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In comprehensive lipidomics studies, accurate quantification is essential but biological and/or clinical relevance is often hindered due to unwanted variations such as lipid degradation during sample preparation, matrix effects and non-linear responses of analytical instruments. In addition, the wide chemical diversity of lipids can complicate the accurate identification of individual lipids. These analytical limitations can potentially be corrected efficiently by the use of lipid-specific isotopically labelled internal standards (IS) but currently such IS mixtures have limited coverage of the mammalian lipidome. In this study, an *in vivo* ^{13}C labelling strategy was employed to explore four species (*Escherichia coli*, *Arthrospira platensis*, *Saccharomyces cerevisiae* and *Pichia pastoris*) as a source of ^{13}C -labelled internal standards (^{13}C -ISs) for more accurate and quantitative liquid chromatography (LC)-mass spectrometry (MS)-based lipidomics. Results showed that extracts from ^{13}C -labelled *P. pastoris* and *S. cerevisiae* contain the highest percentage of uniformly labelled lipids (both 83% compared to 67% and 69% in *A. platensis* and *E. coli*, respectively) and ^{13}C -labelled *P. pastoris* extract was identified as the optimum source of ^{13}C -ISs for comprehensive data normalisation to correct unwanted variations during sample preparation and LC-MS analysis. Overall, use of a biologically generated ^{13}C -IS lipid mixture of 357 identified lipid ions resulted in significant reduction in the lipid CV% of normalisation compared with other normalisation methods using total ion counts or a commercially available deuterated internal standard mixture. This improved normalisation using ^{13}C -IS was confirmed in a typical lipidomics analysis using a large number of samples (>100+) and long analysis time (>70 h). This study highlights the benefit of an *in vivo* labelling strategy for reducing technical and analytical variations introduced during sample preparation and analysis in lipidomics studies.

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Introduction

Perturbations in lipid turnover and metabolism are associated with several human diseases including diabetes,¹ cancer,² atherosclerosis,³ Alzheimer's,⁴ depression,⁵ and cardiovascular diseases.⁶ Monitoring changes in lipid profiles in biofluids or tissues can provide valuable information to improve our understanding of different pathological conditions and has the potential to guide the therapy more efficiently.⁷

Among the various analytical platforms that can be used for lipidomics analysis, liquid chromatography-mass spectrometry

(LC-MS) has emerged as the most valuable tool to monitor and identify lipid changes⁸ due to its high sensitivity and resolving power, and excellent repeatability.^{9,10} However, the variable recovery of lipids from biological samples and unwanted ion suppression/enhancement of MS signals can impact on the quality of quantitative analyses.¹¹ Systematic and random errors due to lipid degradation during sample preparation, and variations in instrumental response in large-batch analysis can also affect the quality of the data leading to high variability in important biological/clinical outcomes such as biomarker discovery.¹² The development of a reliable normalisation method using isotope dilution mass spectrometry (IDMS) methodology, applicable to the structurally diverse lipid species in a biological sample, would therefore have significant advantages. The use of IDMS can also help with the identification of lipids where unambiguous characterisation can be achieved by matching retention time (RT) and fragmentation patterns of product ion spectra with co-extracted IDMS isotopically-labelled internal standards.¹³

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Recently, several research groups have reported the use of isotopically labelled compounds as internal standard (IS) to minimise the matrix effects in lipidomics analysis.^{14,15} Furman *et al.*¹⁶ biosynthetically produced fifty-four uniformly ¹³C-labelled (U-¹³C) arachidonate oxidation products to evaluate the arachidonic acids and their oxidation products in patients with Alzheimer's disease. However, this approach was focused on only oxidised eicosanoids (C20) and hence is of limited value when considering the whole lipidome. Ideally, in IDMS, one isotopically labelled IS for each lipid or lipid species could be used to improve the reliability and accuracy of untargeted lipidomics analysis. However, most lipids are not readily available in isotopically labelled forms and, although chemical synthesis could potentially be used, it is laborious and costly to generate the required diverse and complex structures. To overcome this problem there has been significant recent interest in the biological synthesis of comprehensive mixtures of isotopically labelled IS *in vivo* for application in lipidomics using microorganisms.¹⁷ Potentially, organisms grown on ¹³C-labelled carbon sources can produce a wide range of isotopically labelled intracellular lipid compounds to be used as a source of IS. Ideally, to obtain a representative set of isotopically labelled compounds as a source of IS, the same organism being studied should also be used to generate the ¹³C-labelled IS mixture. For more complex metabolisms (e.g., mammalian cells), where it is difficult to produce high labelling efficacy due to slow growth and the need for multiple carbon sources required for survival,¹⁸ various microorganisms can be used as a source of IS.¹⁹ This approach has been used for quantitative analysis of intra- and extracellular metabolite concentrations in targeted lipidomics studies in which ¹³C-IS were generated from different organisms such as *Escherichia coli*,²⁰ *Penicillium chrysogenum*,²¹ *Pichia pastoris*.²² A preliminary comparison of the *E. coli* Metabolome Database (ECMDB)²³ and Human Metabolome Database (HMDB)²⁴ revealed 376 common lipids between the two databases under the superclass "Lipids and lipid-like molecules" and "Organic compounds" Kingdoms, while 845 metabolites were found to be in common between the Yeast Metabolome Database (YMDB) of *S. cerevisiae* to the HMDB.^{24,25} This suggests that yeasts may be more suitable for generating the correct range of isotopically-labelled lipids for use in IDMS-based lipidomics applied to mammalian cells and tissues. To the best of our knowledge, only two lipidomics studies have explored the use of isotopically labelled extracts as IS in untargeted analysis of clinical biological samples.²⁶ The LILY strategy (Lipidome Isotope Labelling of Yeast)¹⁷ revealed that *in vivo* labelling of *P. pastoris* enabled the production of 212 lipid standards from 13 lipid classes that can be applied for compound specific quantification and/or for class/RT specific quantification in yeast and human plasma samples by isotope dilution. However, the LILY strategy has not been extended yet to other microorganisms belonging to different kingdoms in order to determine which lipids can be covered for human samples. Also, the use of biosynthesized ¹³C-IS as quality control in untargeted lipidomics studies and their application for batch-to-batch corrections including normalisation of data sets obtained from extended analysis times have not been explored yet. The yeast *Pichia*

pastoris has been widely used to produce biological compounds²⁷ and has been shown to generate similar metabolite and lipid profiles to mammalian cells.¹⁷ *Escherichia coli* has been previously used for small-scale production of uniformly ¹³C-labeled cell extract for quantitative metabolome analysis,^{28,29} but there are no examples of *in vivo* generation of ¹³C-IS to apply in lipidomics study of human samples. Similarly, the lipid profile of *Arthrospira platensis* and *Saccharomyces cerevisiae* have not been explored as a source of ¹³C-labelled lipids IS in complex human samples. Therefore, this work aims to (1) compare the lipidomes of *E. coli*, *A. platensis*, *S. cerevisiae* and *P. pastoris*; (2) select the optimal source of labelled IS mixture that can be employed in quantitative lipids analysis of human plasma samples by isotope dilution and (3) to evaluate the full capability of the biosynthesized ¹³C-ISs in correcting various sample processing and instrumental variations that could arise in typical untargeted lipidomics studies.

Experimental section

Materials

E. coli MG1655 was kindly provided by Dr Nigel Halliday, The Biodiscovery Institute, University of Nottingham (Nottingham, UK). Screened blood plasma (pooled normal, control plasma) was provided by the Nottingham University Hospitals Blood Bank, Queen's Medical Centre Campus, Nottingham University (Nottingham, UK). *S. cerevisiae* CEN.PK 113-7D and *P. pastoris* NCYC175 were supplied by EUROSCARF (Oberursel, Germany) and National Collection of Yeast Cultures (Norwich, UK), respectively. *A. platensis* (spirulina) whole cells (lyophilised powder) unlabelled and labelled (U-¹³C, 97%) and D-glucose (U-¹³C6, 99%) were purchased from Cambridge Isotope Laboratory (Massachusetts, USA). SPLASH LipidoMix standard solution was supplied by Avanti (Alabama, USA) and used as a deuterated internal standard (D-IS) mixture that includes all the major lipid classes at ratios similar to those found in human plasma. More details of chemicals and materials used in this study are given in the ESI.†

Growth conditions

To prepare the unlabelled (¹²C) extracts the studied species were grown as described below. A culture of *E. coli* was cultured overnight in M9 minimal media (prepared according to Cold Spring Harbor Protocols³⁰). The cultures of *S. cerevisiae* and *P. pastoris* were prepared similarly in minimal media according to Verduyn *et al.*³¹ Overnight cultures were diluted with 100 mL of minimal media to an optical density (OD₆₀₀) of 0.1 and grown at 122 rpm at 37 °C (*E. coli*) and 30 °C (*S. cerevisiae* and *P. pastoris*) until they reached the stationary phase. During the method development, filter-sterilised glucose solution was added as the only carbon source to a final concentration of 2% for growing all organisms. The growth rate of the studied species was monitored by measuring the OD₆₀₀ every 2 h for 36 h (*n* = 3).

For the ¹³C uniformly labelled glucose experiments 99% U-¹³C-glucose was used in minimal media preparation instead of ¹²C-glucose, the culture conditions were otherwise identical



to those used for ^{12}C glucose. The effect of different concentrations (0.5, 1, 1.5%, 2%) of ^{13}C glucose was evaluated for the selected species only.

Biosynthesized ^{13}C -internal standard extraction

For each studied species, three biological replicates of cell culture at early stationary phase were extracted. The cellular lipids were extracted using a chloroform/methanol/water mixture according to the Bligh and Dyer method (details are given in the ESI†).³² The chloroform (lower) phase was dried under vacuum at room temperature and the dried sample was reconstituted using 200 μL of LC-MS grade isopropanol and stored at -80°C until analysis.

Lipids extraction from plasma samples

Lipids were extracted from 50 μL of plasma with 500 μL of chloroform/methanol (1 : 2) and 100 μL of IS mixture according to the method described by Bligh and Dyer with small modifications given in the ESI.†³² The organic phase was dried under vacuum at room temperature and reconstituted using 100 μL LC-MS grade isopropanol and stored at -80°C until further analysis.

LC-MS lipidomics analysis

Chromatographic separation was conducted on ACE Excel 2 μm super C18 column (50×2.1 mm, pore size 100 \AA , Advanced Chromatography Technologies Ltd, Scotland, UK) using a Dionex Ultimate 3000 Series UHPLC system (Thermo Fisher Scientific, Hemel Hempstead, UK). The chromatographic conditions were set according to the method of Haoula *et al.*³³ with some modifications (details of the LC experimental conditions are given in the ESI†).

Mass spectrometry was performed on a hybrid quadrupole Orbitrap Q-Exactive MS (Thermo Fisher Scientific, Hemel Hempstead, UK) acquiring data in full scan ion mode and tandem MS/MS in both positive and negative modes using an electrospray ionisation (ESI) source (full details of the MS experimental conditions are given in the ESI†). The data quality obtained from the LC-MS/MS analysis was assessed by determining the percentage coefficient of variation (CV%) in the mean peak intensities of all peaks present in the quality control (QC) samples using a metabolomics approach proposed by Want *et al.*³⁴ The software LipidSearch 4.1.16 (MitsuiKnowledge Industry, Tokyo, Japan) was used for lipid identification. The parameters used for lipid identification and alignment across samples are listed in Tables S1 and S2 of the ESI.† Tox-ID software (Thermo Fisher Scientific, USA) was used to assess the labelling efficacy of the ^{13}C labelling strategy.

Optimisation of the ^{13}C IDMS internal standard source

The extracted lipids from different species (*E. coli*, *S. cerevisiae* and *P. pastoris*) were blended in different ratios to evaluate their suitability for use in normalisation studies where a uniform coverage across the major lipid classes is desirable. The effect of different concentrations (0.5–2.0%) of uniformly labelled ^{13}C

glucose was evaluated on the yield of labelled lipids. Three biological replicates of each condition were prepared and the OD₆₀₀ were recorded each 2 h for 48 h. Samples at 36 h were taken at each ^{13}C glucose concentration ($n = 3$) and their extracts were analysed using untargeted LC-MS to establish a comprehensive lipid profile and relative levels of lipids for each concentration of ^{13}C glucose.

Normalisation methods applied to human plasma sample extracts

For normalisation by total ion current (TIC), ions detected in positive and negative modes were normalised by dividing their peak area by the TIC value corresponding to that mode. To normalise lipid peaks by ^{13}C -IS, lipids ions were normalised by dividing their peak area by the peak area of the U- ^{13}C -labelled internal standard form. For normalisation by the deuterated internal standard (D-IS) mixture (SPLASH), 20 μL of the prepared solution was added and the detected peak area of the deuterated ions was used to normalise ions detected in the same class (D-IS masses used for both positive and negative mode are listed in Table S3†). When one lipid class was not detected or was not included in the SPLASH solution, a deuterated ion from other similar class was used for normalisation. The Friedman test was used to assess the difference between different normalisation methods.³⁵ MetaboAnalyst was used to perform unsupervised principal component analysis (PCA) when applicable to evaluate the similarity between data sets and to assess the impact of different normalisation methods on the expected variation in the analysis.³⁶

Initial investigation of lipidomics normalisation by ^{13}C -IS mixture on human and murine plasma samples. The effect of normalisation by ^{13}C -IS mixture on the lipid profiles of extracted plasma samples was evaluated. Briefly, control (blank) plasma samples (50 μL , $n = 6$) (three sets of samples were used: human plasma, mouse plasma and pooled human plasma) were extracted in the presence of labelled yeast extract (^{13}C -IS, 100 μL) (1 : 2 ratio) and of SPLASH® (20 μL). The pooled human plasma was used to reduce the effect of inter-sample variations so that effects of the normalisation could be assessed on sample extraction and instrumental variations (technical variations). Only lipid ions detected in three or more replicates from the same group were included in the analysis. These unlabelled identified ions were then normalised by TIC, ^{13}C -IS or deuterated lipids from the SPLASH solution and compared to non-normalised data.

The effect of normalisation by ^{13}C -IS on a large set of samples with long analysis time. The effect of normalisation in reducing analytical and technical variations was evaluated. 101 aliquots from a well-mixed, pooled human plasma sample were extracted following addition of ^{13}C -IS to each aliquot as previously described. To test the ability of biosynthesized ^{13}C -IS in reducing variations that could be introduced during long samples analysis, 230 injections of a pooled extract of human plasma samples were analysed over a 77 h analysis time. The ability of biosynthesized ^{13}C -IS in reducing instrumental day to day variations was assessed using 15 replicate injections of



a pooled extract of human plasma analysed on three different days (day 0, 7 and 14). Three replicates from pooled plasma were extracted without ^{13}C -IS and used for lipid identification. Only unlabelled ions whose ^{13}C -labelled form was detected in all replicates were included in the analysis (compound-specific normalisation).

Results and discussion

Evaluation of ^{12}C lipid profiles in different species as a potential source of ^{13}C -IS for LC-HRMS based lipidomics

Table 1 represents the total number of lipids in common with HMDB for *E. coli*, *A. platensis*, *S. cerevisiae* and *P. pastoris*, identified by LipidSearch. Fig. S1 of ESI† represents examples of total ion current (TIC) chromatograms in ESI negative and positive modes and pie charts of the main classes of lipid molecules identified from the four microorganisms.

Table 1 shows that diacylglycerols (DG), phosphatidylethanolamines (PE) and monoacylglycerols (MG) represented the main classes of lipids detected in the four microorganisms. The two prokaryotic microorganisms *E. coli* and *A. platensis* had qualitative and quantitative differences in lipids profiles compared with the two eukaryotes, *P. pastoris* and *S. cerevisiae*. In terms of the total coverage of the human lipidome, *P. pastoris* was significantly better than the other microorganisms with over 300 human metabolome database (HMDB) lipids identified.

For *E. coli*, although preliminary comparison between the *E. coli* metabolome database (ECMDB) and the HMDB revealed 376 common lipids, only 176 were actually found and this could be due to different extraction solvents and analytical methods used for the lipid data in HMDB and ECMDB. The lower number of detected lipid ions in *A. platensis* could be attributed to the fact that the extracted sample was small (5 mg) but this number and its lack of coverage of the relevant mammalian classes of lipids make it less suitable for generating ^{13}C lipids.

Both *P. pastoris* and *S. cerevisiae* revealed a wider range of lipid classes compared to *E. coli* and *A. platensis* with the presence of phosphatidylserines (PS), phosphatidylinositols (PI) and ceramides (Cer) increasing the relevance for mammalian lipid analysis. Although the database research indicated a high number of lipid-like molecules (845) in common between *S. cerevisiae* and human samples, experimental results showed that only 153 lipid ions were in common when HMDB data were compared to *S. cerevisiae* lipid profile (Table 1). However, in the *P. pastoris* extract, 345 lipids were found in common with the HMDB. Triacylglycerols (TG, 32%), DG (14%), PC (14%), PE (11%) and PS (7%) were the most predominant lipid ions in common between *P. pastoris* extract and human metabolome similar to previously reported data.^{17,26,37} TG represent more than one third of the identified lipids in *P. pastoris* extract, a much higher proportion than detected in *S. cerevisiae*, and this agrees with previously published data.³⁷

Optimisation of biologically generated ^{13}C -IS mixture to use for normalisation in lipidomics studies

Biologically generated ^{13}C IS extracts from different species were mixed and compared. The results are summarised in Table 2 and Fig. S5.† The number of lipid ions identified using LipidSearch™ to analyse LC-MS data from the combined extracts of (a) *P. pastoris* and *S. cerevisiae*, and (b) *E. coli*, *P. pastoris* and *S. cerevisiae* increased compared to the single species extract. However, the number of the detected labelled ions in *P. pastoris* alone was comparable to other mixture samples (Table 2).

This could be due to ion suppression during analysis as highly abundant ions from different species could suppress co-eluted low abundant ions or because of the similarity of detected lipids between the samples.³⁸ Although a mixture of the extracts from *P. pastoris* and *S. cerevisiae* could provide a small increase in the number of ^{13}C labelled ions (Table 3), the high cost of growing and co-extracting *S. cerevisiae* and *P. pastoris* makes this procedure less worthwhile for a comprehensive source of ^{13}C IS in the future quantitative lipidomics studies.

The ^{13}C enrichment detected (Table 3) shows that prokaryotic and eukaryotic microorganisms were again different in terms of the % incorporation of ^{13}C during growth with ^{13}C glucose as the sole carbon source. Both *P. pastoris*, and *S. cerevisiae* showed a notably higher level of highly enriched lipids than *A. platensis* and *E. coli*.

The higher percentage of unlabelled (0%) and partially labelled (1–99%) lipid ions in *E. coli* (31%) and *A. platensis* (33%) compared with *P. pastoris* and *S. cerevisiae* has potential to increase the complexity of the sample matrix. Additionally, the cost and limited isotopic purity (97%) of the commercially available ^{13}C -labelled *A. platensis* powder limited its use as a source of ^{13}C ISs. Partly ^{13}C enriched lipids (1–99%, Table 3), despite increasing the complexity of the sample matrix, are unlikely to interfere with quantitation, since there will be no spectral overlays with the corresponding unlabelled analytes. This is because of the mass increment of the additional 1.00335 amu per ^{13}C carbon present in the lipid backbone, introducing a significant mass shift when compared to the unlabelled

Table 1 Total number of lipids extracted from each strain and identified by LipidSearch in positive and negative mode that are in common to HMDB

	<i>P. pastoris</i>	<i>S. cerevisiae</i>	<i>A. platensis</i>	<i>E. coli</i>
Total identified lipids compared to HMDB	345	153	129	176
	<i>n</i> (%)			
DG	50 (14)	26 (17)	32 (25)	73 (42)
MG	13 (4)	9 (6)	7 (6)	27 (15)
PC	49 (14)	26 (17)	16 (13)	—
PE	39 (11)	37 (24)	35 (27)	44 (25)
PS	23 (7)	11 (7)	—	4 (2)
PG	9 (3)	3 (2)	18 (14)	9 (5)
TG	112 (32)	15 (10)	7 (5)	6 (4)
PI	6 (2)	5 (3)	3 (2)	—
LPC	10 (3)	7 (5)	—	—
Cer	12 (4)	2 (1)	3 (2)	4 (2)
Other	22 (6)	12 (8)	8 (6)	9 (5)



Table 2 Comparison between different mixtures of *E. coli*, *S. cerevisiae* and *P. pastoris* to find out the optimal source/sources of IS mixture ($n = 3$)^a

Parameters	<i>E. coli</i>	<i>S. cerevisiae</i>	<i>P. pastoris</i>	ES	EP	PS	EPS
Number of identified lipid ions by LipidSearch™ (mean \pm SD)	187 \pm 2	313 \pm 8	401 \pm 13	334 \pm 3	379 \pm 15	409 \pm 7	418 \pm 8
Numbers of detected ions by Tox-ID (mean \pm SD)	134 \pm 2	253 \pm 3	356 \pm 3	268 \pm 1	325 \pm 4	383 \pm 24	311 \pm 29
Number of lipid ions with ¹³ C-enrichment $\geq 99\%$ (mean \pm SD)	121 \pm 2	206 \pm 6	313 \pm 4	223 \pm 2	288 \pm 4	334 \pm 16	279 \pm 20

^a ES = *E. coli* and *S. cerevisiae* extract mixture. EP = *E. coli* and *P. pastoris* extract mixture. PS = *P. pastoris* and *S. cerevisiae* extract mixture. EPS = *E. coli*, *P. pastoris* and *S. cerevisiae* extract mixture.

Table 3 The % of ¹³C-enrichment in detected lipid ions extracted from *A. platensis*, *E. coli*, *P. pastoris*, and *S. cerevisiae*. The detected lipids are divided in three sets based on the ¹³C-enrichment: unlabelled lipids (0% ¹³C-enrichment), partially labelled lipids (1–99% ¹³C-enrichment) and fully labelled lipids (>99% ¹³C-enrichment)

Species	¹³ C-enrichment in detected lipids		
	0%	1–99%	>99%
<i>Arthrospira platensis</i>	20%	13%	67%
<i>Escherichia coli</i>	25%	6%	69%
<i>Pichia pastoris</i>	10%	7%	83%
<i>Saccharomyces cerevisiae</i>	9%	8%	83%

analyte which can be readily detected by accurate mass spectrometry. The presence of unlabelled (¹²C) lipids in the ¹³C mixture presents a potential problem with quantification, and a correction factor might need to be applied depending on the type of study and quantification carried out. However, in a study where two or more groups are compared, the presence of unlabelled lipids will not hinder the relative quantification because an equal amount of ¹³C IS mixture will be added to all samples.

Since significantly more HMDB lipids were identified from *P. pastoris* compared to the other microorganisms, and more lipids were found to be fully ¹³C labelled in *P. pastoris* (83%) this single species was selected as the best source of biologically generated ¹³C-ISs. Another factor in favour of *P. pastoris* was that this species reached a much higher culture OD than *E. coli* and *S. cerevisiae* (Fig. S2–S4†) indicating that more cells can be grown and therefore potentially a higher concentration of ¹³C lipids/IS mixture can be extracted. Therefore, *P. pastoris* was selected as the optimal source of ¹³C ISs among the studied species.

For further analytical investigation and different uniformly labelled glucose concentrations were tested for cell growth with *P. pastoris*. Fig. 1 shows the growth rate of *P. pastoris* at four different concentrations of U-¹³C glucose (0.5, 1, 1.5 and 2%).

Characterisation and isotopic purity of ¹³C-lipids generated by *P. pastoris* grown with U-¹³C glucose

Table S4† shows the 357 lipid ions identified in *P. pastoris* extract based on accurate fragmentation patterns by

LipidSearch™. The detected lipids cover a wide range of lipid classes including fatty acyls (FA), (*O*-acyl)- ω -hydroxy fatty acids (OAHFA), glycerolipids (MG, DG, TG), glycerophospholipids (cardiolipins (CL), dimethylphosphatidylethanolamines (dMePE), lysophosphatidylcholines (LPC), lysophosphatidylethanolamines (LPE), phosphatidic acids (PA), PC, PE, PG, PI and PS), sphingolipids (Cer, glucosylceramides (CerG1), sphingomyelins (SM) and sphingosine (SO)) in accordance with previously reported *P. pastoris* studies.^{17,26} The MS/MS spectra obtained from unlabelled and labelled extracts (Fig. S6†) clearly indicate a mass shift equivalent to 1.00335 Da increment per carbon atom present in the backbone compared with identified unlabelled (¹²C) lipid ions. The retention times of 246 ¹³C-labelled ions detected in *P. pastoris* extract matched (within ± 15 s) with those of the equivalent ¹²C ions. Furthermore, their detected monoisotopic masses were in agreement with the

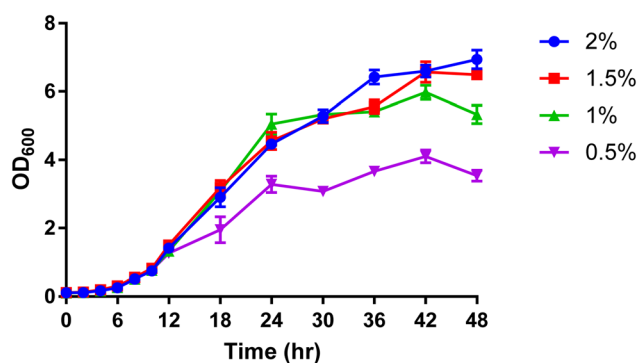


Fig. 1 The effect of U-¹³C glucose concentration (0.5%, 1%, 1.5% and 2%) on the growth rate of *P. pastoris* based on the biomass production measured by the optical density at 600 nm ($n = 3$). The levels of 39 representative lipids identified from different lipid classes was monitored as shown in Fig. 2. Although data showed a significantly lower biomass production with 0.5 and 1% of ¹³C-glucose, the TIC, the number of detected features and the level of selected lipids from the extract of cells grown at 1% ¹³C-glucose were comparable to the extract of cells grown at 2% ¹³C-glucose. Given that a good coverage of ¹³C lipids was obtained with 1% glucose, the small advantage in ¹³C lipid generation using 1.5% or 2% ¹³C glucose (see Fig. 2) was not justified given the significant extra cost involved. Thus, optimal growth conditions for *P. pastoris* in minimal media was confirmed as 1% of the final ¹³C-labelled glucose concentration for 24 h and the ¹³C IS standards generated were used in all the subsequent plasma lipidomic analyses reported below.



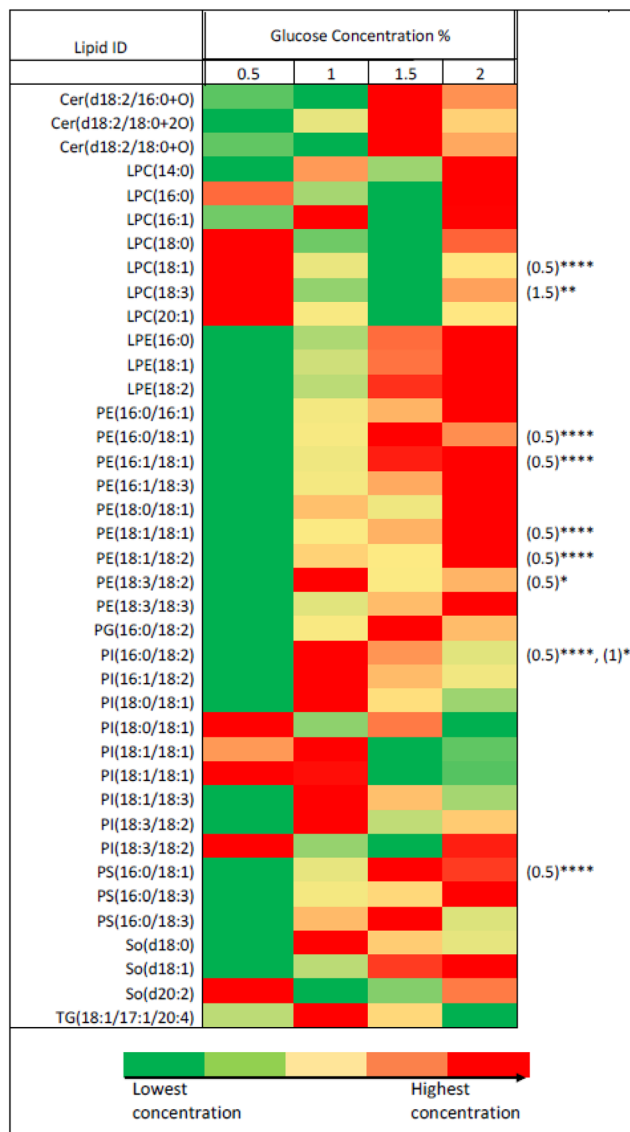


Fig. 2 Heatmap representing the effect of glucose concentration on the level of 39 putatively-identified lipids detected in *P. pastoris* extract based on two-way ANOVA between different glucose concentration compared to 2% concentration. Equal samples from each of the four conditions were collected after 36 h of cultivation (early stationary phase) and the lipid contents of 39 lipids previously putatively identified were explored. *, **, ***, **** represent p -values of ≤ 0.05 , ≤ 0.01 , ≤ 0.001 , and ≤ 0.0001 , respectively. The numbers in the brackets on the right side of the figure represents the condition that is statistically different from 2% based on two-way ANOVA analysis per lipid.

calculated ^{13}C -masses within a mass error of ± 5 ppm, in agreement with Rampler *et al.*¹⁷ The labelling patterns of these ions revealed ^{13}C labelling degree of $\geq 98\%$ (Table S4 of ESI†). Exploring the labelling pattern of identified ions revealed that the other ions were either not labelled or partially labelled. Based on the information on the generated ^{13}C labelled lipids, those with a mass error of < 5 ppm of expected ^{13}C -fully labelled mass detected within RT window of 30 s of unlabelled identified lipid were selected for use in the normalisation studies reported below.

Comparison of three lipidomics normalisation methods using biosynthesized ^{13}C -lipids, total ion count or SPLASH applied to small sample sets of human and mouse plasma

Compound-specific normalisation using ^{13}C labelled IS mixture from labelled *P. pastoris* extract was performed on three sets of plasma samples: six biologically different fasting human plasma samples, six biologically different fasting mice plasma samples and a pooled fasting human plasma sample. The results of the ^{13}C IS normalisation were compared to normalisation by TIC and SPLASH deuterated IS mixture.

Fig. S7† represents the result of compound-specific normalisation of ions ($n = 112$) detected in human plasma samples (Fig. S7A†) and ions ($n = 132$) detected in mouse plasma samples (Fig. S7B†) extracted in the presence of biosynthesized ^{13}C -IS. It appears that normalisation by TIC improves the overall response and an improved CV% was obtained for most of the selected ions. However, we observed that normalisation by ^{13}C -IS or by SPLASH increased the CV% of the selected ions. This was an unexpected result since the introduced ^{13}C -IS mixture was expected to correct analytical and technical variation. To further investigate the efficacy of the introduced normalisation methods we used replicate analysis of a well-mixed pooled human plasma, where the biological variability between samples has been eliminated. Fig. S7C† represents the result of compound-specific normalisation detected in pooled plasma samples extracted in the presence of ^{13}C -IS. Statistical analysis showed a significant increase in the CV% of selected ions after normalisation by all the methods used, suggesting that normalisation had increased rather than decreased inter-sample variation. These unexpected results can be explained by the relatively small set of samples used ($n = 6$) compared to a typical lipidomics experiment which would have a much larger set of samples. Therefore, in the light of these results, the effect of ^{13}C -IS normalisation was further evaluated in an experiment with a larger number of samples and higher inter-individual differences between samples where the level of random error is expected to increase and affect the quality of the data.

Comparison of lipidomics normalisation using biosynthesized ^{13}C -lipids and total ion count applied to a large set of samples and 77 h analysis time

The effect of normalisation by ^{13}C -IS in reducing variations introduced during the extraction of a large set of human plasma samples ($n = 101$) was evaluated. A total of 446 ions were identified in the pooled human QC sample and 142 labelled ions were present in all samples and used for compound-specific normalisation. Fig. 3(A) represents the normalisation results of these ions. As shown, ions normalised by TIC or by ^{13}C -IS showed a significant reduction in CV% compared to that in the raw data. Ions normalised by ^{13}C -IS gave an average CV% of 6.36% compared with those non-normalised data which gave an average of 11.01%. This highlights the efficacy of normalisation by ^{13}C -IS mixture in reducing variations caused during samples preparation especially when a typical size of sample set (101 in this case) is analysed.



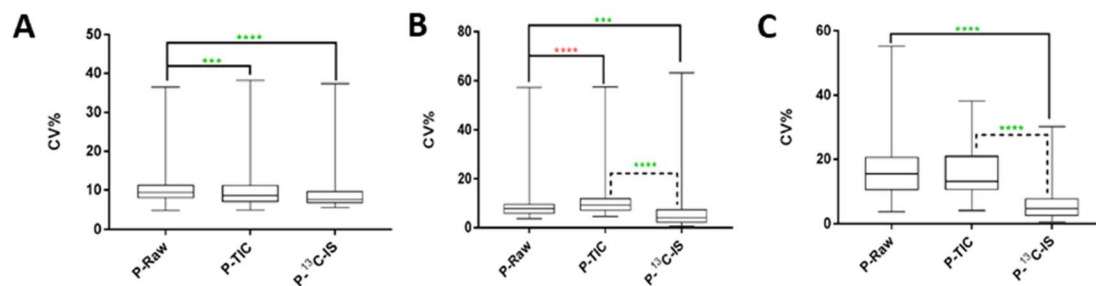


Fig. 3 Coefficient of variance distributions for normalization of (A) pooled human plasma sample repeatedly extracted ($n = 101$) to study the effect of normalization by ^{13}C -IS in reducing variations introduced during extraction of large set of samples; (B) pooled human plasma sample repeatedly injected and analyzed ($n = 230$) to study the effect of normalization by ^{13}C -IS in reducing variations introduced during sample analysis over a long analysis time; (C) pooled human plasma sample extracted and analyzed ($n = 15$) at different days (day 0, day 7 and day 14) to study the effect of normalization by ^{13}C -IS in reducing variations introduced during sample analysis on three separate days (batch to batch variations). (**** $p < 0.0001$, *** $p < 0.001$, where the green color indicates a reduction in overall CV% while the red color indicates an increase in overall CV%, while the lack of comparison between the raw and normalized data indicates no significance).

The effect of normalisation by ^{13}C -IS was further investigated for its ability to correct unwanted variations introduced during long LC-MS analysis ($n = 230$, total analysis time 77 hours). Using the same procedure as for the previous experiment, 100 labelled ions identified in all samples were used for compound-specific normalisation. In this experiment, the calculated CV% shown in Fig. 3(B) reflects variations introduced during sample analytical variations over 77 hours. In metabolomics the

analysis of a large set of samples, for example in cohort studies or samples measured at different time points, is a challenge due to analytical drift caused by metabolite degradation (oxidation or hydrolysis) and/or inconsistency in LC and MS instrumentation (RT, peak shape or variation in MS detector response). Although randomisation of the sample run order is used to reduce the effect of these variations, it cannot be completely removed or compensated without the use of IS. As can be seen

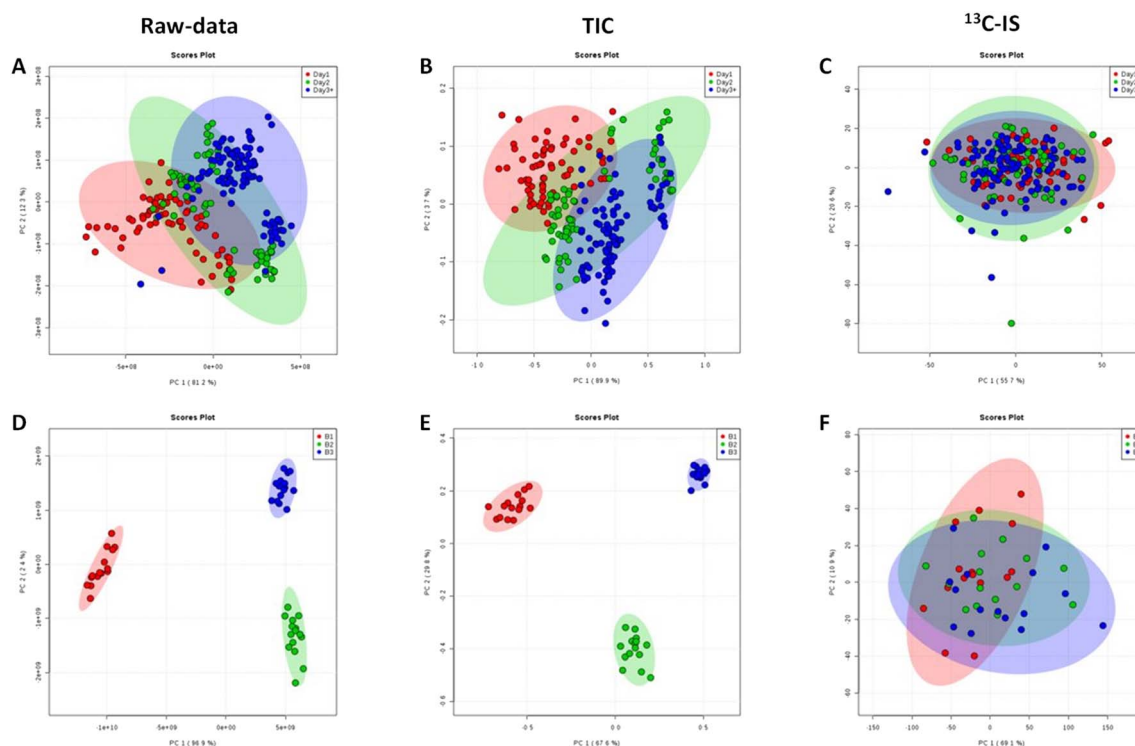


Fig. 4 (A–C) PCA score plots of pooled human plasma sample from blood bank repeatedly injected ($n = 230$) and analysed over 77 hours (red, green and blue colours represent samples run in the first day, the second day and the third day respectively). (D and E) PCA score plots of pooled human plasma samples from blood bank extracted and analysed ($n = 15$) at different days (red, green and blue colours represent samples analysed at day 0, day 7 and day 14 respectively). The PCA show the effect of TIC normalisation (B and E) and compound-specific normalisation by ^{13}C -IS (C and F) compared to raw un-normalised data (A and D). Only common ions between plasma samples and ^{13}C -yeast extract were included in the analysis.



in Fig. 3(B), ions normalised by ^{13}C -IS revealed a significantly lower overall CV%, compared to those in raw data ($p < 0.001$) and normalised by TIC ($p < 0.0001$). In Fig. 3(A) and (B), analytical drifts in instrumental responses were observed over time, leading to a clear separation between injections analysed over 77 hours in PCA scores plot. Similar drifts in QC samples were observed in a large-scale analysis of serum and plasma samples,³⁹ and in urine samples⁴⁰ due to sample instability or unexpected instrumental hardware or software errors. Normalisation by TIC still showed significant variations in instrument responses as can be seen in Fig. 3(A) and (B), whereas normalisation by ^{13}C -IS was able to correct these drifts and remove any difference between injections (Fig. 3(C)).

The effect of normalisation by ^{13}C -IS on data acquired on three separate days was also evaluated. 105 labelled ions were present in all samples and used for compound-specific normalisation. The calculated CV% in raw data shown in Fig. 3(C) reflects variations introduced due to batch-to-batch variations. This type of variation arises from sample handling, preparation and storage, MS performance and column stability over time that collectively affect the results of large-scale studies. It appears that ions normalised by TIC had similar overall CV% compared to that in raw data, whereas ions normalised by ^{13}C -IS had a significantly improved overall CV% (Fig. 3(C)).

Normalisation by ^{13}C -IS can effectively reduce day-to-day variations introduced by the instrument during samples analysis significantly, and this is confirmed by the PCA scores plot where there are clear separations between different batches without normalisation or normalised by TIC (Fig. 4(A), (B), (D) and (E)) but no clusters were observed after normalisation by ^{13}C -IS as shown in Fig. 4(C) and (F).

Furthermore, in order to identify the main lipid species that contributed to the distinctive PCA groupings in Fig. 4 partial least squares discriminant analysis (PLS-DA) was performed, and a variable importance in projection (VIP) value > 1 was selected as a cut off for lipids that were significantly contributing to the grouping. The 15 lipid species that contributed to additional variability in the raw data were decreased to 10 lipids after normalization with TIC and after ^{13}C -IS normalization only 2 lipid species had a VIP greater than 1 as presented in Table S5.† This confirmed that the ^{13}C -IS normalisation had a direct and specific effect in reducing the excess variation of the dataset.

Conclusion

We confirm that a wide range of ^{13}C -labelled lipids can be biosynthesized using the yeast *P. pastoris* using straightforward and simple culture conditions. Compared with *E. coli*, *A. platenensis*, *S. cerevisiae*, the *P. pastoris* lipid profile provided the best match to the lipid profile of human plasma. Using the ^{13}C -labelled IS mixture from *P. pastoris* in typical lipidomics experiments resulted in a significant reduction of unwanted variations due to sample processing and LC-MS analysis, especially where samples are analysed over several days or in separate batches. Furthermore, this study demonstrates that the *in vivo* labelling strategy provides a cost-effective and practical

solution to perform reliable untargeted lipidomics, avoiding expensive or difficult-to-obtain commercial lipid standards. In our work we used a single batch of the biosynthesized ^{13}C -labelled IS mixture, which provided enough material for many hundreds of individual samples. Clearly, there may be batch-to-batch variability in any biosynthesized lipid mixture, so it is important to generate sufficient material to cover the full requirements of any extended lipidomics study. Finally, this normalisation technique could be readily transferred to a variety of biological sample types such as mammalian tissue or other biofluid samples.

Conflicts of interest

The authors declare no competing financial interest.

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