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Published in:
Analytical Chemistry Research

Link to article, DOI:
10.1016/j.ancr.2015.10.001

Publication date:
2015

Document Version
Publisher's PDF, also known as Version of record

Link back to DTU Orbit

Citation (APA):
Khoomrung, S., Martinez Ruiz, J. L., Tippmann, S., Jansa-Ard, S., Buffing, M. F., Nicastro, R., & Nielsen, J. (2015). Expanded metabolite coverage of Saccharomyces cerevisiae extract through improved chloroform/methanol extraction and tert-butyldimethylsilyl derivatization. *Analytical Chemistry Research*, *6*, 9-16. https://doi.org/10.1016/j.ancr.2015.10.001

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Analytical Chemistry Research 6 (2015) 9–16

Contents lists available at ScienceDirect

Analytical Chemistry Research

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

Expanded metabolite coverage of *Saccharomyces cerevisiae* extract through improved chloroform/methanol extraction and *tert*-butyldimethylsilyl derivatization

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ARTICLE INFO

Article history:
Received 21 September 2015
Received in revised form
8 October 2015
Accepted 12 October 2015
Available online 19 October 2015

Keywords:
Metabolomics
Extraction
Derivatization
Saccharomyces cerevisiae

ABSTRACT

We present an improved extraction and derivatization protocol for GC–MS analysis of amino/non-amino acids in *Saccharomyces cerevisiae*. Yeast cells were extracted with chloroform: aqueous-methanol (1:1, v/v) and the resulting non-polar and polar extracts combined and dried for derivatization. Polar and non-polar metabolites were derivatized using *tert*-butyldimethylsilyl (*t*-BDMS) dissolved in acetonitrile. Using microwave treatment of the samples, the derivatization process could be completed within 2 h (from >20 h of the conventional method), providing fully derivatized metabolites that contain multiple derivatizable organic functional groups. This results in a single derivative from one metabolite, leading to increased accuracy and precision for identification and quantification of the method. Analysis of combined fractions allowed the method to expand the coverage of detected metabolites from polar metabolites i.e. amino acids, organic acids and non-polar metabolites i.e. fatty alcohols and long-chain fatty acids which are normally non detectable. The recoveries of the extraction method was found at 88 ± 4%, RSD, N = 3 using anthranilic acid as an internal standard. The method promises to be a very useful tool in various aspects of biotechnological applications i.e. development of cell factories, metabolomics profiling, metabolite identification, ^{13}C-labeled flux analysis or semi-quantitative analysis of metabolites in yeast samples.

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1. Introduction

Yeast is already widely used as a cell factory for the production of biofuels and specialty chemicals, and through metabolic engineering, it is expected to be engineered to produce an even wider range of products in the future, i.e. perfumes, flavors, commodity chemicals, and pharmaceuticals. One of the advantages of using yeast as a cell factory is the large amount of databases and available information about its metabolism [1,2]. In order to engineer yeast metabolism to obtain high production yields, various methodologies i.e. synthetic biology methods [3] and analytical methods for screening wide ranges of metabolites have been developed [4–6]. Metabolomics, comprehensive identification and quantification of a complete set of metabolites in a given organism, has recently become an important tool in biological research [7]. This technology has been suggested for discovery of biomarkers in medical research, to improve production yields of cell factories or gain fundamental insights in the cellular biochemistry. Progress in metabolomics is driven by the development of analytical methodologies such as NMR, GC-, HPLC- or CE-MS and bioinformatic tools. Gas chromatography coupled to a mass spectrometer (GC–MS) has been a method of choice for measuring of various metabolites in several biological specimens [8–11]. Determination of polar

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http://dx.doi.org/10.1016/j.ancr.2015.10.001
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metabolites by GC–MS generally requires chemical modification (derivatization) to reduce the polarity of polar functional groups [12]. The derivatization converts polar functional groups i.e. –COOH, –OH, $-NH_2$, =NH and –SH of those metabolites to non-polar volatile derivatives [12–14]. Derivatization often improves chromatographic separation, reproducibility of the measurement and increases the thermal stability of the analyte. Among the derivatization methods for these metabolites, *tert*-butyldimethylsilylation (*t*-BDMS) is one of the most widely used because of several advantages i.e. less sensitive to the effect of the moisture and better thermal stability compared to trimethylsilylation (TMS) [15]. General products of *t*-BDMS derivatization result in mass increment of the *m/z* 144/functional group. The loss of the *tert*-butyl group [*m/z* M-57] during electron ionization fragmentation is very useful for structure elucidation. The *t*-BDMS derivatization has therefore been widely used in ^{13}C-labeled flux analysis as the original structure from loss of [M-57] contains rich information about the carbon skeleton of the derivative [16]. One of the main drawbacks with *t*-BDMS derivatization is an incomplete derivatization of the metabolites that contain more than one of organic functional groups, resulting in multiple derivative products from one metabolite [12,14,16]. The negative consequence of incomplete derivatization generally precludes precise identification because of the mixture of fully and partially derivatized metabolites and the reliability of quantification of the method. This drawback often prohibits the method to be suitable for semi-quantitative analysis (fold changes in relative to the peak area from the same metabolite) from different phenotypes. In this study, we evaluated and improved several analytical parameters for the determination of metabolites in *Saccharomyces cerevisiae* based on *t*-BDMS derivatization, followed by GC–MS for the low-resolution analysis of metabolites in yeast sample. We introduced and validated a method for increasing the sample throughput using microwave-assisted derivatization and expanded the coverage of detected metabolites by modifying the extraction protocol using $CHCl_3$/MeOH as the extraction solvent.

2. Material and methods

2.1. Chemicals, reagents and standards

All solvents used were analytical grade *N*-*tert*-Butyldimethylsilyl-*N*-methyltrifluoroacetamide with 1% *tert*-Butyldimethylchlorosilane (≥95%) as well as the standards for L-alanine, L-glycine, 2-aminobutyric acid, L-valine, L-leucine, L-isoleucine, L-proline, anthranilic acid, L-serine, L-succinate (disoduim), L-threonine, L-methionine, pyroglutamic acid, L-phenylalanine, malic acid, L-aspartic acid, L-cysteine, L-ornithine, L-glutamic acid, L-asparagine, L-glutamine, citrate, L-tyrosine, L-histidine, L-tryptophan, L-cystathionine, L-arginine, L-cystathionine, L-lysine, palmitoleic acid, palmitic acid, oleic acid with >98% purity and phosphoric acid (49–51% purity) were purchased from Sigma–Aldrich, Germany.

2.2. Sample and sample preparation

2.2.1. Yeast strains and culture conditions

The *S. cerevisiae* strain CEN.PK113-7D was used in this study. Yeast cells were grown in shake flasks containing liquid YPD medium supplemented with 2% glucose, at 30 °C and constant shaking (150 rpm). The medium was inoculated with an initial OD_{600} ~ 0.1. Cells were harvested during the stationary phase after 48 h.

2.2.2. Quenching and washing

Quenching, washing and extraction of the intracellular metabolites was performed as described by Canelas *et* al [17]. Briefly, 5 mL of the sample was withdrawn from the shake flask and quickly added to 21 mL of pure MeOH at −40 °C (in a 50 mL falcon tube which was placed in an ethanol bath). The extracellular metabolites were separated from intracellular metabolites by centrifugation at 2900 g at −20 °C for 5 min. The biomass was kept at −80 °C until further analysis.

2.2.3. Extraction

2.2.3.1. Conventional extraction method. The conventional extraction method was performed according to the known protocol as previously described [17]. 50 μL of anthranilic acid (10 mM) was added to the biomass from the previous step. Then the biomass was re-suspended in a 2.5 mL pre-cooled 50% (v/v) aqueous MeOH, followed by adding 2.5 mL of pre-cooled $CHCl_3$. The mixture was then vigorously shaken for 45 min at −20 °C (2000 rpm) and afterwards centrifuged at 2900 g (at −20 °C) for 5 min (rotor pre-cooled to −20 °C). The upper-phase (H_2O/MeOH) was collected into a new tube. The lower-phase ($CHCl_3$) was re-extracted with 2.5 mL of pre-cooled 50% (v/v) MeOH by vortexing for 30 s. After centrifugation, the upper-phase was collected and pooled together with the first extraction and kept at −40 °C until further analysis.

2.2.3.2. Modification of the extraction method by combining polar and non-polar-fraction. 50 μL of anthranilic acid (10 mM) was added to the biomass fraction and the biomass was extracted with 2.5 mL of pre-cooled 50% (v/v) aqueous MeOH and 2.5 mL of the pre-cooled $CHCl_3$. After shaking for 45 min at −20 °C (2000 rpm), the upper-phase and the lower-phase were collected and pooled together into a new tube. The mixture was then dried in a vacuum concentrator and re-suspended with 250 μL of 50% (v/v) aqueous MeOH and $CHCl_3$, transferred to a silanized GC-vial, then re-dried under vacuum conditions again and kept at −20 °C for further analysis.

2.2.4. Derivatization

2.2.4.1. Conventional derivatization protocol heating. 400 μL of acetonitrile and 100 μL of *t*-BDMS solution were added to the yeast extracts and sonicated for 30 min (30 °C). The sample was then incubated at 80 °C overnight (>20 h) and directly analyzed by GC–MS. Further dilution of the sample was performed when necessary by using acetonitrile as the solvent.

2.2.4.2. Microwave-assisted derivatization. Samples were prepared as above up to the overnight incubation step. The vial was then placed in a microwave vessel (12 cm × 3 cm I.D., 0.5 cm thickness; Milestones Start D, Sorisole Bergamo, Italy) containing 10 mL of Milli-Q water and then sealed with a TFM screw cap (Fig 1.). The vessel was then placed in the PRO-24 medium pressure high-throughput rotor (Milestones Start D, Sorisole Bergamo, Italy) and heated from room temperature to 120 °C within 10 min (800 W) and maintained at 120 °C (600 W) for 2 h in a laboratory microwave (Milestones Start D, Sorisole Bergamo, Italy). After cooling down the sample to room temperature, the samples were injected onto the GC–MS.

2.3. Determination of metabolite derivatives by GC–MS

The metabolite derivatives were separated and analyzed using a Focus GC ISQ single quadrupole GC–MS (Thermo Fisher Scientific, USA). The column was a Zebron ZB-1701 GC column (30 m × 0.25 mm I.D., 0.25-μm film thickness, Phenomenex, Macclesfield, UK). For the analysis, 1 μL of sample was injected in splitless injection (at 220 °C) using helium as the carrier gas with a flow rate of 1 mL/min. The column temperature was set at 50 °C (2 min), and afterwards raised to 150 °C (50 °C/min). Then the

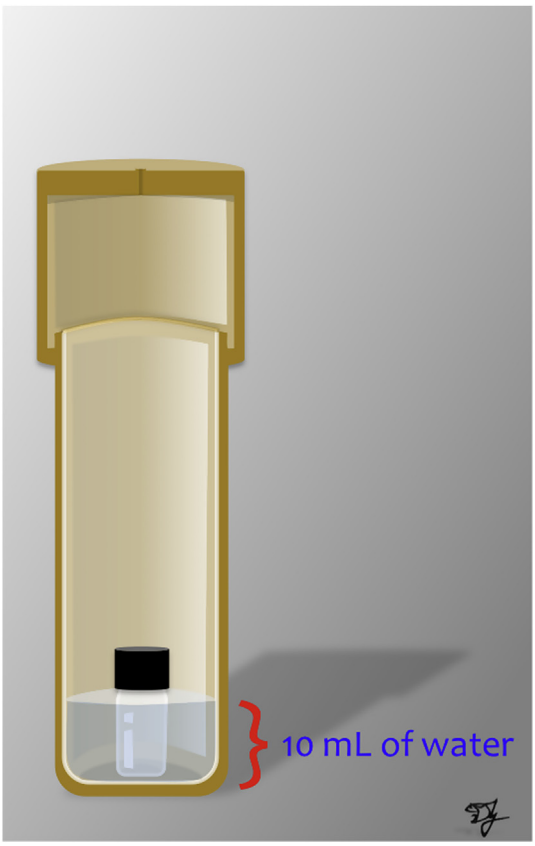

Fig. 1. How a GC vial sits in the microwave extraction container used in this protocol for microwave-assisted derivatization.

temperature was ramped to 300 °C (5 °C/min) and finally held for 3 min. The mass transfer line and ion source were set at 300 and 250 °C. The metabolite derivatives were detected with electron ionization (EI) with 70 eV in scan mode (50–800 *m*/*z*), with a solvent delay of 6 min. The identification of unknown metabolite derivatives from the yeast cells was performed by comparing their retention time and mass spectrum profiles with the synthetic standards or by comparison with the mass spectra library from National Institute of Standards and Technology (NIST). All data processing was performed by a Quan Brower function in the Xcalibur software version 2.2 (Thermo Fisher Scientific).

3. Results and discussion

3.1. Selection of an internal standard

We currently use a single quadrupole GC–MS in our laboratory for routine analysis of various metabolites during the development of microbial cell factories. For semi-quantitative purposes, it is essential to separate the internal standard (IS) from other derivative metabolites during the chromatographic separation. Our first experiment was therefore to determine a suitable IS. The derivatization protocol used in the beginning of this study was adapted from previous work performing the derivatization at 80 °C for 30 min [18]. First, we tested the d-2,3,3,3-alanine (D_4-Ala), which is commonly used as an IS in yeast research [19]. It was possible to derivatize D_4-Ala with the *t*-BDMS method. Using this method, the D_4-Ala derivative eluted at 7.39 min (Fig. 2A) with the molecular ion at *m*/*z*, 321 (Fig. 2B). However, it was not possible to use D_4-Ala as the IS, as it always co-eluted with Alanine (Ala). We tested several possibilities in order to overcome this, such as different column temperature programs, different column stationary-phases i.e. non-polar phase ZB-5 MS (5% diphenyl, 95% polysiloxane), mid-polar phase: ZB1701 (14%-cyanopropylphenyl-86%-dimethylpolysiloxane) or high-polar phase: Zb-50 (50% phenyl 50% dimethylpolysiloxane). However, none of those strategies were successful. Presumably, this could be due to the fact that both D_4-Ala and Ala have very similar physical properties; replacing of hydrogen with a deuterium atom did not significantly change the physical properties of the compound. In a second strategy, we tested the D_6-salicylic (D_6-sal) acid, which is also commonly used as the IS in metabolomics research. It was possible to form D_6-sal derivative (Fig. 2C and D), and the derivative eluted at 13.89 min. However, we did not select D_6-sal as an IS as it was co-eluted with methionine (Met) derivative. On the other hand, an anthranilic acid (Ant) was found to be a suitable candidate to be used as an IS, as it is not synthesised by yeast and possesses properties similar to many metabolites present in yeast. The Ant derivative eluted at 11.49 min (Fig. 2E), while none of the other derivative metabolites eluted in this region. The mass spectrum of Ant derivative (Fig. 2F) shows traces of a molecular ion at *m*/*z* 251 and a strong fragment at *m*/*z* 194 (M-57) which corresponds to the loss of the *tert*-butyl group (also confirmed by the NIST library). This result indicates that the silylation of Ant occurred only in the –COOH functional group, whereas the $-NH_2$ group was not silylated (at 80 °C for 30 min). Further testing of the derivatization conditions (4 h and overnight; Fig. S1) did not yield an additional peak, leading to the conclusion that there is no derivatization occurring with the $-NH_2$ group with *t*-BDMS. This finding is consistent with the previous report by Schoene et, al. 1994 [20] that the ability to silylate NH-functional groups depends on the substitutions neighboring the nitrogen atom, and the direct silylation of aromatic amines is hardly possible without being preceded by an acylation reaction. Based on this, we selected Ant as the internal standard for further method development.

3.2. Derivatization solvent selection

Although many different solvents have been proposed for *t*-BDMS derivatization i.e. Dimethylformamide (DMF) [18,21], toluene [22], acetonitrile (ACN) [22,23], there is no detailed information available that describes the effect of each solvent on the derivatization reaction. Here we tested five solvents commonly used in *t*-BDMS derivatization, ranging from mid polar to non-polar, i.e. ACN, DMF, acetone (AT), ethyl acetate (EA) and hexane (Hex). The efficiency of derivatization using different solvents was evaluated through the mixture of 20 amino/non-amino acids standards derivatized at 80C° for 30 min [18]. In general, ACN (Fig. 3A) was found to be the best solvent for derivatization, in terms of peak shape and intensity, when compared to the other solvents tested. Using ACN as the solvent, however, resulted in incomplete derivatization of Threonine [(Thr1), Fig. 3A: structure in Fig. S2] and Tryptophan [(Trp1), Fig. 3A and B: structure in Fig. S3]. The Thr1 resulted from the silylation of Thr in the –OH and –COOH functional groups, whereas the $-NH_2$ group was not derivatized. As well as in the case of Trp (Fig. 3A and B: structure in Fig. S3), there was no silylation in the amine group that is attached to the benzene ring. To complete the derivatization for all organic functional groups in these molecules required 4 h for Thr (Fig. S4) and overnight incubation for Trp (Fig. S5). Using DMF as the solvent (Fig. 3B), slightly improved the derivatization efficiency of Thr and Trp. Peak tailing or fronting were however, observed in many derivative metabolites i.e. Ala, glycine (Gly), leucine (Leu), iso-leucine (Iso-Leu) or proline [Pro; (Fig. 3B)]. Additionally, the Ant was poorly derivatized when using DMF as the solvent. We therefore preferred

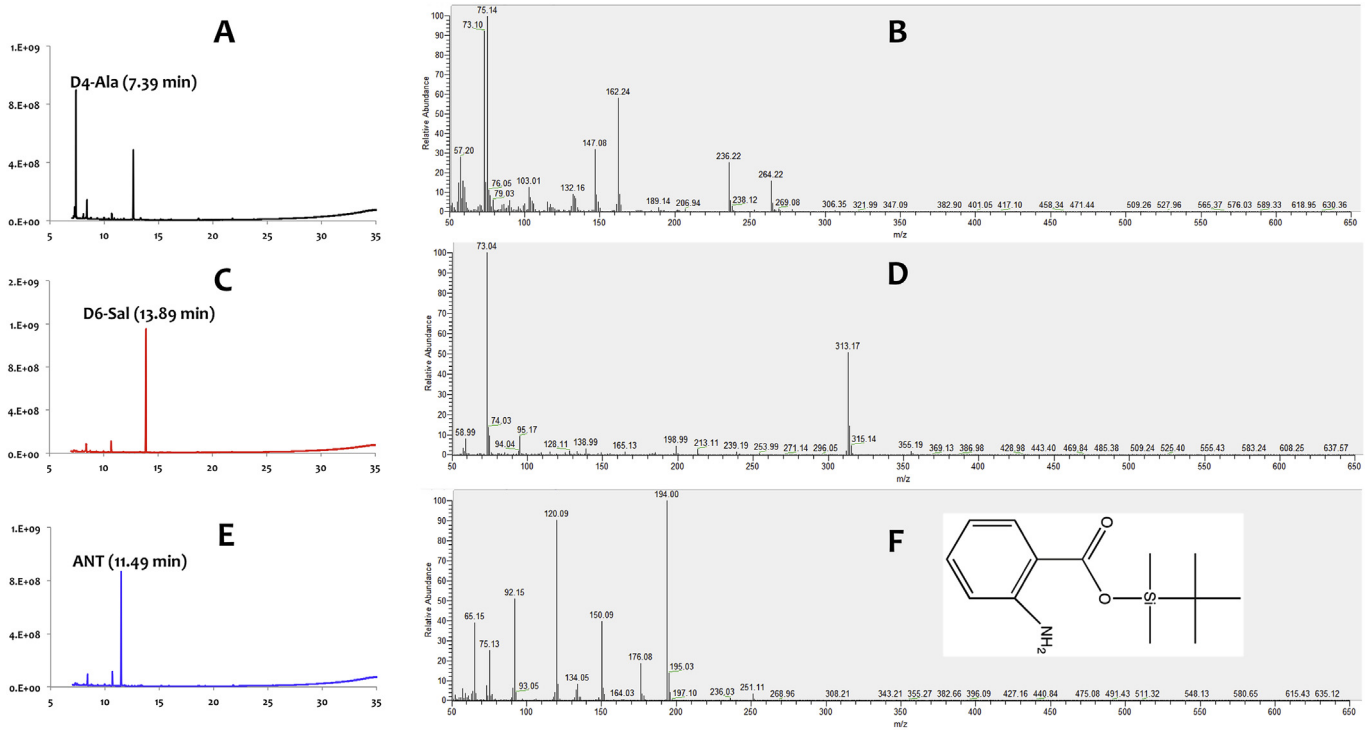

Fig. 2. Chromatograms and mass spectra of potential candidates for internal standards.

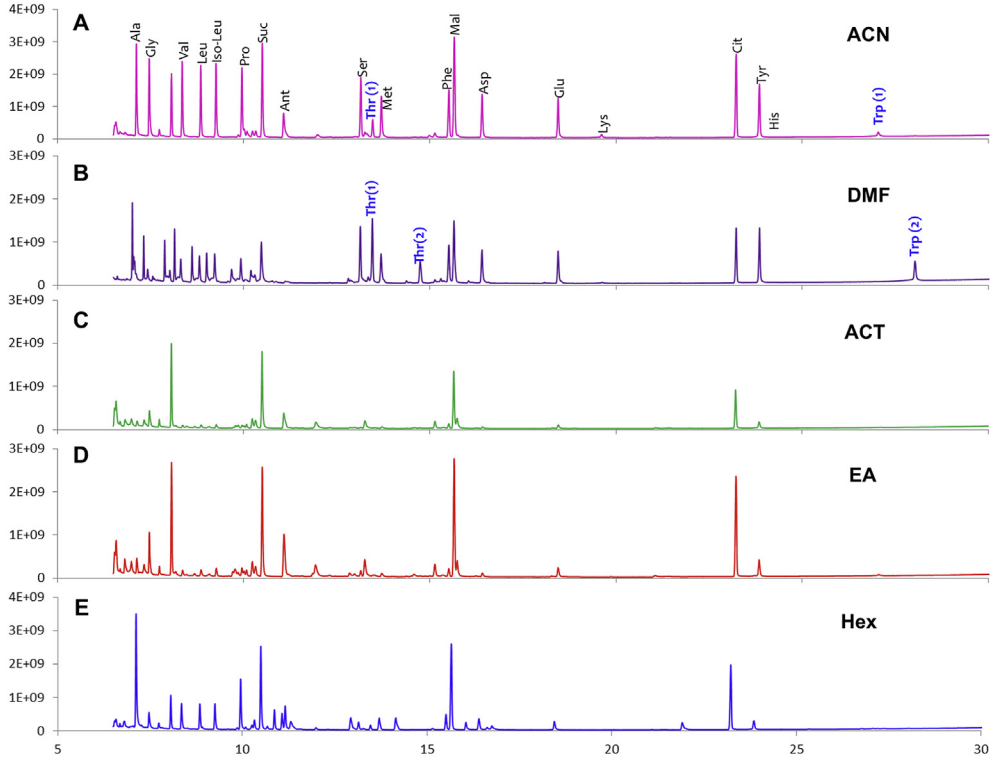

Fig. 3. t-BDMS derivatization of 20 metabolite standards with different solvents Ala = Alanine, Gly = Gylcine, Val = Valine, Leu = Leucine, Iso-Leu = Iso-Leucine, Pro = Proline, Suc = Succinic acid, Ant = Antranlic, Ser = Serine, Thr = Theonine, Met = Methionine, Phe = Phenylalanine, Mal = Malic acid, Asp = Aspartic acid, Glu = Glutamic acid, Lys = Lysine, Cit = Citric acid, Try = Tyrosine, His = Histidine, Trp = Trypophan.

to use ACN as the main solvent for derivatization. To achieve a single derivative when using ACN as the main solvent, we tested the possibilities of mixing DMF with ACN in different ratios. Increasing the percentage of DMF in the ACN (20–100%: see Fig. S6)

enabled complete derivatization of both Trp and Thr. However, as for the use of DMF as the sole solvent, the peak shapes of some derivatives interfered with an increased amount of DMF, we finally decided to use only ACN as the single solvent for the derivatization.

To obtain the optimum derivatization time for each metabolite, we derivatized 31 synthetic standards individually for 4 h and overnight (>20 h). The derivatization temperature was fixed at 80 °C. The 30 metabolites standards were selected according to the most dominant metabolites present in *S. cerevisiae*. Derivatization of most of the metabolites could be completed after 4 h as there were only single derivatives and no incomplete derivatized metabolites observed in the chromatograms. Glycerol was partly derivatized after 4h and the derivatization efficiency could be further improved by increasing the incubation time to overnight (>20 h). It was not possible to detect arginine after *t*-BDMS derivation due to its conversion to ornithine (Fig. S7) as previously reported by other studies [12,13].

3.3. Improved chemical derivatization of t-BDMS by microwave-assisted derivatization

We recently successfully used microwave technology to improve the sample preparation time for fatty acid methyl esters and lipid extraction [4,5]. Within the same basic workflow, we carried out the derivatization reaction in a GC-vial, which was immersed in 10 mL of water (Fig. 1). We used Trp as the model compound for the optimization parameters, as it requires the longest time to complete its derivatization. We found that the derivatization of Trp was completed within 10 min at 160 °C (S8-A). However, performing derivatization at this high temperature often resulted in deforming the shape of the GC cap, which led to a loss of analytes. On the other hand microwave-derivatization at 120 °C did not show any evidence of damage to the GC cap.

The time needed to complete derivatization of Trp at 120 °C was found to be at least 60 min (Fig. S8 B). As the amount of metabolites in the yeast samples may vary from sample to sample and this could influence the derivatization time, we chose 2 h (120 °C) as the optimal condition. We further tested the microwave derivatization method (2 h) with some selected synthetic standards individually and did not observe incomplete derivatization of the metabolites when using this new method. Finally we compared the derivatization by the microwave method (120 °C at 2 h) with the traditional heating (80 °C overnight) method. There was no significant difference in the profiles of metabolite derivatives obtained from microwave-assisted derivatization when compared with traditional heating (Fig. 4). However, the utilization of microwave for sample preparation dramatically reduced the *t*-BDMS derivatization time as well as reported in the case of TMC derivatization [24], as the reaction temperature inside the closed-vessel can be raised above the solvent boiling point, resulting in faster reaction time compared to traditional heating [25].

3.4. Recovery of the internal standard and expanding the coverage of detected metabolites in yeast samples by GC–MS

We investigated the recovery of the method by spiking Ant (0.5 μmol) into the mixture of extraction solvents (50% H_2O–MeOH: $CHCl_3$ 1/1) and performing extraction using a previously reported protocol [17]. The polar fraction (H_2O–MeOH) was dried under vacuum conditions, followed by *t*-BDMS microwave-assisted derivatization for 2 h and subsequently analyzed by GC–MS. Only 26% of spiked Ant (N = 6) was recovered from the polar fraction (compared with direct derivatized Ant without extraction). We assumed that the low recovery could be due to the fact that Ant was partly trapped in the $CHCl_3$ fraction, as part of Ant

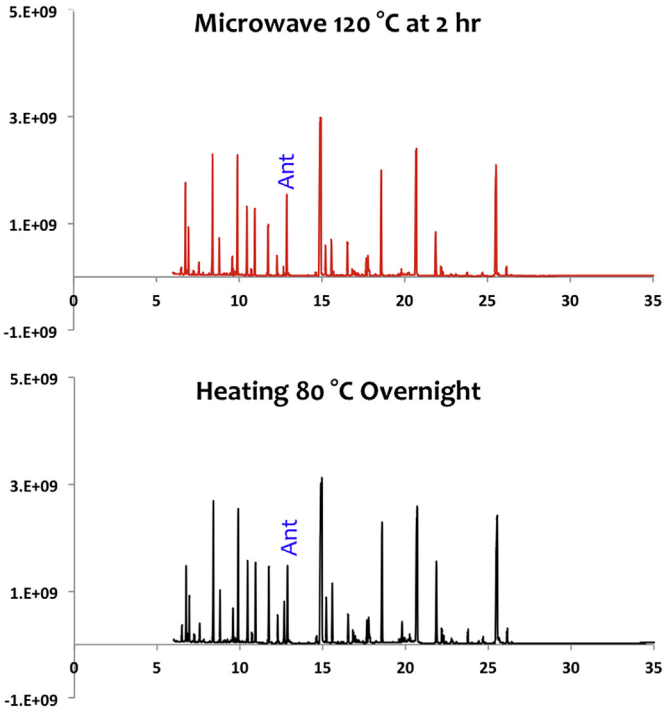

Fig. 4. Comparison of the profiling of amino/non-amino derivatized with microwave-assisted derivatization and traditional heating. The samples were 10 mg of yeast *S. cerevisiae*.

structure contains a non-polar benzene ring. Improving the recovery of the method could be achieved by using a more polar IS than Ant. This, however, would result in the absence of other non-polar metabolites that may be in the $CHCl_3$ phase. Finally, we conducted three different experiments for metabolite profiling in *S. cerevisiae* that derived from different fractions after extraction with H_2O–MeOH: $CHCl_3$.

First, we extracted *S. cerevisiae* with the mixture solvent of H_2O–MeOH: $CHCl_3$ with spiking of Ant (0.5 μmol). The polar fraction (H_2O–MeOH) was dried under vacuum conditions, followed by *t*-BDMS derivatization and analyzed by GC–MS. The non-polar fraction (from the same sample) was dried, derivatized using *t*-BDMS. For the last experiment, we re-modified the extraction protocol by collecting both polar and non-polar factions and combining them together. The combined fractions were then further derivatized and analyzed by GC–MS. Analysis of the polar fractions (Fig. 5A), shows recoveries of Ant at 22 ± 5% (N = 3), which was consistent with the recovery of Ant (72 ± 5%; N = 3) found in the non-polar fractions (Fig. 5B). For polar fraction, we identified 28 metabolites, where 25 metabolites were confirmed by using synthetic standards (see full list of identified metabolites in Table 1). For the non-polar fraction, we identified 7 metabolites, mostly long-chain fatty acids such as 16:1n-7, 18:1n-9 and 18:0 (also confirmed by synthetic standards) or fatty alcohols. The fatty acids detected in the non-polar fraction were originally from free fatty acids, as *t*-BDMS derivatization only work by substitution of an active hydrogen atom of –COOH with *tert-butyldimethylsilyl*, and not with an ester bound in bound fatty acids. Additionally, the method could serve as a simple, rapid and selective method to identify free fatty acids from bound fatty acids in the lipid mixture. The separation and identification of free fatty acids in lipid mixture normally requires a long process and a very complicated protocol.

In general, metabolites found in the non-polar fraction are normally not reported when the extraction is performed based on

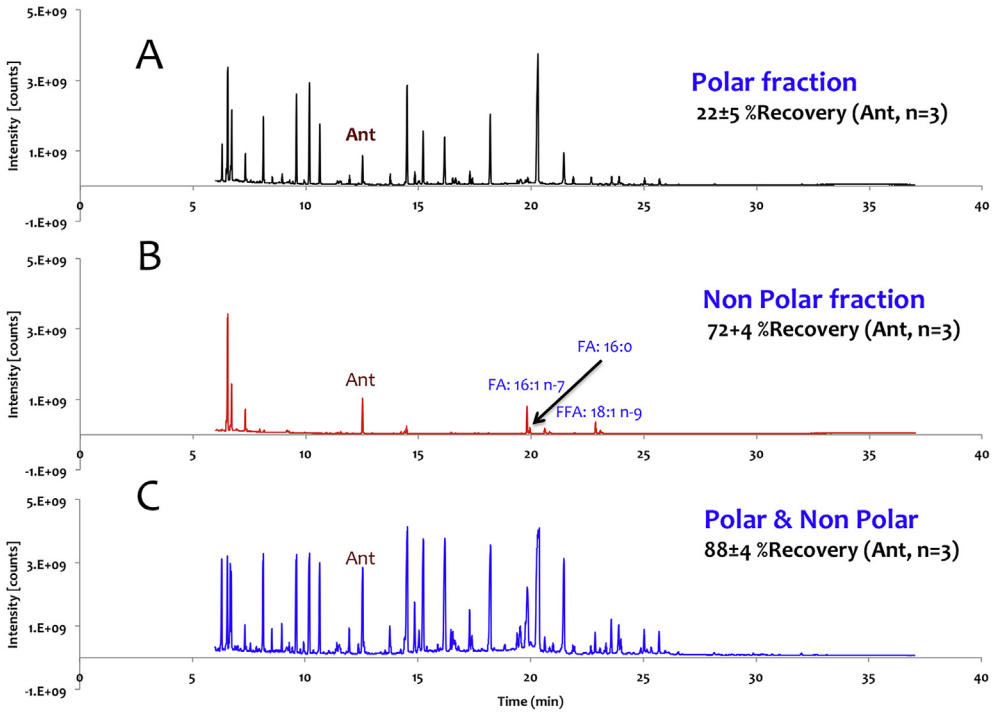

Fig. 5. Chromatographic separation of metabolites derived from different extraction fractions A: Polar fraction, B = Non-polar fraction, C = combination of polar and non-polar fraction.

$CHCl_3$/MeOH, and only the MeOH fraction is further analyzed [26,27]. This could simply be explained by the fact that these long-chain fatty acids or fatty alcohols are hydrophobic molecules and thus partition into $CHCl_3$ rather than aqueous-MeOH. Analysis of the combined fractions (Fig. 5C) resulted in good recoveries of Ant (88 ± 4%; N = 3) and the base line in the chromatogram was remarkably clean compared to that found in polar fraction. The analysis of this fraction resulted in 49 peaks (Table 1) using GC–MS detection. From all the detected metabolites, we confirmed identities of 28 metabolites using known standards, 7 metabolites were putatively identified by NIST library matching and 13 peaks were unidentified. Furthermore, analysis of Trp from the polar fraction generally resulted in poor reproducibility (even from the same sample), which was not the case observed in combined fractions. The peak intensity of Trp from the combined fraction was significantly greater (4-fold, N = 3) than that in the polar fraction. As the structure of Trp contains both of polar organic functional groups (−NH_2 and −COOH) and non-polar region as an indole functional group (aromatic), we presume that extraction of Trp using $CHCl_3$/MeOH would result in the presence of Trp in both polar and non-polar fractions. The quantity of Trp in each fraction is unlikely to be easily controlled. This makes it difficult to obtain good reproducibility when detecting Trp derived from only one of the fractions. The same situation was observed in the case of other metabolites that contain two different polarities of organic functional groups in the same molecule, i.e. Tyr. These results indicate that combining the polar and non-polar fractions does not only expand the coverage of detected metabolites, but also significantly improves the reproducibility and sensitivity of the method.

Performing analysis of polar and non-polar fractions from the same sample for metabolomics profiling has previously been conducted in the case of *Arabidopsis thaliana* [28]. However, the two fractions were derivatized and analyzed separately by GC–MS, resulting in more than 300 metabolites, both polar and non-polar. Analysis of polar and non-polar fractions performed in the same analysis, especially in the yeast sample, has never been reported. Furthermore, it is known that more than 1000 compounds can be present in *S. cerevisiae* [29]. However, the number of metabolites in the yeast sample can vary greatly depending on several factors i.e. background strain, cultivation conditions or sampling state. For example, we did not detect glycerol from this study because the yeast sample was harvested at a stationary phase (48 h), which is known not to contain glycerol.

3.5. Sensitivity and stability of GC–MS for quantitative analysis

It is known that the mixture of $CHCl_3$/MeOH is an efficient combination solvent for lipid extraction and many other non-volatile molecules. These lipids normally cause problems regarding the stability of GC–MS when analyzing them without appropriate derivatization. To address this question, we performed an experiment by analyzing a series of four Ant derivative (N = 5) and derivatized samples from combined fractions (N = 25) sequentially. The result (Fig. 6) shows a slight drop in sensitivity (observed from the intensity of Ant derivative) of the instrument when increasing the number of measurement samples. The reproducibility of the measurement of Ant derivative in each set of runs was excellent (%RSD ≤ 3; N = 5). Acetonitrile is an efficient solvent to perform *t*-BDMS derivatization, and also widely used in the extraction of various metabolites [30] [31] because of its excellent ability to dissolve polar metabolites. On the other hand, lipids tend to dissolve poorly in acetonitrile, as we have observed during the sample preparation, where there was residue of lipids in the vial after addition of acetonitrile (during the derivatization step). Based on this evidence, we presumed that injecting derivatized samples from combined fractions that did not contain most lipid species allowed instruments to main stability and sensitivity during a large number of measurements. Results indicate that the method is highly reproducible and suitable for the use of quantitative or semi-quantitative analysis.

Table 1
Identification of metabolites from in *S. cerevisiae* from different fractions after liquid–liquid extraction.

Order	Retention time (min)	Metabolite	MeOH fraction	$CHCl_3$ fraction	MeOH + $CHCl_3$
1	8.15	L-Alanine	✓		✓
2	8.52	L-Glycine	✓		✓
3	8.96	[a]Butanoic acid	✓		✓
4	9.62	L-Valine	✓		✓
5	9.94	Unknown	✓		✓
6	10.17	L-Leucine	✓		✓
7	10.65	L-Isoleucine	✓		✓
8	11.95	Succinate	✓		✓
9	12.52	Anthranilic acid	✓	✓	✓
10	13.74	Unknown	✓		✓
11	14.50	Phosphoric acid	✓		✓
12	14.84	L-Serine	✓		✓
13	15.06	[a]2-Pyrrolidone-5-carboxylic acid	✓		✓
14	15.21	L-Threonine	✓		✓
15	15.40	L-Methionine	✓		✓
16	15.88	[a]Salicylic acid	✓		✓
17	16.15	L-Pyroglutamic acid	✓		✓
18	16.45	[a]3,5-Disilaheptane, 4-(5-ethoxypyridin-2-yl)-2,2,3,3,5,5,6,6 octamethyl-		✓	✓
19	16.55	Unknown	✓		✓
20	16.79	Unknown	✓		✓
21	17.28	L-Phenylanine	✓		✓
22	17.40	D-Malic acid	✓		✓
23	18.19	L-Aspartic acid	✓		✓
24	18.86	L-Cysteine	✓		✓
25	19.39	[a]3-hydroxy-3-methylglutaric acid	✓		✓
26	19.52	2-amino-4-nitrophenol	✓		✓
27	19.82	Cis-9-Hexadecenoic acid (16:1-n7)		✓	✓
28	19.86	L-Ornithine	✓		✓
29	20.30	L-Glutamic acid	✓		✓
30	20.60	Unknown		✓	✓
31	20.81	[a]1-Octadecanol		✓	✓
32	21.00	[a]D,L-Homocysteine			✓
33	21.48	L-Lysine	✓		✓
34	21.87	L-Asparagine	✓		✓
35	21.92	Unknown		✓	✓
36	22.67	Unknown	✓		✓
37	22.87	Cis-9-Octadecenoic acid (18:1n-9)		✓	✓
38	23.07	Octadecanoic acid (18:0)		✓	✓
39	23.57	Unknown	✓		✓
40	23.90	L-Glutamine	✓		✓
41	24.01	Unknown	✓		✓
42	24.89	Unknown	✓		✓
43	25.03	Citrate	✓		✓
44	25.35	Unknown	✓		✓
45	25.70	L-Tyrosine	✓		✓
46	26.54	Unknown	✓		✓
47	28.13	Unknown	✓		✓
48	29.88	L-Tryptophan	✓		✓
49	32.00	L-Cystathionine	✓		✓

[a] The metabolites identified by the National Institute of Standards and Technology (NIST) mass spectra search but not verified with synthetic standards.

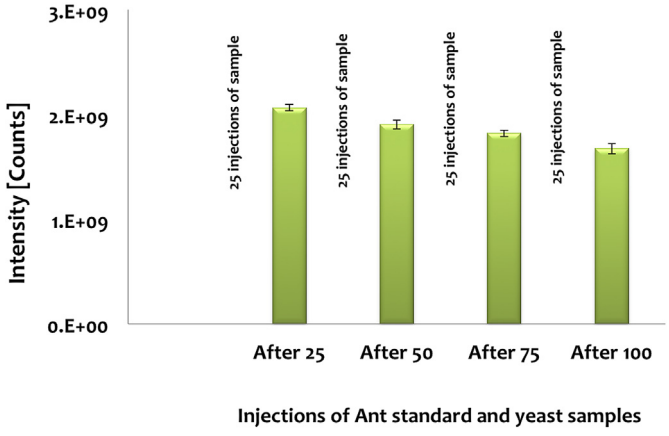

Fig. 6. Stability and reproducibility of the GC–MS obsurved by injecting Ant derivative (N = 5) after 25 injections of sample from combined fractions.

4. Conclusion

In conclusion, we report an improved protocol for derivatization based on *t*-BDMS and for extraction based on $CHCl_3$/MeOH, which is suitable for analysis of metabolites in yeast samples. From this study, we did not observe partially derivatized analytes, which is the major problem when experimenting with *t*-BDMS derivatization. Analysis of combined fractions was shown to be an alternative metabolomics method for sample preparation owing to its increasing accuracy and precision of the measurement. This method will potentially become a highly relevant tool for metabolite profiling in yeast cells being developed as efficient cell factories. Because amino acids and other related metabolites represent key intracellular metabolites that are closely linked to many organic acids, rapid and easy analysis of these metabolites will have a significant impact on our ability to metabolically engineer yeast. We are also confident that the method can be applied to metabolite analysis from other organisms.

Acknowledgments

We would like to thank to David Jullesson for the artwork, Dr. Partho Sen, Dr. Francesco Gatto, Dr. Alastair Ross, Dr. Thomas Moritz and Dr. Eugene Fletcher for comments and constructive discussion. This work has been financially supported by BioVacSafe (http://www.biovacsafe.eu/), the Novo Nordisk Foundation and the Knut and Alice Wallenberg Foundation.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ancr.2015.10.001.

Note

The authors declare no completing financial interest.

Conflict of interest

There is no conflict of interest.

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