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Quantifying the extended energy metabolome of industrially important microorganisms (*Saccharomyces cerevisiae)* using ultra-performance liquid chromatography with mass spectrometry

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A R T I C L E I N F O

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A B S T R A C T

This study has developed a new targeted methodology for the separation, detection, and quantification of metabolites from the wider energy metabolome of industrially important microorganisms such as *Saccharomyces cerevisiae* in a single analytical sample. This has been achieved using UHPLC-MS technology in HILIC mode. Absolute concentrations of metabolites nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide reduced (NADH), nicotinamide adenine dinucleotide phosphate (NADP), nicotinamide adenine dinucleotide phosphate reduced (NADPH), flavin adenine dinucleotide (FAD), adenosine-monophosphate (AMP), adenosine-diphosphate (ADP), and adenosine-triphosphate (ATP) were determined in a single extraction and analytical methodology.

This study demonstrated the development of a rapid, statistically robust, and reproducible methodology through regression calibrations of standard samples from 0.1 to 100 μMol providing a correlation value of $r^2 = >0.98$ for all metabolites. Sample preparation, extraction and analytical methodologies used showed high accuracy, sensitivity, and recovery. With an LOD and LOQ for the targeted analysis of metabolites from the wider energy metabolism in a single sample and analytical run with the lowest LOD of 0.055 nMol (±0.002) and lowest LOQ of 0.167 nMol (±0.006). This method was then applied to *Saccharomyces cerevisiae* cell culture to evaluate the methodology in industrially used microbial cultures. Results obtained have been statistically determined to be robust and reproducible through recovery analysis using deuterated and isotopically labelled internal standards AMP-^{15}N, ADP-^{15}N and ATP-d_{14}.

1. Introduction

Increasing interest is being shown in using products from microbial processes to produce chemicals and fuels to replace those produced from fossil hydrocarbons. However, to reach the volumes required at an economically viable cost point, significant improvements in yield and carbon efficiency need to be achieved. There are a number of strategies that can be applied to achieve this goal based on a robust understanding of the energy metabolome of industrially exploited microbes to direct these efforts. To gain this knowledge of microbial energy metabolome, quick, robust, and high throughput analytical methods of determining the widest possible aspect of the energy metabolome are required (Fig. 1).

The energy metabolism is a fundamental part of all organisms to maintain biological processes such as cellular respiration, reproduction, and molecular synthesis. There are three major biochemical pathways imperative to these functions, the glycolytic pathway, the tricarboxylic acid pathway, and the pentose phosphate pathway [1–5].

The first major pathway, glycolysis, is a common source of energy for these actions to transpire that occurs in both aerobes and anaerobes. Although commonly described as the metabolism of glucose, other simple sugars can serve as an input [4]. The metabolism of glucose or hexose begins in the cytoplasm with glycolysis, whereby a singular glucose molecule is catabolised into two pyruvate molecules. This linear metabolic pathway produces two ATP (adenosine-triphosphate) molecules and two NADH (nicotinamide adenine dinucleotide reduced) molecules. In both aerobic and anaerobic conditions, the pyruvate molecules undergo carboxylation, whereby one ATP molecule and one water molecule activate a pyruvate carboxylase enzyme which results in the formation of oxaloacetate, ADP (adenosine-diphosphate),

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phosphate, and an additional hydrogen molecule [4–6].

Aerobic respiration, a second major source of cellular energy, occurs within the mitochondria via the tricarboxylic acid (TCA) cycle [7]. This begins with the decarboxylation of the pyruvate molecule and the transfer of electrons which results in the reduction of NAD (nicotinamide adenine dinucleotide) to form NADH. Finally, co-enzyme A is added to produce acetyl-CoA [6,7].

In the continuation of the TCA cycle, additional metabolites are formed, reduced, and used in the electron transport chain to generate further products for cellular activity. Specifically, FAD (flavin adenine dinucleotide) and the reduced form of $FADH_2$ (flavin adenine dinucleotide reduced) play a major role as enzyme cofactors in crucial tasks involved in energy metabolism, cell respiration, iron uptake, and reduction, as well as the delivery of electrons to the electron transport chain of the mitochondria [8,9].

The third major source of cellular energy is the pentose phosphate pathway (PPP). This pathway generates NADP (nicotinamide adenine dinucleotide phosphate) used for the biosynthesis of macromolecules required for nucleic and amino acids and NADPH (nicotinamide adenine dinucleotide phosphate reduced) used for the generation of superoxide (O_2^-) and the breakdown of hydrogen peroxide (H_2O_2).

Discovering the variety of physiological and regulatory roles these nucleotides play within cells has fuelled the pursuit of analysing the metabolic pathways in several different models of organisms such as yeast [10].

Although NAD/NADH and NADP/NADPH are structurally similar, both redox pairs have critical but specific metabolic functions which result in great chemical diversity and an even greater range in concentrations present in cells. Therefore, the development of specific yet sensitive methodologies for the detection and quantification of these metabolites that can be transferred to a variety of specimens is imperative [11].

At present, we have access to techniques that are capable of studying tens to hundreds of metabolites in complex biological samples [12]. Subsequently, there are several approaches to metabolomics research that differ depending on the end objective [13,14]. There have been many successes in quantifying parts of the NAD+metabolome using nuclear magnetic resonance (NMR) [15–19] and high-performance liquid chromatography paired with ultraviolet to visible light spectroscopy (HPLC-UV/Vis) [20,21]. However, since its development by Waters in 2004, Ultra-Performance Liquid Chromatography paired with Mass Spectrometry (UPLC-MS) is by far the most commonly used form of technology for the profiling of metabolites [22] (Fig. 2).

A large proportion of current methods for the separation and

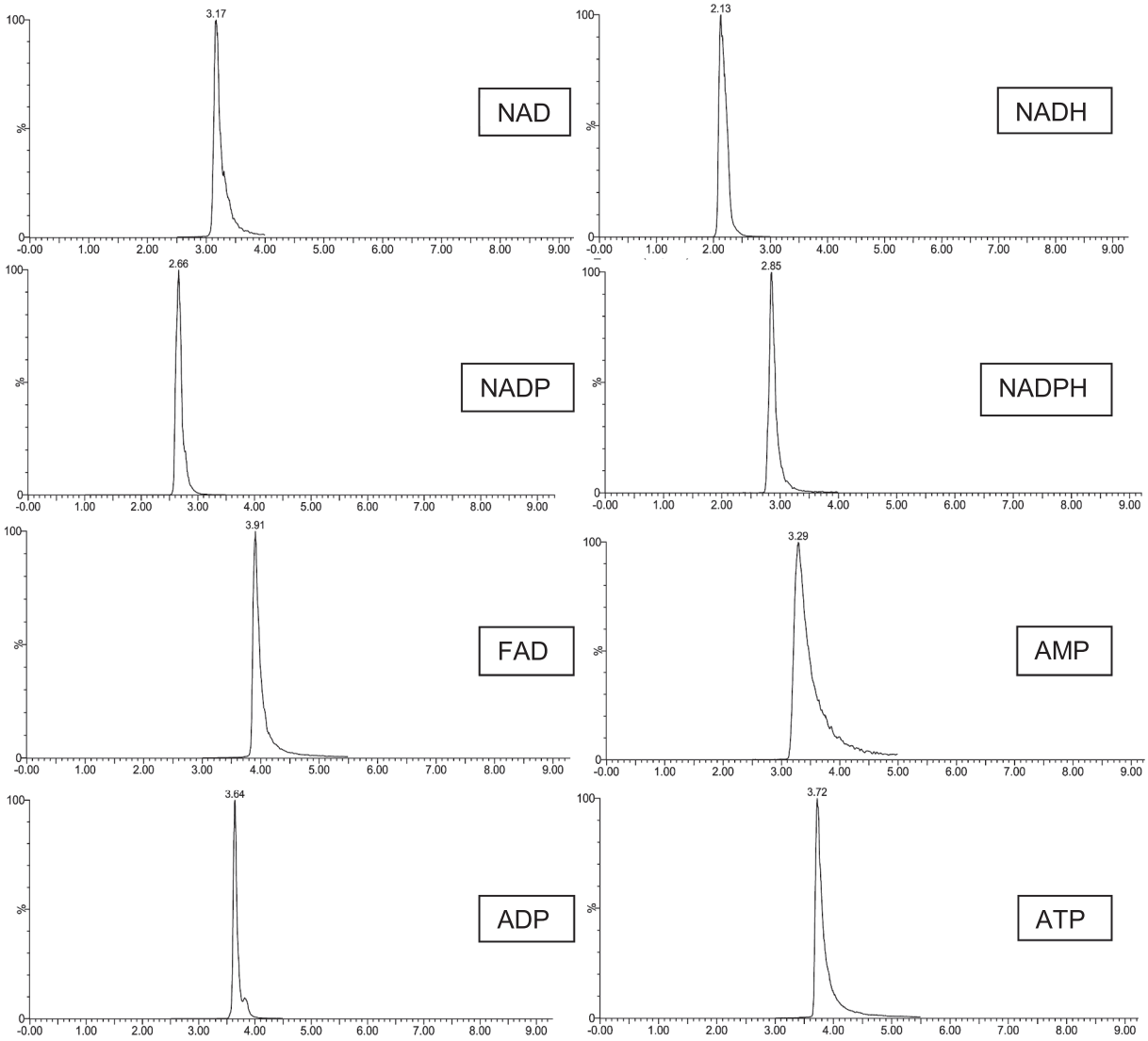

Fig. 1. Total ion chromatograms (TIC) of 100 μMol analyte mixture of NAD, NADH, NADP, NADPH, FAD, AMP, ADP, and ATP dissolved in a buffer of 50 % acetonitrile and 50 % ultrapure water. Detected using parameters specified in Section 2.4. and 2.5.

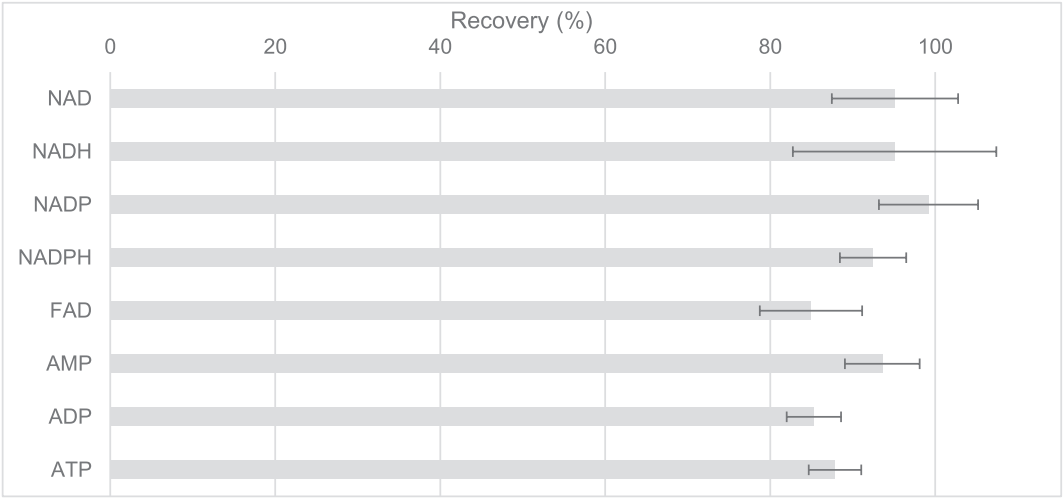

Fig. 2. Matrix ionisation effect completed on known laboratory grade standards at 100 μMol to assess for signal suppression or enhancement.

quantitation of metabolites in biological and environmental samples have relied upon the separate processing and then combined analysis of redox pairs. For example, an acidic extraction for oxidised compounds such as NAD and NADP, and a separate basic extraction for the reduced compounds NADH and NADPH [23–25].

However, by using Zwitterionic columns (Z-HILIC) which contain sulfoalkylbetaine functional groups, the stationary phase is not affected by the pH of the mobile phase [26,27]. Therefore, allowing for fine-tuning of the mobile phase to best suit the polarity of the analytes to elute in one chromatographic run.

This paper reports on the development of a single sample methodology for the quenching, extraction, detection, and quantification of metabolites from the extended energy metabolome of *Saccharomyces cerevisiae* using UHPLC-MS instrumentation in HILIC mode. It is based on using the freeze–thaw cycle method for quenching and extraction of targeted metabolites and their separation using UHPLC-MS. The detection and optimisation of these metabolites are achieved by using a triple quadrupole MS tool in conjunction with TargetLynx and MassLynx software. This protocol removes the necessity for separate sample processing and analysis of metabolites across the Krebs, TCA, and PPP cycles. Providing a sensitive and highly reproducible methodology for eight nucleotide species and their fragmented ions.

2. Materials and methodology

2.1. Chemicals, consumables and biological Materials

All chemicals and standards were purchased from Sigma Aldrich

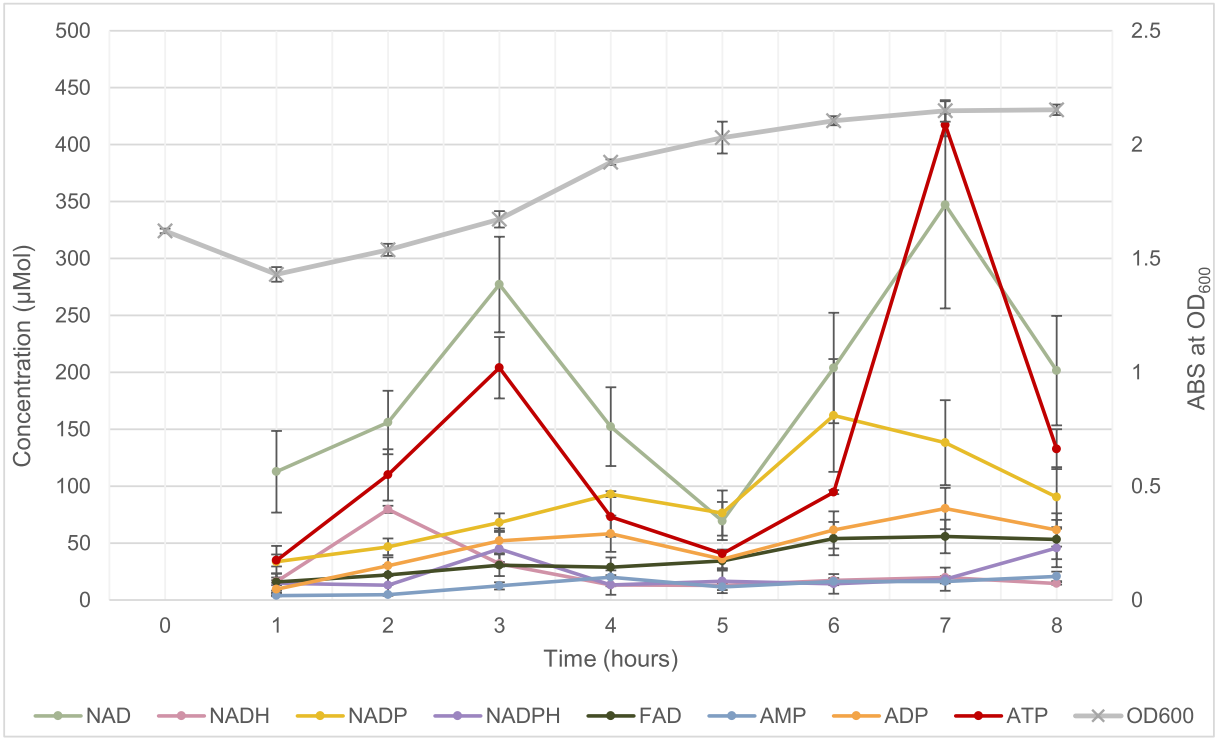

Fig. 3. OD_{600} of *Saccharomyces cerevisiae* over 8 h with target analytes NAD, NADH, NADP, NADPH, FAD, AMP, ADP, and ATP determined using optimised quenching, extraction, detection, and analysis protocols.

suitable for UHPLC-MS analysis. This includes the solid form of metabolites; NAD (100 % purity), NADH (98 % purity), NADP (>97 % purity), NADPH (>97 % purity), FAD (>95 % purity), AMP (>95 % purity), ADP (>95 % purity), and ATP (>99 % purity).

Internal standards include AMP-^{15}N (>95 % disodium salt and > 98 % ^{15}N), ADP-^{15}N (>95 % disodium salt and > 98 % ^{15}N), and ATP-d_{14} (>95 % disodium salt and > 98 % d).

Chromatography consumables include ammonium bicarbonate at 99.5 % purity, a 28 % ammonium hydroxide solution at 99.99 % purity, and formic acid at 98–100 % purity. UHPLC-grade solvents include acetonitrile at 99.9 % purity and methanol at 99.9 % purity. Ultra-pure deionised water was obtained from the Elga Veolia PURELAB® flex 2 UV water delivery system.

Yeast from *Saccharomyces cerevisiae* type 1 YSC1 cells were bought in dried form from Sigma Aldrich (Fig. 3).

2.2. Preparation of standards

Individual stock solutions of the target analytes NAD, NADH, NADP, NADPH, FAD, AMP, ADP, and ATP were created at a concentration of 1000 μMol in a buffer of 50:50 acetonitrile and ultrapure water. The stock solutions were stored in −80 °C conditions for up to 1 month.

In preparation for the construction of a calibration curve, separate compound serial dilutions of 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 5.0, 10.0, 25.0, 50.0 and 100.0 μMol were prepared and stored at 4 °C for up to 1 h before analysis. Combined compound serial dilutions of 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 5.0, 10.0, 25.0, 50.0 and 100.0 μMol were also prepared by combining and diluting the stock solutions and were also stored at 4 °C for up to 1 h prior to analysis.

2.3. Preparation of Saccharomyces cerevisiae

Yeast cells (Baker's yeast) were cultivated in a yeast peptone dextrose (YPD) broth containing 2.5 g yeast extract, 5 g peptone, 5 g dextrose and 5 g d-glucose as the source of carbon. In a 250 mL glass bottle, 100 mL of the broth was added along with 1 g of yeast cells. The sealed Duran bottle is put into complete anaerobiosis by bubbling through CO_2 at a rate of 450 mL/min for 30 min prior to the addition of the cell culture. Fermentation was performed under oxygen-limited conditions for 24 h in a shake incubator at 30 °C and 250 rpm.

2.3.1. Quenching

Per sample, 3 mL of fermentation culture was filtered through a 0.45 μM pore size, 25 mm diameter, nylon Corning® syringe filter (Sigma Aldrich). Immediately after filtration, yeast cells were washed with 5 mL of a −40 °C 60 % methanol and ultrapure water quenching solution. Analysis of cell mass recovery was performed in triplicate using the Hach DR3900 Laboratory Spectrophotometer at an optical density of 600 nm for cell density replication.

2.3.2. Extraction

Metabolite extraction recovery was assessed using three different extraction solutions. For comparison as to the most effective extraction method, yeast cells were collected as per the quenching methodology and extraction was performed in triplicate [20–30].

All follow the same initial process of post quenching, the 5 mL solution containing the yeast cells is transferred to a 5 mL Eppendorf and centrifuged at 13,000 rpm at −20 °C for 5 min to form a cell pellet. The quenching supernatant is removed, and the pellet is suspended in 1 mL of:

)a) 80 % methanol 20 % ultrapure water at −80 °C.
)b) 40 % acetonitrile, 40 % methanol, 20 % 0.05 M formic acid at 4 °C.
)c) 40 % acetonitrile, 40 % methanol, and 20 % 0.1 M formic acid at 4 °C.

Each sample is agitated for 15 min to form a homogenised solution and stored at −80 °C for 20 min before being agitated for a final time for 5 min. The entire sample is filtered through a 0.22 μM pore size, 25 mm diameter, nylon Corning® syringe filter (Sigma Aldrich).

Samples using extraction method A containing methanol are suitable for UHPLC-MS analysis without further processing. Samples using extraction methods B and C require further sample processing with the addition of 10 mM ammonium bicarbonate for pH stabilisation and UHPLC-MS compatibility. The total processing time for all three methods remains the same with an approximate total extraction time of < 50 min.

2.4. UHPLC-MS conditions and optimisation

The instrumentation used consists of the Waters Acquity UPLC H-Class PLUS system fitted with a binary solvent manager, sample manager, 6-capacity column manager, and PDA detector. Paired with the Xevo TQ-S Cronos triple quadrupole mass spectrometer.

The column specification is the Atlantis Premier BEH Z-HILIC column (95 Å 1.7 μm, 2.1 mm x 150 mm) coated with a zwitterionic functional group of sulfobetaine, fitted with an Atlantis Silica HILIC VanGuard Cartridge (100 Å, 3 μm, 2.1 mm x 5 mm) column protection.

Due to the polar to highly polar nature of the metabolites of interest, reversed-phase chromatography has proved to be the most effective in the separation and analysis. Specifically, C18 chemistry columns and polar functionalities. One such material popular in metabolomics is a Sulfobetaine stationary phase. These contain negatively charged sulphonate and positively charged quaternary ammonium groups as a 1:1 M ratio, equalling a zwitterion. With their net zero surface charge, zwitterionic stationary phases have been shown to accumulate a relatively thick layer of adsorbed water from the mobile phase, which makes them strongly retentive for polar analytes. Most of the available zwitterionic stationary phases are available in the form of a HILIC (hydrophilic interaction liquid chromatography) column. The combination of high retentivity and a net zero surface charge has made zwitterionic hydrophilic interaction liquid chromatography (Z-HILIC) columns a popular choice for the separation of complex mixtures in metabolomics.

The column chemistry and chromatographic methodology was assessed through separation, resolution, peak symmetry and tailing of all compounds at a concentration of 100 μMol. Peak symmetry and peak tailing values have been presented in Table 3. An ideal range for peak symmetry is a value between 0.9 and 1.2 or 1.5, with all compounds apart from AMP and ATP within this range. While peak tailing is noted for AMP and ATP, the calculated A_s values are within the acceptable limit of 2.0 so are therefore not concerning [55].

Peak identification was performed using the Waters MassLynx V4.2. Analyte quantification was performed using TargetLynx. Optimisation was performed using Intellistart tuning software, also part of the MassLynx V4.2 software package.

Argon was used as the collision gas and nitrogen was used as the nebuliser gas. Spectra were acquired using MRM (multiple reactions monitoring) with dwell times calculated using the MassLynx auto dwell function.

A serial dilution from the stock solution was created at a concentration of 100 μMol per analyte and directly injected into the Xevo TQ-S Cronos triple quadrupole mass spectrometer. The direct infusion using Intellistart tuning software automatically determines the most effective instrumentation parameters for optimum analyte detection.

Tables 1 and 2 indicate the mass of the compounds as well as the tuning parameters automatically identified as being optimum for the detection of the three largest fragment ions.

2.5. Chromatographic conditions

All samples were injected at a volume of 10 μL. Mobile phase A is 15 mM ammonium bicarbonate in ultra-pure water and mobile phase B is

Table 1
Optimised MS conditions for the detection and quantification of target analytes using Intellistart software as part of the MassLynx V4.2. package.

MS1 Resolution (ΔM)	0.75
MS2 Resolution (ΔM)	0.75
Cone Voltage Range (V)	2–100
Collision Energy Range (V)	2–80
Lowest Fragment Mass (g/Mol)	150.00
ESI Mode	Positive (+)
Capillary (kV)	3.00
Source Temperature (°C)	150
Desolvation Temperature (°C)	350
Desolvation Gas Flow (L/hr)	650
Cone Gas Flow (L/hr)	50
Low Mass Resolution 1 (au)	10.0
High Mass Resolution 1 (au)	15.0
Low Mass Resolution 2 (au)	9.4
High Mass Resolution 2 (au)	15.0
Multiplier (V)	0.5
Syringe Pump Flow (μL/min)	10.00

Table 2
All eight metabolites in standard form and three isotopes labelled/deuterated internal standards for Intellistart MRM analysis. With parent mass, mass fragments, ESI mode, cove voltage, and collision energy optimisation.

Compound	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Cone (V)	Collision (V)
NAD	664.08	428.04	52	20
		524.02	52	12
		542.09	52	8
NADH	665.12	302.06	52	30
		514.07	52	18
		649.12	52	14
NADP	743.07	508.02	48	22
		604.00	48	14
		622.01	48	10
NADPH	745.09	302.13	52	26
		320.08	52	34
		729.04	52	10
FAD	785.16	348.12	70	12
		359.18	70	38
		439.11	70	14
AMP	348.02	250.06	14	18
		178.06	14	36
		214.03	14	24
ADP	427.03	178.15	38	32
		232.08	38	22
		348.12	38	12
ATP	506.99	216.08	44	38
		348.13	44	10
		410.04	44	8
AMP-^{15}N	397.07	379.03	36	16
		295.10	36	18
		257.00	36	16
ADP-^{15}N	477.01	222.92	46	24
		337.01	46	14
		319.00	46	18
ATP-d_{14}	539.07	202.69	8	40
		258.94	8	20
		302.88	8	22

15 mM ammonium bicarbonate in acetonitrile. Both are made from a stock solution of 150 mM ammonium bicarbonate by diluting 11.858 g of ammonium bicarbonate in 960 mL of ultra-pure water with the addition of 40 mL of ammonium hydroxide to stabilise the ammonium bicarbonate and acetonitrile matrix.

The use of ammonium bicarbonate is an important addition to this chromatographic method. As a volatile buffer with a high buffering capacity, it allows for the maintenance of a stable pH during ESI analysis, a very important facet when analysing polar to highly polar metabolites. The addition of ammonium hydroxide is used to stabilise the inorganic salts of the ammonium bicarbonate and acetonitrile matrix.

Table 3
All eight metabolites at a concentration 100 μMol with calculated peak symmetry and peak tailing factor as stipulated by RSC Analytical Methods Committee.

Analyte	Peak Symmetry (A_s)	Tailing Factor (T)
NAD	1.30	1.15
NADH	1.36	1.18
NADP	1.13	1.06
NADPH	1.27	1.13
FAD	1.27	1.14
AMP	0.98	0.99
ADP	1.42	1.21
ATP	1.91	1.45

The column temperature remains constant at 30 °C. The mobile phase program runs from 0 to 5 min with a flow rate of 500 μL/min and mobile phase A starts at 10 % to 35 % and mobile phase B starts at 90 % to 65 %. This configuration is then held for 1 min. Mobile phase A is reduced to 10 % and mobile phase B to 90 % over 2 min. The flow rate is increased to 1000 μL/min at the 8-minute mark and for 1.5 min mobile phases A and B are held. The same composition is held for a further 0.5 min but at a reduced flow rate of 500 μL/min. The total run time is 10 min where initial instrumentation conditions are reverted at 8 min and held for 2 min to equilibrate the column. A blank injection is run at the same gradient and flow rate specifications after every sample injection to ensure the sample loop and column are free from contaminants.

2.6. Method validation

Statistical analysis was completed using IBM SPSS Statistics using the ANOVA test with a significance level of $\alpha = 0.05$ (95 %), as well as the Bonferroni correction method with outliers included.

2.6.1. Precision and accuracy

Instrument reproducibility was determined through intraday analysis of serial dilutions with 10 repeats of both individual samples and combined metabolite samples to assess variability and effect.

Chromatographic methodology robustness was determined through the interday analysis of total standard preparation procedures, and the analysis of a calibration curve construction performed on 5 separate occasions. The results obtained from all analyses were then compared to determine if there is any statistical difference in the quantitation and mass-to-charge data obtained from intraday single and combined metabolite samples compared to interday combined samples.

2.6.2. Linearity and sensitivity

The sensitivity of the methodology is determined by calculating the limit of detection (LOD) and limit of quantification (LOQ). These values have been calculated in the results section using the peak signal-to-noise values for each compound and the following equations:

$$LOD = \frac{(3.3.\delta)}{S} LOQ \frac{10.\delta}{S}$$

where σ is the standard deviation and S is the slope of the calibration curve.

2.6.3. Recovery and matrix effect

The quenching method's ability to arrest cellular metabolic activity was assessed through the analysis of known concentrations of standards and their subsequent redox-paired metabolite. This was completed on the redox pairings of NAD/NADH, NADP/NADPH, AMP/ADP, and ADP/ATP. Individual standard metabolite samples were processed following the quenching methodology and the recovery of the metabolite was analysed compared to the expected value from the buffer as well as the detection of the redox pairing.

Analysis of the extraction method was assessed through internal standard recovery. Yeast cells were spiked with 25 μMol of AMP-^{15}N, ADP-^{15}N, and ATP- d_{14} prior to the extraction process. All three different processes were compared for internal standard recovery using the equation below as well as the highest concentration of metabolites recovered to determine the most effective method.

$$\frac{(Found\ Concentration - Blank\ Sample\ IS\ Concentration).100\%}{Added\ Concentration}$$

Finally, matrix ionisation was also determined using internal standard recovery and comparing metabolites made up in buffer compared to quenching and extraction solution effects.

3. Results and discussion

3.1. Linearity and sensitivity

Using the TargetLynx software, a calibration curve was created using the peak area of the metabolites of each calibrator versus its concentration. The calibration curve was constructed using 11 calibration points with 3 injections and 3 repeats.

The quantified compound summary report of metabolite calibration includes linear regression values and the calibration graph. The coefficient of determination value is excellent, with all cases, apart from ATP-d_{14} (0.987), above > 0.990.

The hypothesis for the marginally lower linear regression value for ATP-d_{14} is an individual data point determined using TargetLynx at the concentration of 100 μMol. While the individual value is not statistically significant, it has contributed to the overall power model. This is believed to be due to instrumentation variation as each sample point is taken as a repeat measure from a single sample. However, the r^2 value of ATP-d_{14} is still above the acceptable r^2 value of > 0.95.

All regression model graphs generated demonstrate an even distribution of calibration points above and below the residual line with no statistically notable outliers which would indicate there is any systemic error.

The calibration curve is calculated using the linear regression model, which is demonstrated with the strong r^2 values, and is the best fit for the data obtained. This is primarily due to the robust methodology, the use of repeat measures with 11 different concentration calibration points and not exceeding the saturation point of the UHPLC-MS instrumentation which will often exhibit non-linear behaviour. The statistical analysis further confirms to robust nature of the methodology and reproducibility through non-statistically significant differences in results between repeated analytical methods. This includes the LOD and LOQ values obtained for each metabolite.

As the MassLynx software does not automatically detect the limit of detection (LOD) and limit of quantification (LOQ), these have been calculated manually using the equations referenced in section 2.6. The peak area and signal-to-noise values have been taken from the smallest and largest concentrations on the calibration curve and reported as nMol.

The sensitivity of this methodology reflective in the LOD and LOQ values is greater compared to studies of a similar nature. When comparing to Røst *et al.*, 2020 [52], LOD and LOQ values fall between 0.80 nMol and 12.00 nMol for NAD, NADH, NADP, NADPH and FAD, with the study unable to detect adenosine molecules. Similarly, this can be seen with other referenced studies with a much larger difference in LOD and LOQ values [47–49,51,52]. A 2017 study conducted by Bustamante *et al.* [50] analyses the same metabolites as this report and does provide more sensitivity for a select few. However, are unable to reliably detect and quantify the important redox pairing of NADPH and AMP. Furthermore, the total LC-MS runtime is 30 min compared to this study of 10 min. Similarly, a 2013 study conducted by Trammell and Brenner [40] provides more sensitivity for certain metabolites but is unable to quantify FAD and uses two separation methods, an acid 23.4-minute gradient and an alkaline 32.3-minute gradient.

3.2. Intra-day accuracy and precision

As the majority of the method development was conducted using single analyte samples, it was imperative to ensure that sufficient statistical tests were conducted to ensure there was no statistically significant difference when analysing samples that were combined in a matrix. This was completed by analysing intraday and interday combined samples as calibration points against data obtained from individual samples during calibration.

The peak area responses were statistically compared to one another to determine if there was any significant difference in the data obtained between metabolites for repeat analytical runs. Data is processed using the aforementioned statistical parameters and a summary is presented in Table 4.

Linear regression values for intra-day analysis for all compounds provide a value of > 0.98 with the exception of AMP which has an r^2 value of 0.93. While this value is marginally lower, this is suspected as due to instrument error from individual data points as these were repeated injections from a single sample. Furthermore, the ANOVA statistical analysis results provide a value of greater than 0.98 for all compounds including AMP. Therefore, as the p-value is greater than the alpha (the 95 % level of significance), the hypothesis is accepted that the averages between runs are assumed to be equal. In other words, the difference seen between samples in each run is not large enough to be statistically significant.

These results are further compared to the data obtained from individual sample analysis completed for calibration. All data obtained indicates a p-value greater than α (95 % confidence interval), therefore, the average of both groups is considered equal with no significant statistical difference.

3.3. Inter-day reproducibility and robustness

Interday standard sample analysis is first assessed for reproducibility over the five days and then robustness when compared to the calibration data. A calibration of 7 data points is constructed of 0.1, 0.5, 1.0, 5.0, 10.0, 50.0 and 100.0 μMol with 3 injections and 10 repeats. The data is reported in Table 5 and is assessed for variance between injection repeats and days compared to the average, *2 standard deviation and *3 standard deviation (Table 6).

The r^2 values for all compounds provide a value of > 0.98, showing excellent linear regression for inter-day analysis. Furthermore, no data exceeds *3 standard deviation of the average expected values.

The analysis of 10 different combined metabolites within a single sample indicates that the difference between combined metabolite injections is not large enough to be statistically significant. The final comparative series of statistics is between the results obtained on 10

Table 4
Target metabolites and internal standards retention time, correlation coefficient value (r^2), limit of detection (LOD) and limit of quantification (LOQ) from calibration.

Analyte	RT (min)	R^2	LOD (nMol)	LOQ (nMol)
NAD	3.17	0.999	0.072 ± 0.002	0.217 ± 0.005
NADH	2.66	0.994	0.105 ± 0.021	0.317 ± 0.064
NADP	3.91	0.997	0.110 ± 0.027	0.332 ± 0.081
NADPH	3.65	0.998	0.276 ± 0.019	0.837 ± 0.058
FAD	2.12	0.998	0.055 ± 0.002	0.167 ± 0.006
AMP	2.86	0.998	10.388 ± 2.304	31.480 ± 6.982
ADP	3.28	0.994	0.428 ± 0.094	1.297 ± 0.284
ATP	3.72	0.997	0.627 ± 0.138	1.901 ± 0.420
AMP-^{15}N	3.09	0.997	12.078 ± 6.078	36.600 ± 18.418
ADP-^{15}N	2.60	0.990	6.908 ± 2.834	20.933 ± 8.587
ATP-d_{14}	3.30	0.987	45.653 ± 40.870	138.342 ± 123.850

Table 5
Analysis using ANOVA of Intra-day and Inter-day analysis of combined analyte samples at 100 μMol. Intra-day analysis was completed as 10 repeats in a single day and statistically compared against the calibration values. Inter-day analysis was completed over 5 days.

Analyte	Intra-day Analysis		Inter-day Analysis	
	ANOVA P-Value	r^2	AVG r^2	AVG ANOVA P-Value
NAD	0.982	0.993	0.991	0.975
NADH	0.998	0.984	0.984	0.926
NADP	0.996	0.998	0.991	0.936
NADPH	0.992	0.999	0.996	0.953
FAD	0.996	0.983	0.989	0.934
AMP	0.978	0.926	0.988	0.911
ADP	0.999	0.995	0.996	0.982
ATP	0.989	0.999	0.989	0.946

Table 6
Method validation of quenching ability to arrest metabolic activity. Assessed using laboratory standards of recovery and redox pair metabolite detection.

Compound	Redox Pair	Actual Recovery (μMol)	Recovery %	Redox Recovery (μMol)
NAD >	NADH	47.92 ± 1.88	95.84 ± 3.92	0.39 ± 0.04
NADH >	NAD	50.37 ± 5.68	100.75 ± 11.27	0.57 ± 0.07
NADP >	NADPH	46.64 ± 3.54	93.28 ± 7.59	0.06 ± 0.06
NADPH >	NADP	49.47 ± 1.95	98.94 ± 3.04	1.27 ± 0.08
ADP >	AMP	45.11 ± 4.77	90.21 ± 10.57	0.51 ± 0.19
ATP >	AMP	47.27 ± 5.19	94.53 ± 10.98	0.67 ± 0.05
ATP >	ADP	47.27 ± 5.19	94.53 ± 10.98	3.88 ± 0.37

separate analyses of individual metabolite samples compared with 10 separate analyses of combined multi-component metabolite samples. The results for this indicated that there is no significant, difference between single sample injections (M = 308908.9, SD = 610655.2) and combined sample injections (M = 309473.3, SD = 631905.1). Since the f statistic is 0.00575 which falls within the 95 % region of acceptance and the p-value was 0.934 which is greater than α of 95 %, the hypothesis cannot be rejected. Therefore, it can be assumed that there is no difference in calibration results obtained between single metabolite samples and multi-component metabolite samples using this chromatographic methodology.

As reported, there is no statistical difference between the data sets, therefore I can confidently state that the methodology has been robustly analysed using laboratory standards and is suitable to be used for further analysis.

3.4. Matrix effect

Components within the matrix or buffer the analyte is suspended in can lead to ion suppression or enhancement properties of the primary signal response. This is particularly notable for compounds with a large mass and/or strong polarity.

Due to polar to highly polar nature of the target analytes, the matrix ionisation effect is assessed through the use of internal standard recovery and the slope of calibration curves of known standards in 50 % acetonitrile and 50 % ultrapure water buffer compared to standards through the cell extraction process.

Signal suppression was observed for all metabolites with ≤ 15 % for FAD, ADP, and ATP; with the remaining analytes and internal standards falling within ≤ 10 %. Therefore, it was concluded that there is a non-statistically significant difference in the quantification of matrix effect.

3.5. Quenching method validation

A crucial stage in sampling for metabolomics is the quenching process. This process is designed to rapidly inhibit cellular enzymatic and metabolic activity to obtain the most accurate quantifications of the metabolites present upon analysis.

When assessing the most appropriate methodology to adopt for intracellular and metabolite extraction, quenching should be performed as quickly as possible without damaging the cell membrane to prevent the leakage in intracellular metabolites and, therefore a loss in concentration. Furthermore, redox pairs such as NAD/NADH and NADP/NADPH are approximately one mass unit apart. Therefore, the rapid quenching of nucleotides is important to prevent misidentification of these metabolites. The quenching solution used in this methodology is an adaptation of the original de Koning and van Dam protocol and a freeze–thaw cycle [11,28–35].

Metabolic quenching ability was determined through the analysis of known standards, assessing their stability and redox conversion after 48 h.

Using the 1000 μMol stock solution for NAD, NADH, NADP, NADPH, AMP, ADP, and ATP; 100 μL was pipetted directly into 900 μL of buffer to make a 100 μMol solution in individual Eppendorf's. Samples are processed following the quenching protocol in section 2.3.1. and stored for 48 h at 4 °C. Each 100 μMol analyte is reconstituted with buffer and analysed following the chromatography method in Section 2.5. The expected concentration of the target analyte is determined based on the calibration values and conversion of the analyte to a redox pairing is calculated.

Analyte recovery from the quenching solution is observed to be greater than 90 % for all metabolites. The quenching ability has also been deemed appropriate due to the very low detection levels of redox-paired metabolites. Furthermore, the concentration of redox metabolite for all compounds is observed to be insignificant.

3.6. Extraction method validation

The aim of the extraction process is to separate target metabolites from within the intracellular membrane of microorganisms without destroying them and minimising loss. Chemical lysis for intracellular metabolite extraction is the preferred methodology compared to mechanical disruption. This is generally a result of greater control over modifying protocols to optimise methods for specific bacteria strains when compared to the limitations mechanical lysis offers. Furthermore, this extraction procedure causes minimal degradation to the cell envelope while keeping the dilution factor of the sample to a minimum.

The accuracy and effectiveness of the extraction methodology are determined through the greatest concentrations of targeted metabolites recovered, as well as the recovery of known concentrations of internal standards. The general acceptable recovery range of an internal standard should be ± 20 % compared to calibrations.

Additionally, it is important to address the phenomena of matrix suppression and matrix enhancement. It is crucial to perform a thorough analysis of their impact on the quantitative accuracy of results. This understanding will aid in the optimisation of extraction methods.

Ideally, there should be an internal standard representing all target metabolites, however, this has not been possible due to the unavailability and feasibility of suitable products. Therefore, extraction method recovery and efficiency is assessed alongside target analyte recovery using AMP-^{15}N, ADP-^{15}N, and ATP-d_{14} to determine the most appropriate extraction method.

Extraction processes chosen for analysis include methanol and ultrapure water (80:20) extraction method conducted at −80 °C. Acetonitrile, methanol, and 0.05 M formic acid in ultrapure water (40:40:20) extraction method at pH 3.7 conducted at 4 °C. Acetonitrile, methanol, and 0.1 M formic acid in ultrapure water (40:40:20) extraction method at pH 3.4 conducted at 4 °C. Prior to the addition of the extraction

solution, Yeast samples are spiked with 25 μMol of internal standards AMP-^{15}N, ADP-^{15}N, and ATP-d_{14}, and samples are processed using the methodology from section 2.3.3.

Table 7 indicates the average calculated concentration from the yeast cell extraction and Table 8 indicates the internal standard recovery for Recovery 1 reference 80 % methanol, Recovery 2 0.05 M formic acid and Recovery 3 0.1 M formic acid.

Recovery method 1 yields a maximum concentration of 14.20 μMol (±3.80) with NAD and a minimum concentration of 0.16 μMol (±0.03) with NADPH.

The recovery rates for the internal standards were 70 % for AMP-^{15}N, 124 % for ADP-^{15}N, and 77 % for ATP-d_{14}. None of these values fall within the acceptable 20 % deviation range. Furthermore, AMP-^{15}N and ATP-d_{14} exhibited matrix suppression of up to 30 %, while ADP-^{15}N demonstrated matrix enhancement by 24 %. Consequently, the results were deemed unsuitable for accurate analysis of the targeted metabolites.

Recovery method 2 yields a maximum concentration of 83.32 μMol (±7.76) with NAD and a minimum concentration of 0.23 μMol (±0.03) with NADPH.

The recovery rates for the internal standards were 87 % for AMP-^{15}N, 94 % for ADP-^{15}N, and 99 % for ATP-d_{14}, all of which fall within the acceptable 20 % deviation range, indicating excellent recovery. Matrix suppression was minimal, with the highest observed value being 13 %. These findings suggest that the extraction method is highly suitable for accurate analysis.

Recovery method 3 yields a maximum concentration of 76.72 μMol (±12.08) with NAD and a minimum concentration of 0.28 μMol (±0.05) with FAD.

The recovery rates for the internal standards were as follows: 72 % for AMP-^{15}N, 118 % for ADP-^{15}N, and 92 % for ATP-d_{14}. While both AMP-^{15}N and ATP-d_{14} demonstrated matrix suppression, only ATP-d_{14} remained within the acceptable 20 % range. ADP-^{15}N showed matrix enhancement, though it also fell within the accepted 20 % range.

Based on the analysis of extraction methodologies, Extraction Method 2 (acetonitrile: methanol: 0.05 M formic acid) was selected as the most suitable. This method demonstrated an internal standard recovery range of 87 % to 99 %, indicating high accuracy and precision with minimal matrix suppression. Additionally, it consistently yielded some of the highest concentrations of metabolites in Saccharomyces cerevisiae samples.

Often extraction procedures are lengthy and can prove to be unnecessarily dangerous i.e. use of perchloric acid. For example, the use of the popular chloroform extraction process which increases total sample processing time by at least 12 h [11,25–47], and the addition of perchloric acid in quenching methodologies which often destroys the cell envelope leading to no distinction between intra and extracellular metabolites [45].

Method adaptation has been performed based on Canelas *et al.*, 2009; Rabinowitz *et al.*, 2007; Lu *et al.*,2018 [11,32,39,53,54]. The length of time the extraction process takes remains very similar with referenced methodology approximately 25 – 60 min. However, this methodology utilises the entire sample through fast filtration opposed to solely supernatant analysis.

Table 7
Target metabolites NAD, NADH, NADP, NADPH, FAD, AMP, ADP, and ATP recovery from extraction method trials with error margins.

Analyte	Recovery Method 1		Recovery Method 2		Recovery Method 3	
	AVG (μMol)	± (μMol)	AVG (μMol)	± (μMol)	AVG (μMol)	± (μMol)
NAD	14.2	3.8	83.32	7.76	76.72	12.08
NADH	0.41	0.08	0.51	0.07	0.86	0.15
NADP	1.24	0.28	7.4	1.35	0.72	0.12
NADPH	0.16	0.03	0.23	0.03	0.51	0.14
FAD	0.85	0.15	2.59	0.29	0.28	0.05
AMP	3.05	0.06	16.04	3.87	17.95	3.36
ADP	3.22	0.63	14.5	1.53	5.14	0.66
ATP	0.97	0.16	11.21	3.81	1.82	0.54

Table 8
Internal standard AMP-^{15}N, ADP-^{15}N, and ATP-d_{14} recovery from extraction method trials with error margins.

Analyte	Recovery Method 1		Recovery Method 2		Recovery Method 3	
	AVG (%)	± (%)	AVG (%)	± (%)	AVG (%)	± (%)
AMP-^{15}N	70.15	11.93	86.99	5.86	71.94	3.11
ADP-^{15}N	123.75	6.39	94.4	8.69	118.03	1.64
ATP-d_{14}	77.11	9.42	99.64	7.76	92.26	13.55

3.7. Method application

The optimised quenching, extraction, detection, and analysis protocols were applied to further *Saccharomyces cerevisiae* samples to determine the intracellular concentration of metabolites of the wider energy metabolome at varying growth points.

Methodology follows the same described in Section 2.2.-2.6. Yeast culture samples were taken every hour for the first 8 h of the growth cycle as well as an optical density measurement was taken to track growth at OD_{600}. Each sample was then analysed to determine intracellular concentrations of target metabolites of the wider energy metabolome (Table 9).

As the cell culture growth is performed under anaerobic conditions, only glycolysis can occur. Therefore, it is expected that nucleotides NAD, NADP and ATP are relatively high in abundance compared to their redox pairs and intermediates NADH, NADPH, and AMP which is reflected in the observed data. Within available comparative literature, intracellular concentrations of NAD have been reported to be within the region of 99 μM to 3000 μM [24–27,42,46,47]. The only comparable study found within the Yeast Metabolome Database (YMDB) for the anaerobic growth of *S.cerevisiae* reports an intracellular concentration of 950 ± 150 μM [48]. Compared to NADH with available comparative literature reporting 6.7 μM to 1420 μM [14,24,25,27,34,46] and the YMDB 500 ± 0 μM [49].

Similarly with the redox pairing NADP: NADPH, reported concentrations of NADP metabolites range from 1.26 μM to 50 μM with no comparable reference on the Yeast Metabolome Database (YMDB) [24,44,48]. Compared to NADPH reported concentrations ranging from 126.9 μM to 780 μM, with no comparable reference on the Yeast Metabolome Database (YMDB) [24,44,48].

There is no reported data available from YMDB however, what is available suggests an expected FAD concentration range of 19 μM to 87 μM [46,47]. Intracellular concentrations of AMP, ADP, and ATP are often reported together, as is the case referencing data obtained from the YMDB. In similar studies, intracellular concentrations of AMP are reported at 200–250 ± 170 μM, ADP reported at 530–1400 ± 800 μM and ATP reported at 1600–2650 ± 1750 μM [50–52].

All targeted metabolites with similar studies with intracellular concentrations reported are comparable with the concentrations reported in this study.

4. Concluding remarks

A relatively simple, fast, and accurate methodology for the determination of nucleotide concentrations from the wider energy metabolome of *Saccharomyces cerevisiae* using a zwitterionic column and UHPLC-MS instrumentation was developed and robustly validated. This is, to the best of our knowledge, the only methodology that does not require samples to be pre-spiked with the metabolites intended for detection and quantification. While these methodologies do work for laboratory samples, they do not accurately validate extraction and detection methodology for larger-scale processes beyond test tube size.

Table 9
Average calculated concentration of NAD, NADH, NADP, NADPH, FAD, AMP, ADP, and ATP with error margins from Saccharomyces cerevisiae samples taken at hour intervals over 8 h.

Time Stamp (h)	AVG Calculated Concentration (μM)							
	NAD	NADH	NADP	NADPH	FAD	AMP	ADP	ATP
1	112.64 ± 35.85	16.26 ± 1.11	33.65 ± 13.85	14.83 ± 1.83	15.84 ± 7.59	3.83 ± 0.52	9.50 ± 3.53	34.74 ± 5.26
2	155.91 ± 27.87	79.63 ± 3.13	46.74 ± 7.32	12.99 ± 1.01	21.97 ± 8.68	4.58 ± 0.82	30.11 ± 7.37	109.87 ± 22.55
3	277.11 ± 41.98	31.79 ± 1.60	67.94 ± 8.16	44.98 ± 15.71	30.55 ± 9.51	12.40 ± 3.16	51.94 ± 10.75	204.02 ± 26.97
4	152.28 ± 34.59	13.11 ± 1.04	92.99 ± 2.60	13.21 ± 2.68	28.70 ± 8.68	19.88 ± 6.12	58.21 ± 15.91	73.12 ± 17.57
5	69.39 ± 16.72	12.79 ± 1.02	76.38 ± 19.83	16.55 ± 1.05	34.26 ± 8.43	11.56 ± 2.80	35.94 ± 8.03	40.64 ± 3.69
6	203.79 ± 48.52	17.16 ± 1.16	162.11 ± 49.53	14.16 ± 0.86	53.95 ± 14.61	16.14 ± 3.13	61.53 ± 16.32	94.77 ± 1.78
7	347.12 ± 90.99	19.72 ± 1.21	138.18 ± 37.33	18.28 ± 1.71	55.80 ± 14.75	16.20 ± 1.60	80.34 ± 18.14	417.28 ± 9.99
8	201.48 ± 48.09	14.57 ± 1.06	90.33 ± 26.36	45.72 ± 16.89	53.11 ± 17.25	20.72 ± 4.20	61.53 ± 14.62	132.50 ± 17.49

This method relies upon the polar chemically bonded internal chemistry of the BEH HILIC column for the detection, separation, and quantification of polar to highly polar metabolites, within one methodology. Furthermore, this method used adapted established quenching and extraction protocols for the processing of *Saccharomyces cerevisiae* cell cultures.

The major advantages of this methodology include the runtime of 10 min per sample, as well as the use of a single triple quadrupole MS detection unit, which is standard equipment in many laboratories, and the use of widely available labelled isotopic or deuterated internal standards.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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