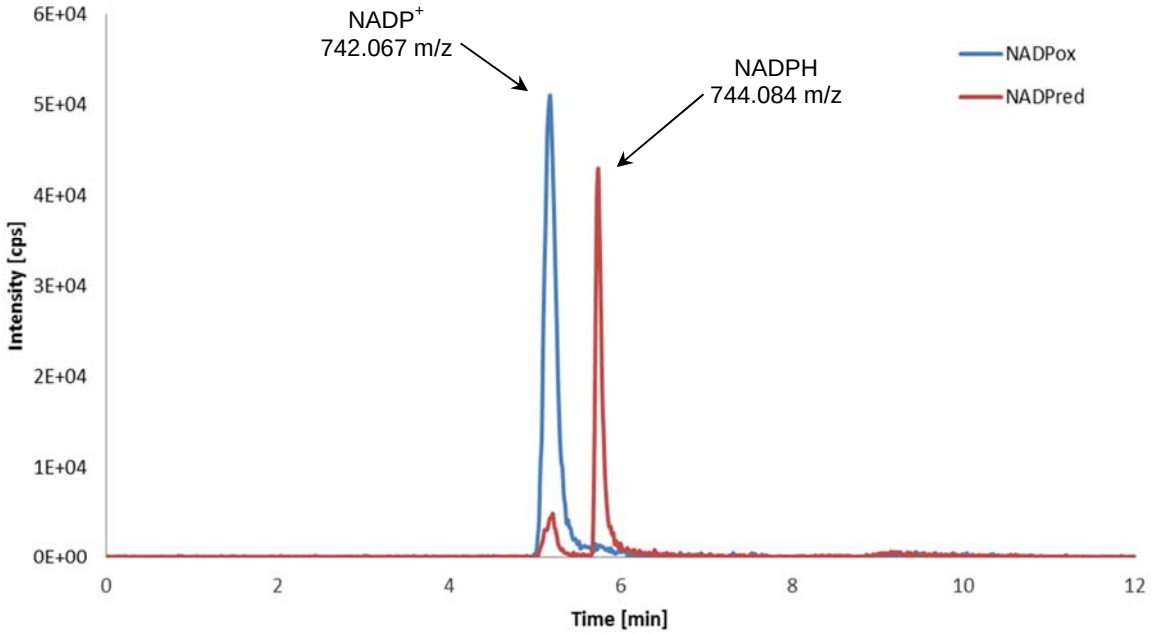

Figure 7: Reversed-phase separation of a standard mix of $NADP^+$ and NADPH (5 µM each) using an Atlantis T3 column (2.1 x 150 mm, 3 µm particle size, Waters). The signal was detected using an Agilent 6210 series Time-of-Flight mass spectrometer with electrospray ionization coupled to an Agilent 1200 series binary HPLC system.

The LC method was coupled with TOF-MS for method development as well as qualitative measurements such as stability evaluations. The LC-QQQ-MS system with its higher sensitivity and extended linear dynamic range, however, was used for the quantification of $NADP^+$ and NADPH. A calibration based on mixed standard solutions of $NADP^+$ and NADPH

in 5 mM ammonium acetate buffer (pH 8.0) was performed on both LC-MS systems. The respective calibration curves are shown in Figure 8, Figure 9, Figure 10 and Figure 11. Linear regression was used to fit a linear function to the measurement points. R^2 values of greater than 0.99 in all cases indicate a very good fit and confirm the linear correlation between the sample concentration and the respective signal response. The determination of the sample content was then based on the equation describing this linear function, which was used to convert the response obtained from a certain sample into the respective concentration of $NADP^+$ or NADPH. The limit of detection (LOD) and limit of quantification (LOQ) was also determined for both compounds on both LC-MS systems based on the noise height observed in the analysis of a 1 µM standard of the respective compound. The resulting values are given in Table 6. In comparison, the LOD and LOQ values for the LC-QQQ-MS system were much lower than those determined on the LC-TOF-MS system. This confirms the higher sensitivity of the LC-QQQ-MS system and its suitability for quantification.

Table 6: Analytical characteristics of the presented LC-ESI-MS method for the LC-TOF-MS and LC-QQQ-MS systems.

	Time-of-flight mass spectrometer				
Analyte	Retention time [min]	Capacity factor k'	LOD[1] [nM]	LOQ[2] [nM]	RSD[3] (N = 5)
$NADP^+$	5.2	2.74	160	540	6.04%
NADPH	5.7	3.10	240	780	6.99%
	Triple quadrupole mass spectrometer				
Analyte	Retention time [min]	Capacity factor k'	LOD [nM]	LOQ [nM]	RSD (N = 5)
$NADP^+$	3.5	1.08	8	25	3.4%
NADPH	4.9	1.92	30	100	4.5%

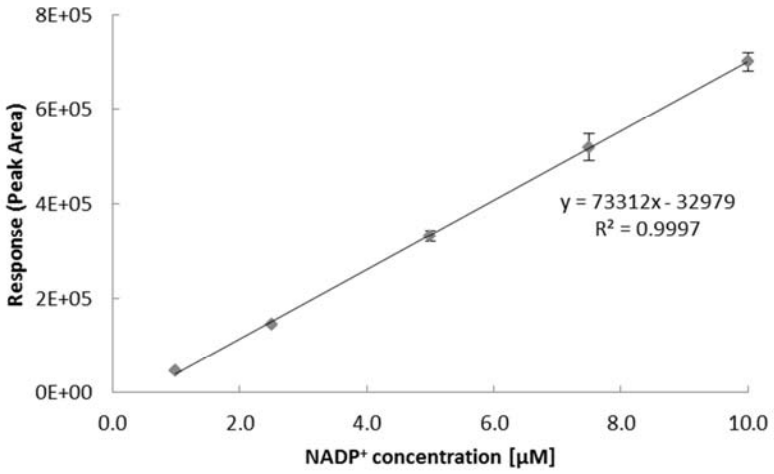

Figure 8: Calibration curve for the quantification of $NADP^+$ in the range of 1-10 µM in ammonium acetate buffer (pH 8.0) using the presented reversed-phase LC-ESI-QQQ-MS method recorded in triplicates on a Thermo TSQ Vantage Triple Quadrupole mass spectrometer.

[1] LOD … limit of detection
[2] LOQ … limit of quantification
[3] RSD … relative standard deviation

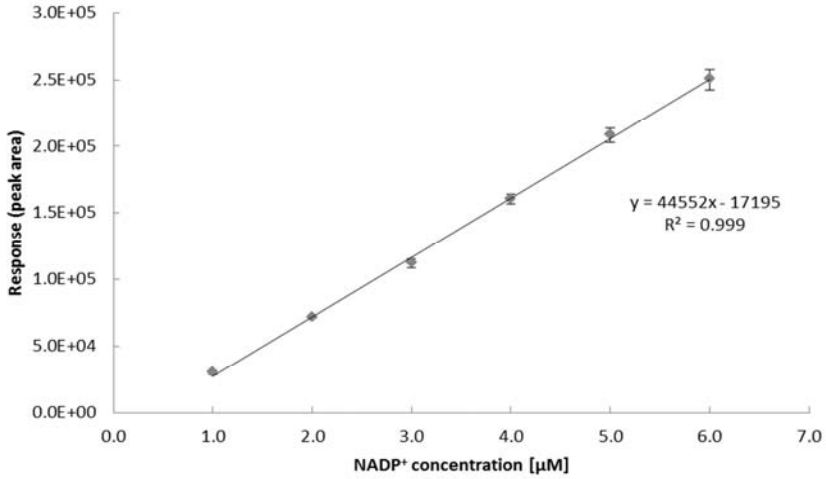

Figure 9: Calibration curve for the quantification of $NADP^+$ in the range of 1-6 µM in ammonium acetate buffer (pH 8.0) using the presented reversed-phase LC-ESI-TOF-MS method recorded in triplicates on an Agilent 6210 series series mass spectrometer.

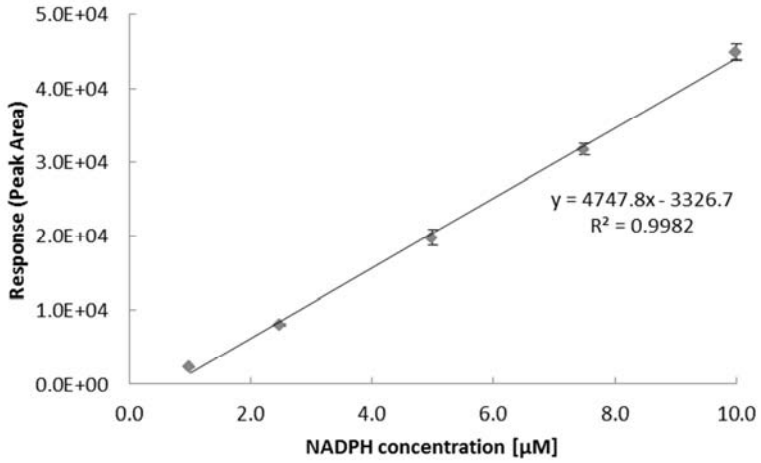

Figure 10: Calibration curve for the quantification of NADPH in the range of 1-10 µM in ammonium acetate buffer (pH 8.0) with the presented reversed-phase LC-ESI-QQQ-MS method recorded in triplicates on a Thermo TSQ Vantage Triple Quadrupole mass spectrometer.

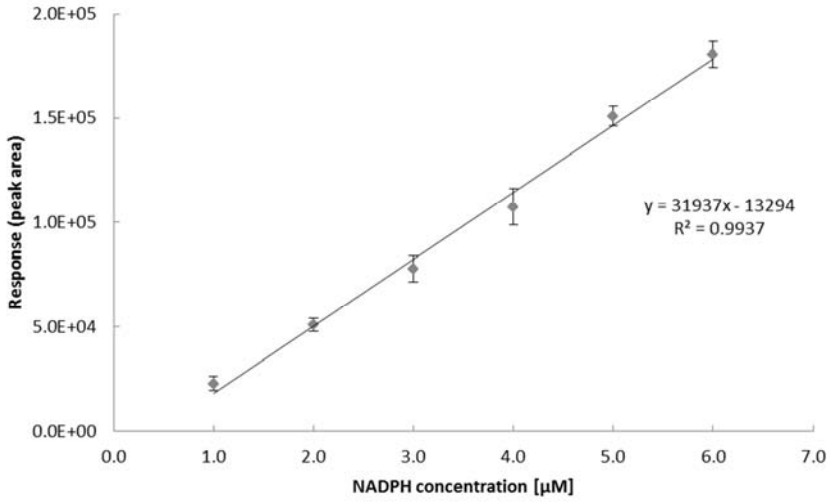

Figure 11: Calibration curve for the quantification of NADPH in the range of 1-10 µM in ammonium acetate buffer (pH 8.0) with the presented reversed-phase LC-ESI-QQQ-MS method recorded in triplicates on a Thermo TSQ Vantage Triple Quadrupole mass spectrometer.

Internal standardization was not used in this work. As explained in 2.3, a suitable internal standard for this work would be isotopically labeled $NADP^+$ and NADPH. Unfortunately, these compounds are not commercially available due to their inherent instability and laborious synthesis. A uniformely ^{13}C-labeled cell extract of *Pichia pastoris* is used for similar measurements, as published by Neubauer *et al.* [30]. Analysis of this extract in an appropriate dilution showed that the NADP levels are generally low. Especially the signal obtained for $U^{13}C$-NADPH was very low (estimated concentration 2 µM) in the tested aliquot. This may be a consequence of the drying step in the boiling ethanol extraction procedure applied in the preparation of the uniformely ^{13}C-labeled cell extract [30]. As illustrated in more detail in 4.3.1, the drying of the extract under reduced pressure causes substantial losses of both forms of NADP. The signal obtained for $U^{13}C$-$NADP^+$ was up to 10 times higher (estimated concentration 7 µM). This does not reflect the ratio of NADPH and $NADP^+$ that is to be expected in biological samples according to literature (see 2.1). There are several possible reasons for this observation, which probably all contributed to the final result. First of all, NADPH is consumed in the cell as a response to oxidative stress (see 2.1), which may occur during sampling. Secondly, the extraction solvent used (75% v/v ethanol) is not buffered, so that pH-mediated oxdiation is to be expected to some degree (see 4.2). Last but not least, the ethanolic extract as well as the dried extract is usually stored for several days or weeks. While this is adequate for other compounds, NADP is highly instable in solution, as is described in 2.2. Considering that the extract would be furtherly diluted in the sample when used as internal standard, the resulting signal will be below the detection limit of the method. All these factors make an extract prepared according to [30] unsuitable as internal standard for the analysis of $NADP^+$ and NADPH. Nonetheless, the situation may be different when such an extract is prepared in conditions optimized for NADP.

In the present work, true internal standardization could not be performed for the reasons named above. In order to still have a tool for monitoring and assessment of the general measurement and instrument performance, $^{15}N_5$-5'AMP was added to all samples. However, since $^{15}N_5$-5'AMP is more stable than NADPH, it was not used as internal standard but rather as a monitoring tool. Data was only used for further evaluation, when the signal obtained for $^{15}N_5$-5'AMP was in an accecptable range.

4.2 Investigation of stability

For evaluation of the pH-stability of NADP standards, 5 µM standard solutions of $NADP^+$ and NADPH were prepared separately in 5 mM ammonium acetate buffers adjusted to pH 4.0, pH 6.0 and pH 8.0 respectively. The results of the repeated measurement within a time frame of 8 h are shown in Figure 13 and Figure 12. The samples were kept at 6℃ in the thermostated autosampler during the experiment.

At pH 4.0, the oxidized $NADP^+$ was found to be stable, with values ranging from 85.8% to 101.2% of the initial response and a relative standard deviation (RSD) of 4.9% within 11 injections in 8 hours (Figure 13). As expected, NADPH showed a very different behaviour in these conditions. During 8 hours, significant degradation was observed (Figure 12). In the last injection, the signal was only 18.1% of the initial response. The resulting half-life time of NADPH was calculated as 176.2 min at pH 4.0 in a 5 mM ammonium acetate buffer. As the NADPH signal decreased, another peak arose in the chromatogram directly after the original peak (not shown). The extracted MS spectrum shows that this peak corresponds to the same mass, which in combination with the fact that this unknown compound is slightly more retained on the stationary phase points towards a conformational change in the NADPH molecule, induced by the low pH. Because of the apparent degradation, pH 4.0 is not in the desired working range, therefore the appearance of this isoform was not investigated furtherly.

$NADP^+$ was also stable at pH 6.0 in this experiment, with values ranging from 96.2% to 109.4% (Figure 13). The relative standard deviation was 4.4% in 10 injections during 8 hours. The more sensitive NADPH showed an improved stability at this pH, but the signal still showed a significant decrease to 77% of the initial response in 8 hours (Figure 12), corresponding to a half-life time of more than 800 min in a 5 mM ammonium acetate buffer at pH 6.0. At this pH, conformational changes or molecular rearrangements resulting in the appearance of either of the masses at a different retention time in the chromatogram were not observed.

The best results were obtained in a 5 mM ammonium acetate buffer at pH 8.0. $NADP^+$ was stable in these conditions (Figure 13), with values ranging from 97.4% to 110.7% and an RSD of 4.5% in 17 injections during 8 hours. In contrast to any other pH range tested, NADPH was found to be stable during 8 hours at alkaline pH (Figure 12). The values ranged from 99% to 111% of the initial response, resulting in an RSD of 4.5%.

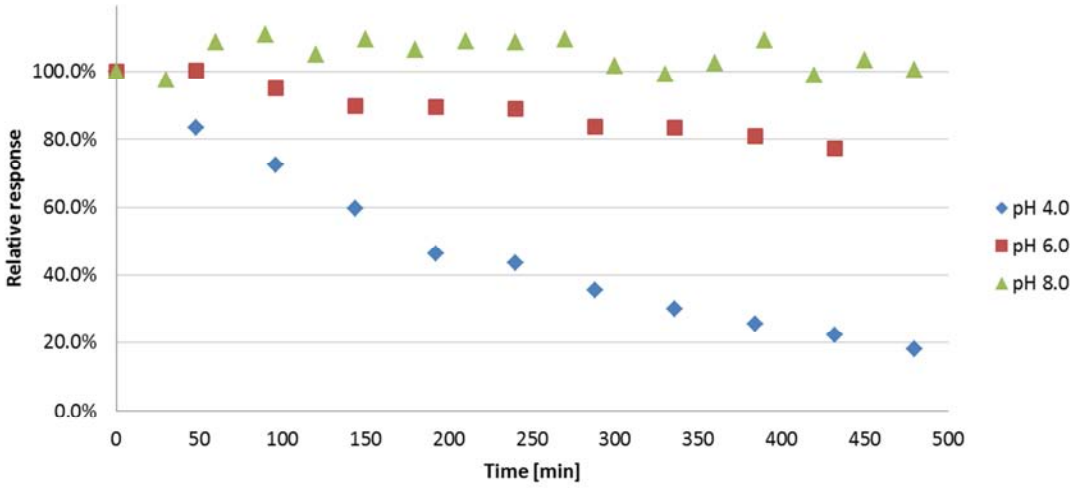

Figure 12: Investigation of the influence of pH on the stability of NADPH. 5 µM standard solutions of NADPH were prepared separately in 5 mM ammonium acetate buffers adjusted to pH 4.0, pH 6.0 and pH 8.0, respectively and analyzed repeatedly during 8 hours using an Agilent 6210 series ESI-MS-TOF coupled to an Agilent 1200 series binary HPLC system.

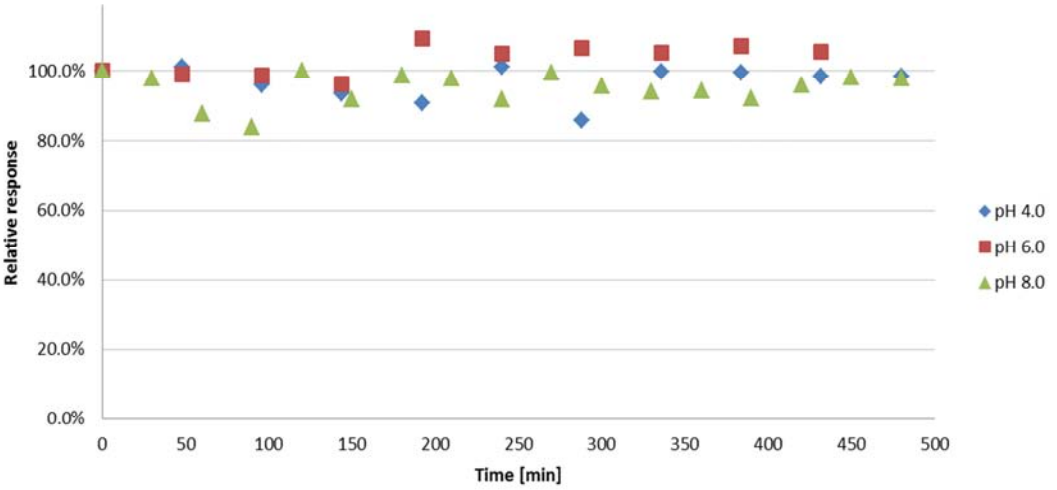

Figure 13: Investigation of the influence of pH on the stability of $NADP^+$. 5 µM standard solutions of $NADP^+$ were prepared separately in 5 mM ammonium acetate buffers adjusted to pH 4.0, pH 6.0 and pH 8.0, respectively and analyzed repeatedly during 8 hours using an Agilent 6210 series ESI-MS-TOF coupled to an Agilent 1200 series binary HPLC system.

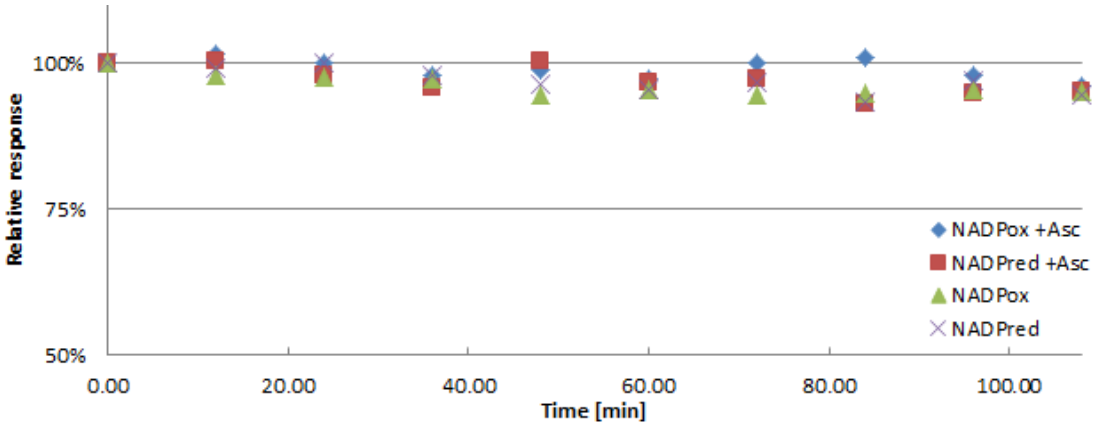

Figure 14: Evaluation of the influence of ascorbate on the stability of a mixed standard of $NADP^+$ and NADPH in 10 consecutive injections, corresponding to a time frame of approximately 2 hours. For comparison, one standard mix was prepared in 5 mM ammonium acetate buffer at pH 8.0 and another one in the same buffer but with the addition of ascorbate to a final concentration of 50 µM.

All these findings are in accordance with the conclusions that Wu *et al.* drew in 1986 from an investigation of the influence of solvent pH, temperature and ionic strength on the stability of NADPH, where a 5-fold reduction of the rate constants of degradation was found for every pH unit increase between pH 3 and 7.5 while for higher pH values, the results indicated that degradation occurs by different mechanisms and thus showed different kinetics [6]. The influence of temperature on the stability of NADPH was characterized by a 2.7 fold increase in the rate of degradation for every 10℃ increase in temperature [6].

In addition to the choice of buffer pH for optimum standard stability, the effect of the addition of ascorbate as antioxidant was evaluated. Ascorbic acid is a water-soluble ketolactone that contains two ionizable hydroxyl groups, of which one is deprotonated in physiological conditions [50]. Ascorbate ($AscH^-$) readily donates one or two electrons, which makes it an excellent electron donor and very effective antioxidant [50]. Ascorbate was added to a 5 mM ammonium acetate buffer adjusted to pH 8.0 in an excess of approximately 10 times with respect to the analyte concentration. Mixed standards containing $NADP^+$ and NADPH in this solvent were analyzed in 10 consecutive injections. Comparison with the measurement of standards without ascorbate showed that ascorbate does not influence standard stability during 10 consecutive injections, corresponding to a time frame of approximately 2 hours (Figure 14). The values varied between 94.6 and 98.0% of the initially detected signal from a freshly prepared standard without ascorbate. No significant difference, i.e. only a minor improvement in stability was seen in the solutions with ascorbate, with a recovery of 96.2 – 101.5% of the initial signal. Similarly, it was assessed that degassing the ammonium acetate buffer by ultrasonication has no significant influence on the standard stability (not shown). This indicates that in standard solutions, the reduction and oxidation of NADP is stabilized in the buffer at pH 8.0, even when no other antioxidant measures are taken. Based on these results, a 5 mM ammonium acetate buffer at pH 8.0 was chosen as suitable buffer for standard preparation.

4.3 Metabolite extraction

As described in 2.4, metabolite extraction and sample preparation are maybe the most critical aspects of the analytical workflow. An inappropriate extraction protocol may not only fail to give access to the metabolite pool in question but may also promote a loss of extracted metabolites in unfavourable conditions. Especially for sensitive metabolites like $NADP^+$ and

NADPH, a detailed study of the compatibility and suitability of various extraction protocols is necessary.

In this work, five different approaches to the extraction of $NADP^+$ and NADPH were tested. In two separate experiments, both the extraction recovery and the extraction efficiency were determined and evaluated to find an extraction and sample preparation protocol that is suitable for the subsequent LC-MS analysis. The parameters evaluated in these studies were (a) the metabolite recovery, i.e. the stability of the two compounds in the extraction conditions, (b) the extraction efficiency, i.e. the success of cell lysis, and (c) the compatibility with the subsequent LC-MS analysis. The following figures lead through the five principal extraction procedures that were tested. All these extraction procedures are listed and described also in 3.3.1.

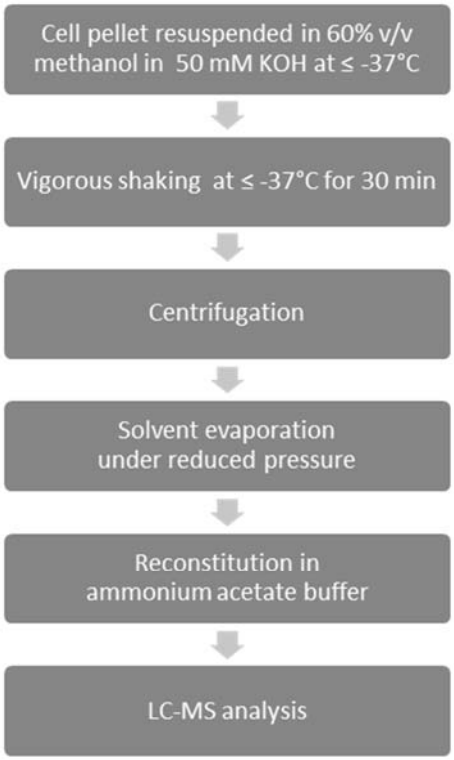

Figure 15: Schematic workflow of the alkaline cold methanol extraction using 60% methanol in 50 mM KOH as extraction solvent. LC-MS analysis was performed according to 3.5.1.

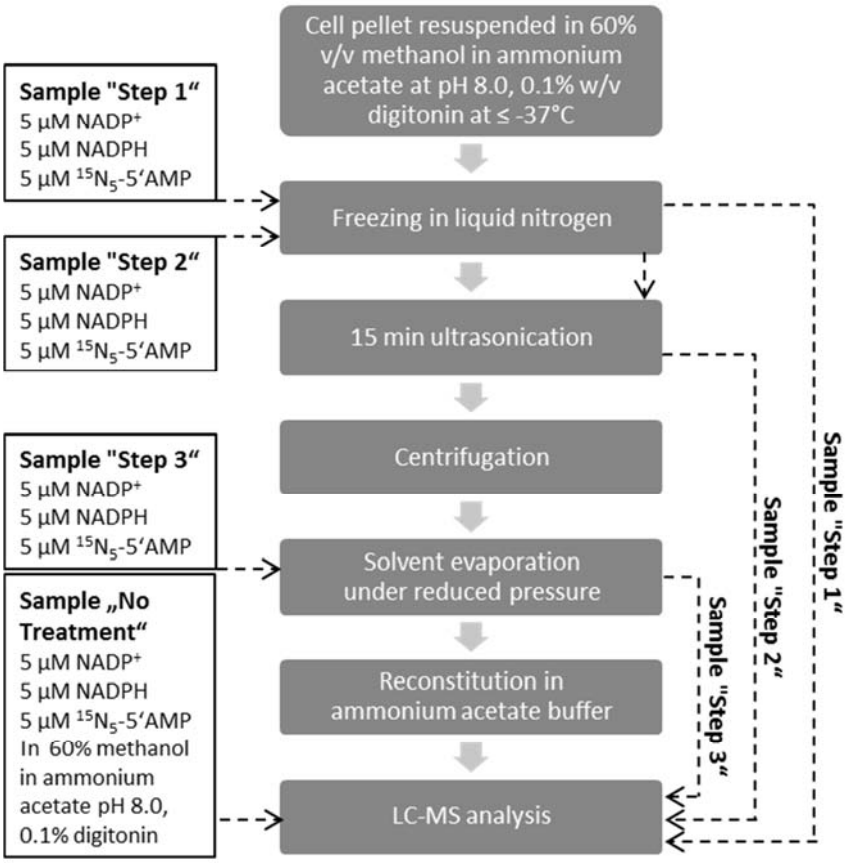

Figure 16: Schematic workflow of the freeze-thawing extraction procedure. The extraction solvent, was 60% v/v methanol in 5 mM ammonium acetate pH 8.0 with or without 0.1% w/v digitonin. LC-MS analysis was performed according to 3.5.1. Broken lines indicate the sample treatment for the determination of the standard recovery.

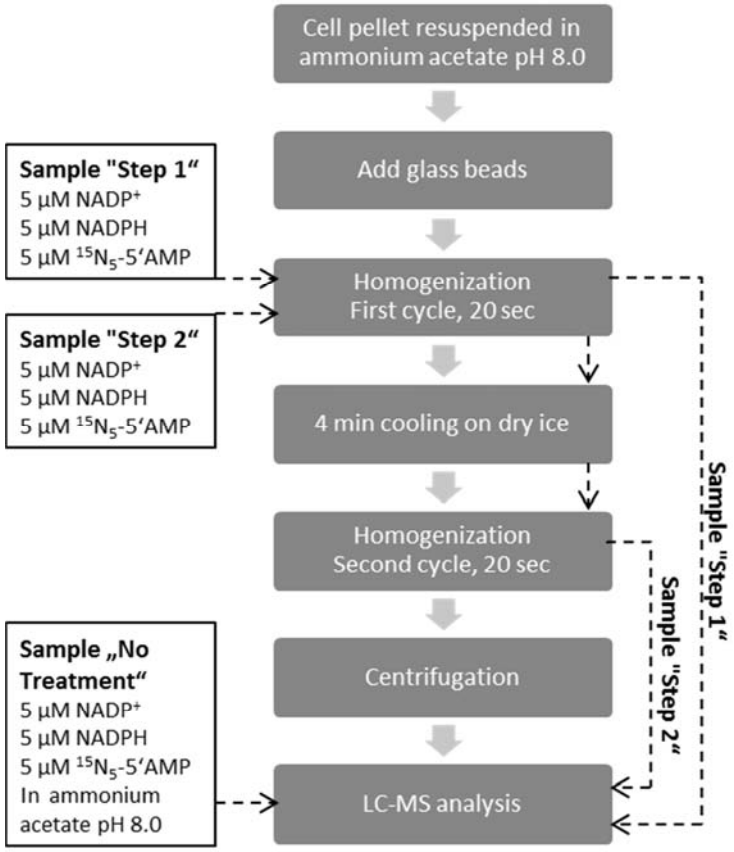

Figure 17: Schematic workflow of the mechanical cell lysis and extraction prodedure. The extraction solvent was 5 mM ammonium acetate pH 8.0 with or without 0.1 mM GSH and 1 mM GSSG as redox buffer. LC-MS analysis was performed according to 3.5.1. Broken lines indicate the sample treatment for the determination of the standard recovery.

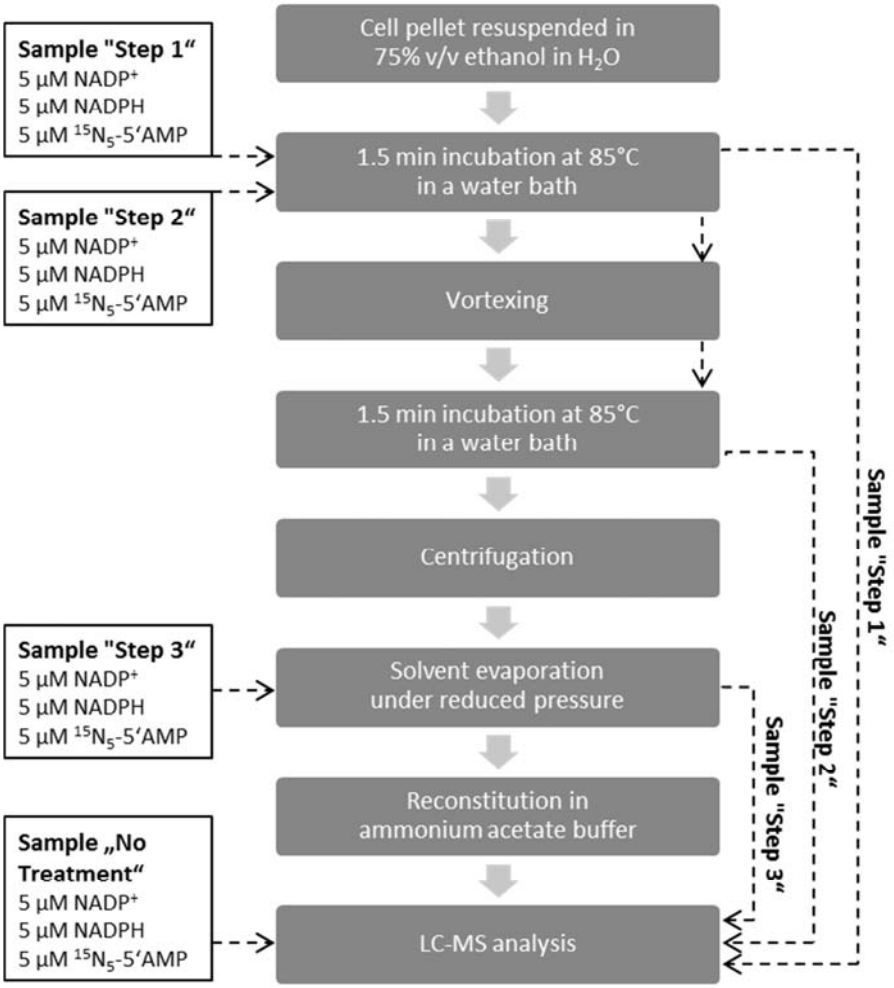

Figure 18: Schematic workflow of boiling ethanol extraction procedure. The extraction solvent, was 75% v/v ethanol in H_2O. LC-MS analysis was performed according to 3.5.1. Broken lines indicate the sample treatment for the determination of the standard recovery.

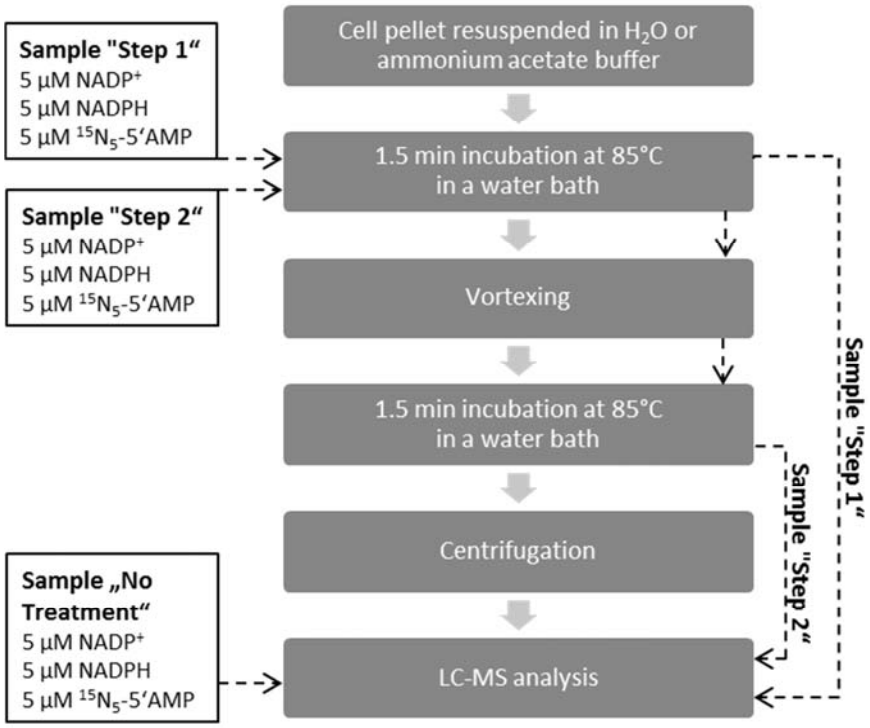

Figure 19: Schematic workflow of the hot aqueous extraction procedure. The extraction solvent, was 100% H_2O or 5 mM ammonium acetate, pH 8.0. LC-MS analysis was performed according to 3.5.1. Broken lines indicate the sample treatment for the determination of the standard recovery.

4.3.1 Extraction recovery

In order to monitor the stability of $NADP^+$ and NADPH during the tested extraction procedures, a standard recovery experiment was performed for four different extraction protocols, namely the freeze-thawing extraction with methanol, boiling in ethanol, aqueous hot extraction and mechanical lysis using glass beads. Compound stability information about the individual steps of each protocol allows the identification of critical steps that introduce either a source of greater uncertainty or loss of the target analyte. The extraction protocol using cold methanol and shaking at ≤-37℃ was not included in this study since preliminary experiments had shown that the high salt content of the extracts interferes with the chromatographic separation on the chosen stationary phase. Additionally, flow injection delivers the analyte to the ion source of the MS without chromatographic separation, where such a high salt content would cause signal suppression and falsified results.

Separate 5 µM mixed standard solutions were prepared for the critical steps listed in Table 3. For each of these steps, fresh standard solutions were analyzed in triplicates after treatment in flow injection MS analysis as described in 3.3.2. By comparing the signal obtained after treatment with the signal obtained from a standard without treatment, specific losses during the respective steps of the protocol can be identified. The schematic views of the evaluated extraction protocols in the figures above also show the samples investigated in this recovery study and how they were treated. The averaged results are given in Figure 20 for $NADP^+$ and in Figure 21 for NADPH.

As an example for cold extraction, a freeze-thawing protocol using buffered 60% methanol with the addition of Digitonin as extraction solvent (see Figure 16) was examinated. Figure 20 and Figure 21 show that both forms of NADP are relatively stable in these conditions. Thawing was performed at room temperature and by ultrasonication in separate samples (bars 2 and 3 in Figure 20 and Figure 21). The recoveries for the oxidized $NADP^+$ were 96.8 ± 3.1% for thawing at room temperature and 89.8 ± 1.0% for thawing by ultrasonication. Comparison of the two values indicates that ultrasonication causes a minor loss of $NADP^+$. Overall, the reduced NADPH seems to be less sensitive to the freezing and subsequent thawing, as indicated by the recovery of 103.9 ± 4.4% for thawing at room temperature and 98.2 ± 2.8% for thawing by ultrasonication. However, during the last step of the protocol, the drying of the extract under reduced pressure, only 65.2 ± 0.2% of $NADP^+$ and 49.8 ± 1.3% of NADPH could be recovered. This indicates that this final step of the protocol is the most critical step in this protocol that significantly decreases the extraction yield. Suitable internal standards would allow for the correction of this effect and accurate quantification despite

these losses. However, since isotopically labeled NADP standards are not available due to instability, this problem hinders accurate quantification.

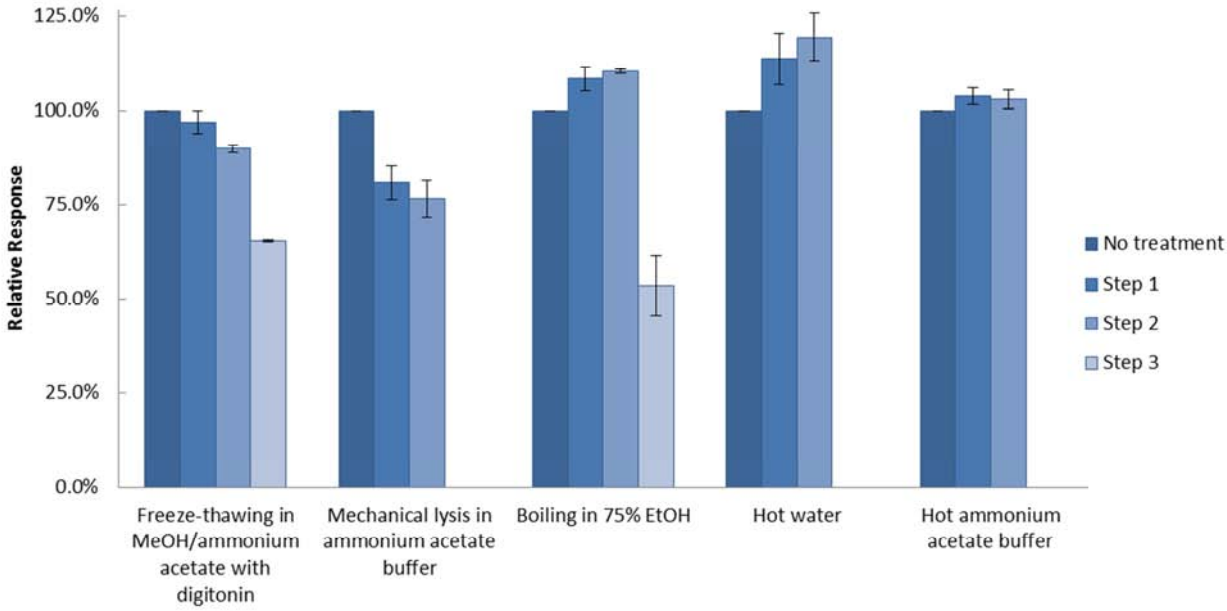

Figure 20: Investigation of the standard recovery for $NADP^+$ in 5 different extraction protocols. Each bar corresponds to a certain step of the extraction protocol (see Table 3). Step 3 (drying under reduced pressure) was not necessary for all extracts. Separate 5 µM standard solutions in the respective extraction solvent were prepared in triplicate for each step of the respective protocol. After treatment, the samples were analyzed by flow injection using a Thermo TSQ Vantage ESI-QQQ-MS.

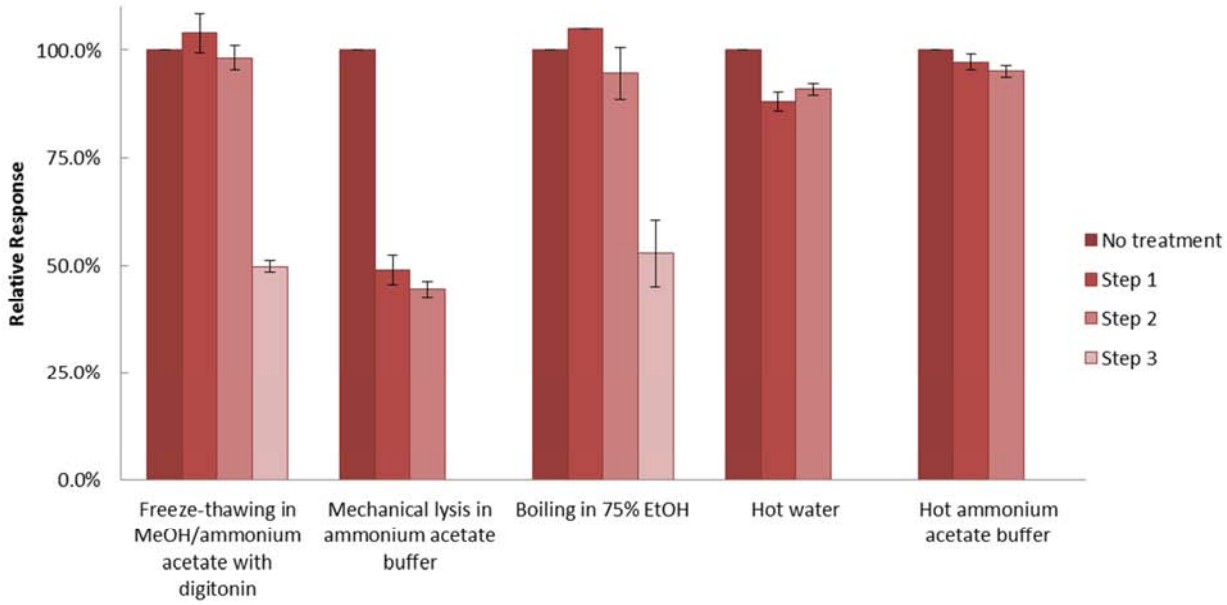

Figure 21: Investigation of the standard recovery for NADPH in 5 different extraction protocols. Each bar corresponds to a certain step of the extraction protocol (see Table 3). Step 4 (drying under reduced pressure) was not necessary for all extracts. Separate 5 µM standard solutions in the respective extraction solvent were prepared in triplicate for each step of the respective protocol. After treatment, the samples were analyzed by flow injection using a Thermo TSQ Vantage ESI-QQQ-MS.

During the mechanical cell lysis and extraction protocol (see Figure 17), the two NADP forms showed different behaviour, as can be seen in Figure 20 and Figure 21. While 80.8 ± 4.4% of the oxidized $NADP^+$ was still detectable after the first homogenization cycle, nearly 50% of the reduced NADPH was lost in the same step (49.1 ± 3.4% recovery). While the loss of $NADP^+$ is probably mostly due to the mechanical forces such as shear stress involved in this type of cell lysis, this doesn't seem to be the only explanation for the loss of NADPH. Mechanical cell lysis according to this protocol is performed by mixing the sample at high speed, which creates turbulences and also introduces a lot of air into the sample. Given that NADPH is sensitive to oxidation, this in combination with the shear forces seems to result in the observed metabolite loss during cell lysis. The number of cycles and thus the duration of homogenization however does not seem to have a major influence in this protocol. The standard recovery after two cycles à 20 s was comparable to the one after only one cycle, with 76.5 ± 4.8% of the initial $NADP^+$ and 44.5 ± 1.9% of the initial NADPH response, respectively. An additional drawback of this protocol is the fact that a close control of the temperature is not possible during the homogenization cycles. This is a possible problem in biological samples, since cell lysis includes the release of enzymes, proteins and other cell constituents into the extract. The samples are cooled on ice or dry ice immediately after each cycle, but the overall conditions in combination with the absence of any organic solvent allow some enzymes to stay active, which may add to the previous losses of NADPH by enzymatic consumption. The presence of proteins in the extract is also a possible problem for the chromatographic separation, where they may interfere with the interactions between the analyte and the stationary phase or cause signal suppression in MS analysis. Since the solvent for this extraction protocol does not contain any organic solvent, it is in principle compatible with the desired LC-MS system, but the problems described above prevail.

The three "hot" extraction protocols shown in Figure 18 and Figure 19 were highly similar in their recovery pattern, as can be seen in Figure 20 and Figure 21. Both $NADP^+$ and NADPH were surprisingly stable during the whole procedure. This was not expected since the available data about the stability of NADP suggests otherwise [6] [7]. However, there does not seem to be a significant loss of either form of NADP. $NADP^+$ was recovered to 110.4 ±0.6% after 4.5 min of boiling in ethanol, 119.4 ± 6.2% in water and 103.0 ± 2.5% in 5 mM ammonium acetate buffer (pH 8.0) as extraction solvent. NADPH recoveries were 94.5 ± 6.0% in ethanol, 90.9 ± 1.4% in water and 95.0 ± 1.5% in 5 mM ammonium acetate buffer (pH 8.0). These results in addition with the positive aspects of high temperatures like enzyme inactivation justify the use of this protocol for the extraction of $NADP^+$ and NADPH as for example in [29], [30] and [31]. Of course, for analysis according to 3.5.1 the sample must be free of organic solvent (as described in 4.1.2), so the ethanolic extract was dried under

reduced pressure. The standard recovery for this step was comparable with the freeze-thawing extract, with a loss of approximately 50% for $NADP^+$ and NADPH, respectively.

Based on these results, either buffered or un-buffered hot water extraction seems most favorable for the extraction of NADP from *Pichia pastoris* for subsequent LC-MS analysis. The ethanolic boiling protocol showed a much higher variability in the standard recovery experiment and has the additional drawback that the ethanolic solvent has to be evaporated prior to analysis with the presented separation method.

4.3.2 Extraction efficiency

A set of homogenous *Pichia pastoris* cell pellets of 10 mg cell dry-weight from a fed batch culture was used for this experiment. Four different extraction solvents and in total nine different variants of extraction protocols were tested on this sample set in three replicates each. The averaged results for the total NADP, i.e. the sum of reduced and oxidized NADP from the LC-MS measurement of these extracts are shown in Figure 22.

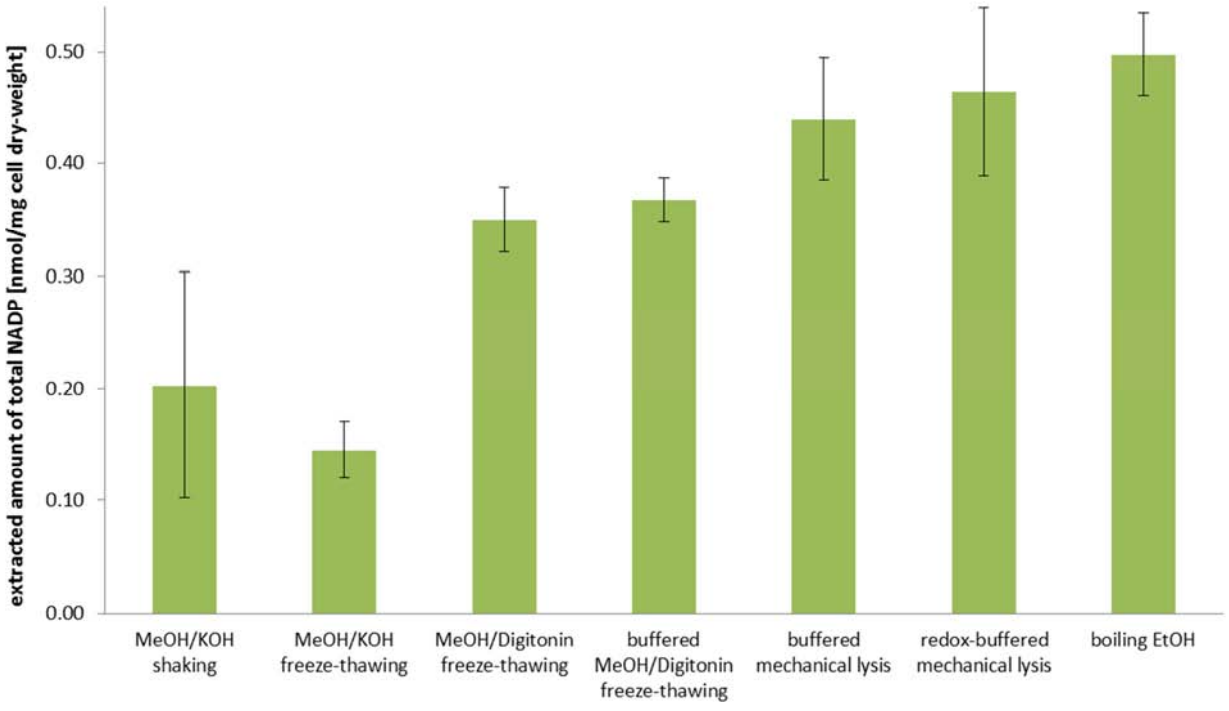

Figure 22: Investigation of the extraction efficiency for $NADP^+$ and NADPH using different extraction protocols. A homogenous sample set of *Pichia pastoris* was used for this study. Each protocol was tested in triplicates and the extracted amount of each compound was related to the amount of biomass used for the extraction. The presented results for the total NADP, i.e. the sum of oxidized and reduced NADP, are averaged values with the standard deviation represented by the error bars.

The available data about the temperature-dependent stability of NADPH (see section 2.2) suggests the use of a cold extraction protocol. These protocols are typically carried out at very low temperatures, e.g. -40℃ or even lower, with methanolic extraction solvents. In this work, several variants of cold extraction protocols were evaluated. Reports and data in literature (see 2.2) as well as the findings presented in 4.2 indicate that extraction should be performed in alkaline conditions to ensure stability of the reduced form of NADP. Potassium hydroxide (KOH) is one of the standard chemicals used in alkaline cell lysis. In the extraction protocols applied here, it was combined with methanol in an extraction solvent consisting of 60% v/v methanol in H_2O with 50 mM KOH. This mixture was used both in a shaking (see Figure 15) and a freeze-thawing protocol (see Figure 16). In comparison with the results from the other extraction procedures, the extraction yields from these two protocols were outstandingly low, with less than 0.2 nmol of NADP extracted per mg cell dry-weight. Moreover, the results show a low reproducibility and a high relative standard deviation of 49.7% for the shaking at -40℃ and 17.3% for the freeze-thawing protocol. Moreover, the chromatographic separation suffered from shifts in the retention time of both compounds and partial peak splitting, both of which were probably caused by the high pH that may have induced further deprotonization of the compounds. The high salt content may also have caused signal suppression in MS analysis, which explains the low yield in comparison to the other protocols.

Freeze-thawing according to Figure 16 was also used in two other extraction methods, but with a slightly different solvent consisting of 60% v/v methanol and Digitonin, a mild non-ionic detergent that permeabilizes the cell membrane. For one assay, the methanolic extraction solvent was buffered with 5 mM ammonium acetate, pH 8.0, while the other one was unbuffered. Compared to the alkaline extracts, the extraction yield was significantly higher with this solvent. Furthermore, the relative standard deviation was much smaller (8.2% for the unbuffered solvent and 5.2% for the buffered solvent), which indicates good reproducibility. Overall, buffering the extraction solvent did not significantly improve the extraction yield.

Another typical variant of extraction protocols is mechanical cell lysis with glass beads. While this method is thought to be very efficient in lysing cells, it involves vigorous mixing and introduces a lot of oxygen into the solution, which is not favourable for the stability of oxidation-sensitive compounds like NADPH. Nevertheless, the yield obtained from this extraction was among the highest ones achieved in the whole experiment. For comparison, the same protocol was performed with a slightly different extraction solvent, where glutathione was used as a redox buffer. The extraction solvent contained 0.5 µM GSSG and 50 µM GSH in addition to 5 mM ammonium acetate. An aliquot of the extract was filtrated to

remove proteins immediately after the second cycle. The overall NADP yield from these extractions was not significantly higher than the one from the samples that were not redox-buffered, but the standard deviation was much higher. Moreover, NADPH could not be detected from these samples (not shown). An interference of the redox buffer is in principle possible, since glutathione and NADPH interact in biological samples. The reduced form of glutathione, GSH, is easily oxidized by reactive oxygen species (ROS) and forms the dimeric oxidized form GSSG. The enzyme glutathione reductase uses NADPH as cofactor for the reduction of GSSG to GSH [1] and may be present in the cell extract.

Somewhat surprisingly, considering data about the stability of NADPH, the highest yield of NADP was achieved with boiling ethanol extraction. Moreover, the standard deviation was 7.4% and thus among the smallest of all methods. In fact, the evaluation of the standard recovery presented in 4.3.1 shows that both compounds are stable in all steps of the protocol, except the final solvent evaporation step in which losses of up to 50% were observed. This extraction protocol is indeed well-established for the extraction of various intracellular metabolites from yeast cells in similar metabolomics studies. The benefits of this method are an effective cell lysis within a relatively fast and simple procedure and the inactivation of enzyme activity due to the high temperature. The major drawback of this method in the context of this work is the necessity of an additional solvent evaporation step, since the chromatographic system does not tolerate any organic fraction in the injection solvent. As mentioned above, this solvent evaporation step, typically performed under reduced pressure, introduces losses of up to 50% for both compounds and lengthens the sample preparation procedure.

In summary, the highest extraction yields for the total NADP were achieved with the procotols using mechanical cell lysis and high temperatures. The fact that these yields are similarly high suggests that near-total extraction was achieved, i.e. these protocols give access to the entire NADP pool of the cell. However, proof for this is hard to find. The cold extraction protocols resulted in lower yields, indicating a less effective cell lysis or an instability of either or both forms of NADP in the extraction conditions. Considering the standard recovery experiments presented in 4.3.1, where the mechanical cell lysis introduced substantial losses, hot extraction was the method of choice for further extraction experiments. To avoid a solvent evaporation step in the protocol, 5 mM ammonium acetate at pH 8.0 was used as extraction solvent. The resulting procedure is shown in Figure 19.

4.4 Evaluation of suitability

In order to test the ability of the presented method to reflect the NADP levels of the cell, two strains of *Pichia pastoris* were compared in a suitability study. One was a wild-type strain and the other a strain overexpressing glucose-6-phosphate dehydrogenase, an enzyme that catalyzes the reduction of $NADP^+$ to NADPH. Due to this overexpression, the cells of the overexpression strain should in theory yield higher NADPH levels than the wild-type. The samples were prepared as described in 3.2.2 and 3.3.1.5. Since the three samples were randomly taken from four independent culture flasks per strain, they can be considered as three biological replicates for each strain. The LC-MS measurement according to 3.5.1 was then performed in triplicates, corresponding to three analytical replicates per biological replicate.

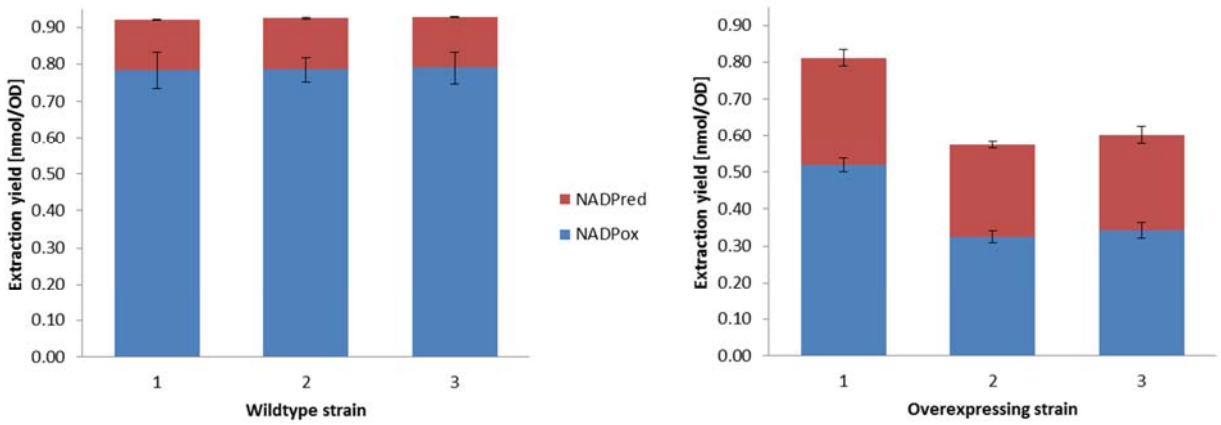

Figure 23: A wild-type strain was compared to a recombinant strain of *Pichia pastoris*. The recombinant strain overexpresses glucose-6-phosphate dehydrogenase and should thus contain more NADPH than the wild-type strain. The numbers 1-3 indicate the three biological replicates that were analyzed independently. The results shown are biomass-corrected and averaged over the results of the triplicate LC-MS/MS measurement with the error bars indicating the observed standard deviation.

Figure 23 shows the results from the LC-MS measurement of the three samples per strain. As can easily be seen, a difference in the relative abundance of $NADP^+$ and NADPH was observed. While in the wild-type strain, the ratio NADPH/$NADP^+$ was 0.18, the NADPH content was higher relative to the measured $NADP^+$ in the overexpression strain, resulting in an average ratio NADPH/$NADP^+$ of 0.69. This is what was expected, considering that the overexpressed glucose-6-phosphate dehydrogenase in the recombinant strain promotes the reduction of $NADP^+$ to NADPH.

The error bars in Figure 23 give additional information about the quality of the measurement. The overall yield of $NADP^+$ and NADPH is represented by the total height of the bar for each sample. In general, the extraction yield was consistently higher in the wild-type than in the

overexpression strain. This observation is most likely based on the negative effect that any overexpression has on the biomass yield and growth of an organism. The presented results have been corrected for the different cell density in the cultures between wild-type and recombinant strain. Within the same cultivation time, the overexpression strain had only grown to an OD of 6, while the wild-type reached an OD of 10. This already indicates a lower biomass yield and also seems to affect the intracellular NADP level.

For the wild-type strain, the overall yield was highly similar within the investigated sample set, with a relative standard deviation of only 0.3%. The results obtained from the samples of the overexpression strain were more divergent, with a relative standard deviation of 19.6% within the investigated samples. A closer look on Figure 23 reveals that the first sample yielded more NADP than the other two. This observation most likely has a biological reason, since the three samples represent three biological replicates, i.e. independent samples. From a biological point of view, this variation is not out of the ordinary, since the intracellular NADP levels are subject to fluctuations on a regular basis. From an analytical point of view, the signal for $^{15}N_5$-AMP that is used as monitoring tool in this LC-MS analysis and was added to the samples prior to extraction, showed no abnormalities. This allows the assumption that the sample was correctly treated and analyzed and that the difference in the resulting values is caused by biological variability. Any further clarification of the cause for the divergent results is not the scope of this work.

The repeatability of the measurement results, indicated by the observed relative standard deviation in the triplicate measurement of each sample, was very good for both $NADP^+$ and NADPH. Quite independent of the sample identity, the RSD was below 6.3% for $NADP^+$. For NADPH, the signals were slightly more divergent, with RSDs ranging from 1 to 9.1%. This may be a consequence of the relatively low signal intensity obtained from NADPH (see LOD values given in Table 6), where variabilities have a larger impact than at high signal intensities.

The biological reproducibility of the measurement of $NADP^+$ and NADPH can be evaluated from the overall RSD that was observed within the sample set (N=3) for each strain. The wild-type strain showed excellent reproducibility for both compounds, with an overall RSD of 0.4% for $NADP^+$ and 0.7% for NADPH. The variability was higher for the overexpression strain, with an overall RSD of 27.2% for $NADP^+$ and 8.4% for NADPH. The comparably high value for $NADP^+$ is, as is similarly explained above for the total NADP yield, caused by the higher-than-average amount found in sample 1 of the overexpression strain. Considering the very small size of the sample set and the inherent high variability of complex biological samples, this is still an acceptable result. If this obvious outlier is not taken into consideration, the overall RSD is 4.0% for $NADP^+$, representing a very good reproducibility.

The same samples were simultaneously tested with a commercially available enzymatic test kit (data not shown). Unfortunately, no reasonable results were achieved using the cell extracts from the presented hot aqueous extraction procedure. It seems that the conversion step that is supposed to convert $NADP^+$ to NADPH in order to allow its enzymatic determination did not work in the cell extracts. The assumption is that it is crucial to correctly adjust the conditions for the enzymatic conversion. Since the content of the reagents delivered with the kit is not specified, this is only possible when the extraction protocol defined by the enzymatic test kit is used. As it turns out, this protocol is not effective for the lysis of *Pichia pastoris*, so that overall, this enzymatic test kit is of limited use in this problem. Additional efforts could be made to adapt the sample preparation procedure, however, this was not in the scope of this work.

In summary, the results presented above show that without further improvement that is undoubtedly still necessary, the established workflow, including sample preparation and LC-MS/MS analysis, allows the evaluation of differences in the intracellular NADP levels between different strains. Both the analytical repeatability and the biological reproducibility were good in this experiment and the results reflected the expected differences in the NADP levels between the wild-type and the strain overexpressing glucose-6-phosphate dehydrogenase. However, internal standardization was not used in this experiment, so that a correction for compound-specific losses during sample preparation and analysis was not possible. Since the sample preparation process thus represents a black box and lacks traceability, the conclusions drawn from the presented results cannot be truly quantitative. However, the quality of the measurement and the results presented above indicate that the method is already highly fit for the qualitative evaluation of NADP levels.

5 CONCLUSION

The quantitative determination of $NADP^+$ and NADPH in yeast is a complex task. The main problems are the instability of the analytes and the lack of effective extraction protocols and analytical methods. The few reports in literature about measured intracellular NADP levels are highly divergent, indicating that there is no straight-forward solution to this problem.

The instability of NADPH under various conditions limits the available options for metabolite extraction. The target organism of this work, the yeast *Pichia pastoris*, has a relatively tough cell wall, so that cell lysis can only be achieved in relatively harsh conditions. State of the art in the extraction of intracellular metabolites from yeast for metabolomic studies is boiling in ethanol. In this work, this procedure was compared with cold and mechanical extraction. Hot extraction showed by far the best results concerning the overall extraction yield and the stability of both compounds in the extraction conditions. The extraction solvent of choice was a 5 mM ammonium acetate buffer at pH 8.0, in which $NADP^+$ and NADPH were shown to be stable. The resulting extraction procedure is fast, simple and reproducible.

The cell extracts were analyzed by LC-MS/MS for the determination of $NADP^+$ and NADPH. The state of the art in the chromatographic separation, as published in literature, is ion-pairing chromatography, hydrophilic interaction liquid chromatography or porous graphitic carbon chromatography. Of the separation mechanisms tested in this work, reversed-phase chromatography with 5 mM ammonium acetate pH 8.0 and methanol as mobile phases showed the best results concerning peak separation, retention time stability and compatibility with the desired overall LC-MS system. The stationary phase used was the silica-based column Atlantis T3 from Waters. The packing material of this column is suitable for the use of 100% aqueous mobile phases, like it is necessary for the retention of polar compounds like $NADP^+$ and NADPH. The established chromatographic method was coupled to triple quadrupole mass spectrometry for highly selective and sensitive analysis of the eluting compounds. The detection and quantification limits were in the lower nanomolar range for both compounds in this setup. It was shown that this overall procedure delivers data fit for the evaluation of intracellular NADP levels in yeast. The chosen LC-MS/MS analysis process allows the fast automated analysis of high sample numbers and meets the requirements of metabolomic studies.

The practical experience with the determination of $NADP^+$ and NADPH gained in this work and personal communication with groups working on the same problem showed that in contrast to literature estimations, a much higher intracellular abundance of the reduced

NADPH over the oxidized $NADP^+$ is not observable in biological samples. The highest ratio NADPH/$NADP^+$ reported so far is 4.92 and was observed in a cell extract of *Saccharomyces cerevisiae*. For the same organism, other reported ratios are 0.04, 0.3, 1.3 or 4.23. Even if the different extraction procedures and analytical methods are considered, this variability in the observed NADP levels indicates that there is still a need for further investigation and discussion. The presented analytical workflow (Figure 24) has been shown to be suitable for the task, but still contains some points that are to be critically evaluated. For instance, internal standardization has not been established for this analytical problem. As discussed above, isotopically labeled $NADP^+$ and NADPH would be ideal internal standards but are not commercially available. It will be the task for future work to find a strategy for internal standardization that introduces traceability and a basis for quantitative data evaluation.

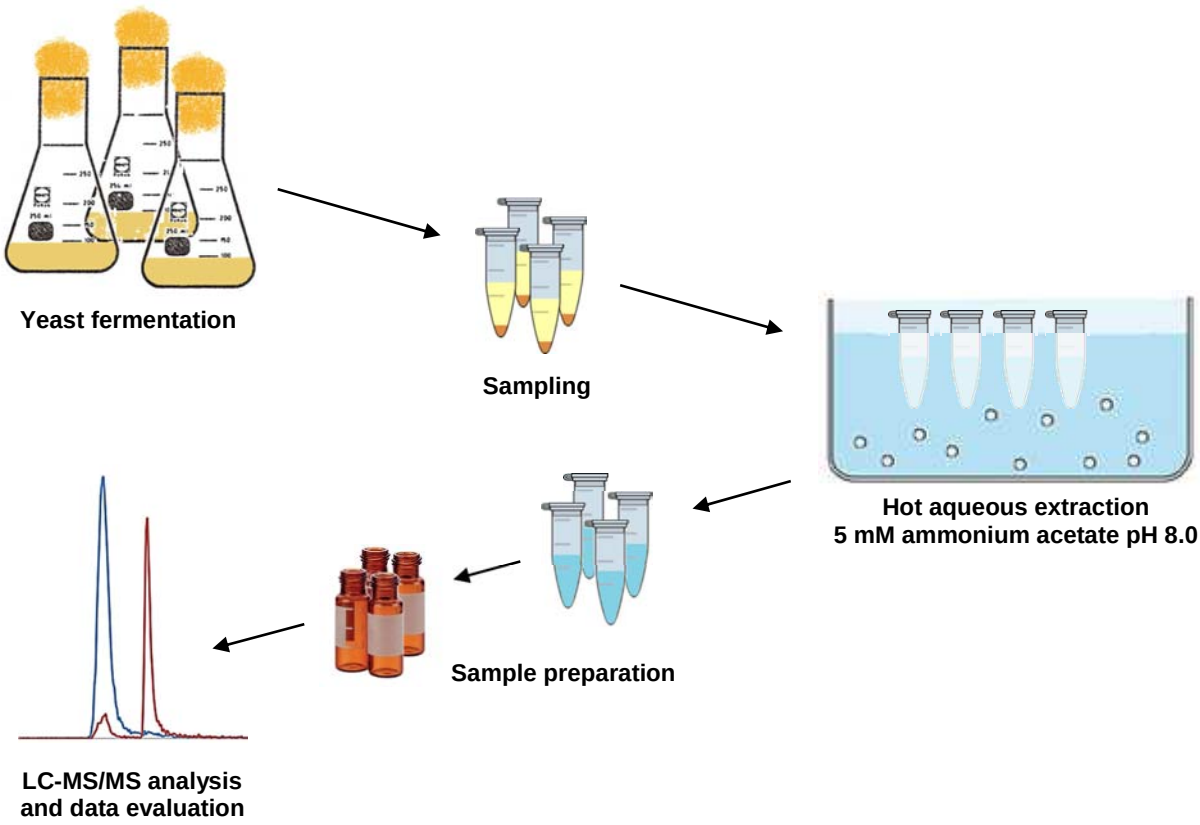

Figure 24: Overview on the established analytical workflow for the determination of $NADP^+$ and NADPH in yeast. The process includes sampling, metabolite extraction, sample preparation and LC-MS/MS analysis.

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