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ARTICLE

Quantitative analysis of 3-OH oxylipins in fermentation yeast

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Abstract: Despite the ubiquitous distribution of oxylipins in plants, animals, and microbes, and the application of numerous analytical techniques to study these molecules, 3-OH oxylipins have never been quantitatively assayed in yeasts. The formation of heptafluorobutyrate methyl ester derivatives and subsequent analysis with gas chromatography – negative chemical ionization – mass spectrometry allowed for the first determination of yeast 3-OH oxylipins. The concentration of 3-OH 10:0 (0.68–4.82 ng/mg dry cell mass) in the SMA strain of *Saccharomyces pastorianus* grown in laboratory-scale beverage fermentations was elevated relative to oxylipin concentrations in plant tissues and macroalgae. In fermenting yeasts, the onset of 3-OH oxylipin formation has been related to fermentation progression and flocculation initiation. When the SMA strain was grown in laboratory-scale fermentations, the maximal sugar consumption rate preceded the lowest concentration of 3-OH 10:0 by ~4.5 h and a distinct increase in 3-OH 10:0 concentration by ~16.5 h.

Key words: negative chemical ionization, heptafluorobutyrate, mass spectrometry, hydroxy fatty acids, miniature fermentation.

Résumé : En dépit de la distribution généralisée des oxylipines chez les plantes, les animaux et les microbes, et le recours à une multitude de techniques analytiques pour étudier ces molécules, on n'a jamais procédé à des analyses quantitatives des 3-OH oxylipines chez les levures. La formation de dérivés d'esters méthyliques d'heptafluorobutyrate et leur analyse ultérieure par chromatographie en phase gazeuse – ionisation chimique négative – spectrométrie de masse ont permis de mesurer pour la première fois les 3-OH oxylipines chez les levures. La concentration de 3-OH 10:0 (0,68–4,82 ng/mg de poids cellulaire sec) de la souche SMA de *Saccharomyces pastorianus* cultivée en fermentations de boissons réalisées en laboratoire était élevée relativement aux concentrations d'oxylipines retrouvées dans les tissus végétaux et les macroalgues. Chez les levures en fermentation, l'initiation de la formation de 3-OH oxylipine a été liée à la progression de la fermentation et à l'initiation de la floculation. Lorsqu'on a cultivé la souche SMA en fermentations de laboratoire, la vitesse maximale de consommation de sucre a précédé la plus faible concentration de 3-OH 10:0 par ~4,5 heures et une hausse marquée de la concentration de 3-OH 10:0 par ~16,5 heures. [Traduit par la Rédaction]

Mots-clés : ionisation chimique négative, heptafluorobutyrate, spectrométrie de masse, acides gras hydroxylés, fermentation miniature.

Introduction

Oxylipins, oxygenated fatty acids, abound in nature and have been identified in numerous different genera and species of yeast (van Dyk et al. 1991; Kock et al. 2000; Smith et al. 2000; Leeuw et al. 2006). In yeasts, as in other life forms, oxylipins can be bioactive and may directly or indirectly mediate a wide variety of cellular processes, including cell signalling, morphogenesis, quorum sensing, gene expression, and interkingdom signalling (Kock et al. 1999; Deva et al. 2001; Nigam et al. 2011; Pohl and Kock 2014). During yeast oxylipin investigations, previous studies have employed numerous experimental techniques and approaches when detecting these compounds. For example, Kock et al. (1991) used radio immuno-assay and gas chromatography – mass spectrometry (GC–MS), while van Dyk et al. (1991) were successful when employing silica thin-layer chromatography (TLC), radio TLC, nuclear magnetic resonance, and GC–MS. More recently, an immunological detection system called Oxytrack was devised that utilized rabbit-raised primary antibodies that were specific for 3-OH oxylipins (Kock et al. 1998). Immunogold labelling coupled with transmission electron microscopy analysis (Smith et al. 2000) and ultra-high performance liquid chromatography – mass spectrome-

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try (Madu et al. 2015) have also been used to detect yeast oxylipins.

Despite the wide variety of analytical techniques that have been employed in yeast oxylipin investigations, most approaches have only been qualitative or semi-quantitative in nature (Kock et al. 2013; Madu et al. 2015). Furthermore, particularly in the case of GC–MS analysis, the difficulty associated with the use of diazomethane has necessitated the development of other methods that still specifically target the unesterified free fatty acid fraction without the use of this reagent. Our earlier study found that 3-OH oxylipins could be detected in *Saccharomyces pastorianus* without diazomethane by employing an ethyl acetate extraction, trimethylsilylation of the total lipid extract, and resolution and detection using GC–MS (Potter et al. 2015). Trimethylsilylation is an effective derivatization approach, as it adds a trimethylsilyl group at the carboxylic acid and β-OH functionalities of 3-OH oxylipins but does not react with esterified fatty acids. Unfortunately, accurate quantitation of trace 3-OH oxylipin levels, as in past studies, was not possible with our earlier method owing to their low relative abundance and the small chromatographic peaks arising from these compounds.

As with other types of yeasts, oxylipins have been detected in industrial fermentation strains of *Saccharomyces cerevisiae* and *Saccharomyces pastorianus*. In these strains, 2 oxylipins in particular, 3-OH 8:0 and 3-OH 10:0, have been identified and are assumed to be bioactive (Kock et al. 2000; Strauss et al. 2005; Potter et al. 2015). In brewing strains, 3-OH oxylipins also formed at flocculation onset and were speculated to have a role in controlling this phenomenon (Kock et al. 2000; Speers et al. 2006; Potter et al. 2015). However, the relationship between 3-OH oxylipin formation and flocculation remains unknown. An inability to quantify oxylipins also prevents us from understanding the exact biological functions of these molecules and any possible effect they may have on the industrially important flocculation phenomenon and the overall beverage fermentation process.

In this study we analyzed yeast 3-OH oxylipins with negative chemical ionization (NCI)–MS analysis of heptafluorobutyrate (HFB) methyl ester derivatives, which has previously been used to assay low levels of hydroxy fatty acids from other biological samples (Stan and Scheutwinkel-Reich 1980). In particular, the level of 3-OH 10:0 was assayed in the SMA strain of *Saccharomyces pastorianus* grown in laboratory-scale fermentations, and density decline was coincidently modelled with accepted statistical techniques for brewery fermentation kinetics. The aim of this manuscript, therefore, is to detail the first quantification of fermenting yeast 3-OH oxylipins with an associated determination of fermentation progression.

Materials and methods

Double grow up and culture

The SMA strain of *Saccharomyces pastorianus*, which had been stored on a yeast extract – peptone – dextrose agar slant, was cultivated using the double grow up approach described in the American Society of Brewing Chemists (ASBC) miniature fermentation assay standard protocol (ASBC 2013). Thereafter, using an abridged version (Potter et al. 2015) of the ASBC standard protocol (ASBC 2013), sufficient wort was prepared (i.e., 3 × 450 mL) to conduct 3 coincident miniature fermentations (i.e., 3 sets of 30 tubes). In a deviation from the standard protocol, 30 taller test tubes (15 cm) per fermentation were utilized so that settled yeast could be collected by vortexing the tube contents.

Sample harvesting and preparation

At each of 12, 24, 36, 48, and 60 h of the fermentation, 6 test tubes were selected for sampling from each of the 3 coincident miniature fermentations. Within each group of 6 tubes, the contents of the first 3 were vortexed to resuspend any settled yeast and were then transferred to a single sterile 50 mL centrifuge tube. The top approximately 2 cm of the remaining 3 tubes were filtered through 7 cm Whatman No. 4 paper for density analysis as previously described (Potter et al. 2015). The same tubes were then vortexed and their contents were transferred to a second 50 mL test tube. Both 50 mL tubes were then centrifuged at 2000*g* for 3 min and the supernatants were discarded. The cell pellets were washed with 5 mL of sterile distilled water, the contents of each tube were pooled, and another round of centrifugation and supernatant disposal was carried out. All the cell pellets were frozen for at least 12 h in a –30 °C freezer and were then lyophilized in a freeze dryer (Labconco FreeZone 2.5 Plus, Kansas City, Missouri, USA) for 55 h. Once the freeze drying process was complete, the samples were returned to –30 °C cold storage until analysis.

Lipid extraction

All glassware used in the lipid extractions was washed and then triple rinsed with dichloromethane to remove traces of contaminants. The solvents used in the extraction steps were reagent grade. In a 40 mL glass test tube approximately 500 mg of lyophilized cell mass was mixed with an equal or greater mass of 425- to 600-μm-diameter acid-washed glass beads (Sigma-Aldrich, St. Louis, Missouri, USA) and 3.6 g of NaCl to help prevent emulsion formation (Salmon and Flower 1982). Chloroform-washed water (10 mL) was then added and all were acidified to pH 3.5–4.5 with 10 μL of 0.25 mol/L citric acid. Ethyl acetate (10 mL) and 3-OH 11:0 (200 ng) (Matreya LLC, Pleasant Gap, Pennsylvania, USA) as an internal standard were also added to each tube. All tubes were vortexed for 4 min to ensure cell breakage and thorough solvent penetration. The tubes were then shaken for 10 min on a bench-top shaker (Burrell Wrist-Action, Pittsburgh, Pennsyl-

vania, USA) and centrifuged for 10 min at 160*g*. In some samples an emulsion formed, and this was dispersed by adding 0.75 mL of cold acetone, swirling the tubes, and centrifuging for a further 4 min at the same speed. After centrifugation, the upper ethyl acetate layers were transferred to a clean test tube containing anhydrous Na_2SO_4. A second extraction using another 10 mL aliquot of ethyl acetate was carried out and the upper ethyl acetate layer was combined with the previous ethyl acetate fraction. The extracts were dried under streaming nitrogen, and the lipids were redissolved in 100 μL of chloroform.

Oxylipin isolation, collection, and purification

To isolate the oxylipin-containing fraction, the total lipid extract from each sampling time was streaked on TLC Silicagel 60 plates (EMD Millipore, Billerica, Massachusetts, USA) along with authentic 3-OH 9:0 and 3-OH 11:0 standards (Matreya LLC). The TLC plates were developed with a mixture of hexane – diethyl ether – acetic acid (80:20:2, by volume). Lipid classes were visualized using 2,7-dichlorofluorescein (0.2% in 96% ethanol) under UV light (254 nm). Each oxylipin-containing band was then scraped and collected in a test tube. Lipids were removed from the silica gel by vortexing the collected material with 3 mL of methanol–chloroform (2:1, *v*/*v*) for 1 min, centrifuging the slurry for a further minute, and filtering the supernatant through Whatman No. 4 filter paper. Thereafter, the solvent was removed under streaming nitrogen.

Preparation of heptafluorobutyrated methyl ester derivatives

To prepare methyl ester derivatives of oxylipins, 1.5 mL of BCl_3–methanol solution (Supelco, Bellefonte, Pennsylvania, USA) was added to each tube, and all tubes were heated at 100 °C for 1 h. After heating, the tubes were allowed to cool to room temperature and 1.5 mL of water and 1 mL of hexane were added. Tubes were then shaken and centrifuged for 1 min at low speed. The methyl-ester-containing hexane layer was transferred to a new glass tube and a second recovery with hexane was carried out to ensure a full extraction. The recovered organic layer was washed 3 times with 1 mL of $CHCl_3$-washed water to remove excess BCl_3 and was dried with anhydrous Na_2SO_4.

To prepare HFB derivatives of methylated oxylipins, the hexane was evaporated under nitrogen and 1.0 mL of isooctane and 100 μL of heptafluorobutyric anhydride (HFBA) (Sigma-Aldrich) were added. All tubes were placed on a heating block at 70 °C for 60 min and were allowed to cool to room temperature at the end of the heating period. Residual heptafluorobutyric acid was eliminated by adding 2 mL of phosphate buffer solution (pH = 8) (Longo and Cavallaro 1996), and the HFB methyl ester derivatives were double extracted with 1.0 mL aliquots of isooctane. Following drying with anhydrous Na_2SO_4, the isooctane was removed under streaming nitrogen and the analytes were redissolved in 200 μL of hexane and were transferred to GC vials.

GC–NCI–MS analysis

GC–NCI–MS analysis was performed using a Trace GC Ultra fitted with a Zebron ZB-35HT column (35% phenyl – 65% dimethylpolysiloxane, 30 m × 0.25 mm ID, 0.25 μm film thickness), a Polaris Q mass spectrometer, and a Thermo Triplus AS autosampler. A splitless injection was employed with a 1.0 μL injection volume at a temperature of 250 °C and a splitless time of 2 min. The initial oven temperature was 60 °C and this was held for 2 min. This was increased by 7.5 C°/min to 250 °C, which was subsequently held for 5 min. Helium at a flow rate of 1.0 mL/min was used as the carrier gas and the transfer line was set at 280 °C.

The MS was optimized for NCI analysis by first tuning in electron impact (EI) ionization mode and then in the positive chemical ionization (PCI) setting with methane as the reagent gas. During both initial EI and PCI tuning, the ion source was set at 200 °C and the emission current at 250 μA. In NCI mode, the ion source was maintained at a temperature of 230 °C and a pressure of approximately 70 mTorr (1 Torr = ~133.3 Pa) with a methane flow rate of 1.2 mL/min. The emission current was set at 100 μA, and the lenses were optimized according to manufacturer instructions. The mass spectrum was recorded in the full-scan mode from *m*/*z* 60 to 600 and in the selected ion monitoring (SIM) mode for quantification. Settings in the SIM mode were as follows: *m*/*z* 358 for the 3-HFB-*O*-10:0 methyl ester analyte $[M - 40]^{\bullet -}$ fragment measured from 14.30 to 15.38 min and *m*/*z* 372 for the 3-HFB-*O*-11:0 methyl ester internal standard $[M - 40]^{\bullet -}$ fragment measured from 15.38 to 32.23 min. The retention times for these derivatives were previously verified using derivatized authentic 3-OH 10:0 and 3-OH 11:0 standards (Matreya LLC). Peak areas were integrated with the Thermo Xcalibur™ Qual Browser Genesis™ peak finding algorithm (Waltham, Massachusetts, USA).

Analytical curve

To account for different responses of the HFB-derivatized methyl ester analyte (3-OH 10:0) and internal standard (3-OH 11:0) in the GC–MS, an analytical curve was constructed. A series of 4 standards were created (Mueller et al. 2006) with each containing 200 ng of internal standard and increasing amounts of the 3-OH 10:0 analyte that encompassed the experimental data range. Analytical standards were prepared in triplicate and carried through the previously described derivatization procedures. HFB methyl ester derivatives of these standard mixes were then injected in a randomized order and analyzed in the same GC–NCI–MS SIM mode. The analytical curve was obtained by plotting the peak area ratio of derivatized 3-OH 10:0/3-OH 11:0 versus the concentration ratio of derivatized 3-OH 10:0/3-OH 11:0 injected. The analytical curve was subsequently evaluated by assessing the

residuals using the Anderson–Darling and Kolmogorov–Smirnov tests for normality and by visual inspection for any apparent heteroscedasticity.

Fermentation modelling

Density attenuation was modelled using the 4-parameter logistic model (eq. 1) developed by Speers et al. (2003) and now used in the ASBC Yeast-14 method (ASBC 2013):

$$(1) \qquad P_t = P_e + \frac{P_i - P_e}{1 + e^{-B(t-M)}}$$

where P_t is the extract density at time t (°P), P_e is the final asymptotic density (°P), P_i is the initial asymptotic density (°P), B is a function of the slope at the inflection point (°P/h), and M is the time to reach the inflection point (h). The model was fit with the Prism Version 5.0 (GraphPad Software Inc., La Jolla, California, USA) software package.

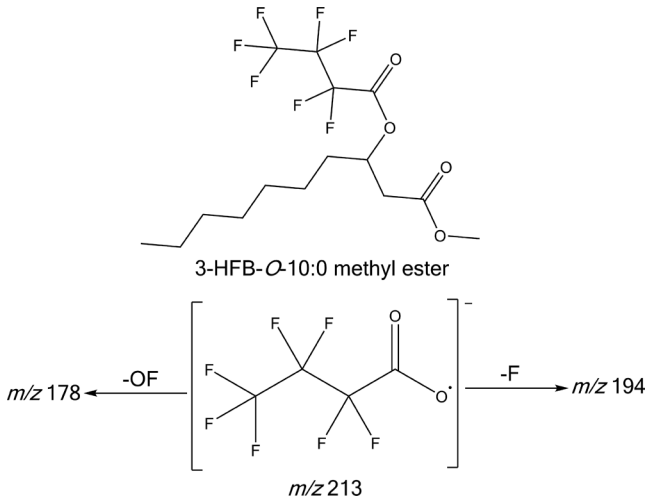
Results and discussion

Analytical methodology

The distribution of yeast cell 3-hydroxy oxylipins varies with growth phase (Kock et al. 2000) and substrate availability (Strauss et al. 2005). Furthermore, these hydroxy fatty acids are believed to originate during β-oxidation or partial β-oxidation and could occur intracellularly (Kock et al. 2007, 2013). In this work, glass beads were added to the extraction system to break apart the cells so that oxylipins would be detected regardless of their location in the cell. The effectiveness of the cell breakage and efficiency of the solvent penetration was verified by purifying the lipid extracts obtained in the presence and absence of the glass beads. In the presence of the beads and with agitation, there were significantly more distinct bands on the TLC plates. Interestingly, in additional preliminary TLC experiments, the R_f for 3-OH 9:0 and 16:0 standards were determined to be 0.033 and 0.27, respectively. Other authors have referred to yeast 3-OH oxylipins as hydrophobic molecules (Leeuw et al. 2006); however, these results suggest that fermentation yeast oxylipins are in fact quite polar, rather than hydrophobic.

During method development in this study, it was repeatedly found that a sulfuric-acid-catalyzed transesterification did not derivatize 3-OH fatty acid standards (Potter et al. 2013). Indeed, an older patent in the literature reported that the OH group on hydroxy fatty acids inhibited the methylation reaction during an HCl and H_2SO_4 acid-catalyzed esterification (Filachione and Fisher 1946). BCl_3 was later identified as a potential acid catalyst for 3-OH oxylipin derivatization and has been used by Miller (1982) in investigations involving bacterial hydroxy fatty acids. Peterson et al. (1965) also suggested that BCl_3 was a successful catalyst, as it helps to keep the reaction medium dry, thereby driving the equilibrium in favour of the products. Furthermore, BCl_3 was found to be a stronger Lewis acid then BF_3, making it more reactive as an acid catalyst (Peterson et al. 1965). BF_3 could have been used to catalyze the methylation of hydroxy fatty acid standards without any alteration of the hydroxy functionality (Morrison and Smith 1964), but Klopfenstein (1971) found evaporative losses were greater with BF_3 compared with BCl_3. Thus, BF_3 was avoided and BCl_3 was utilized in this experiment.

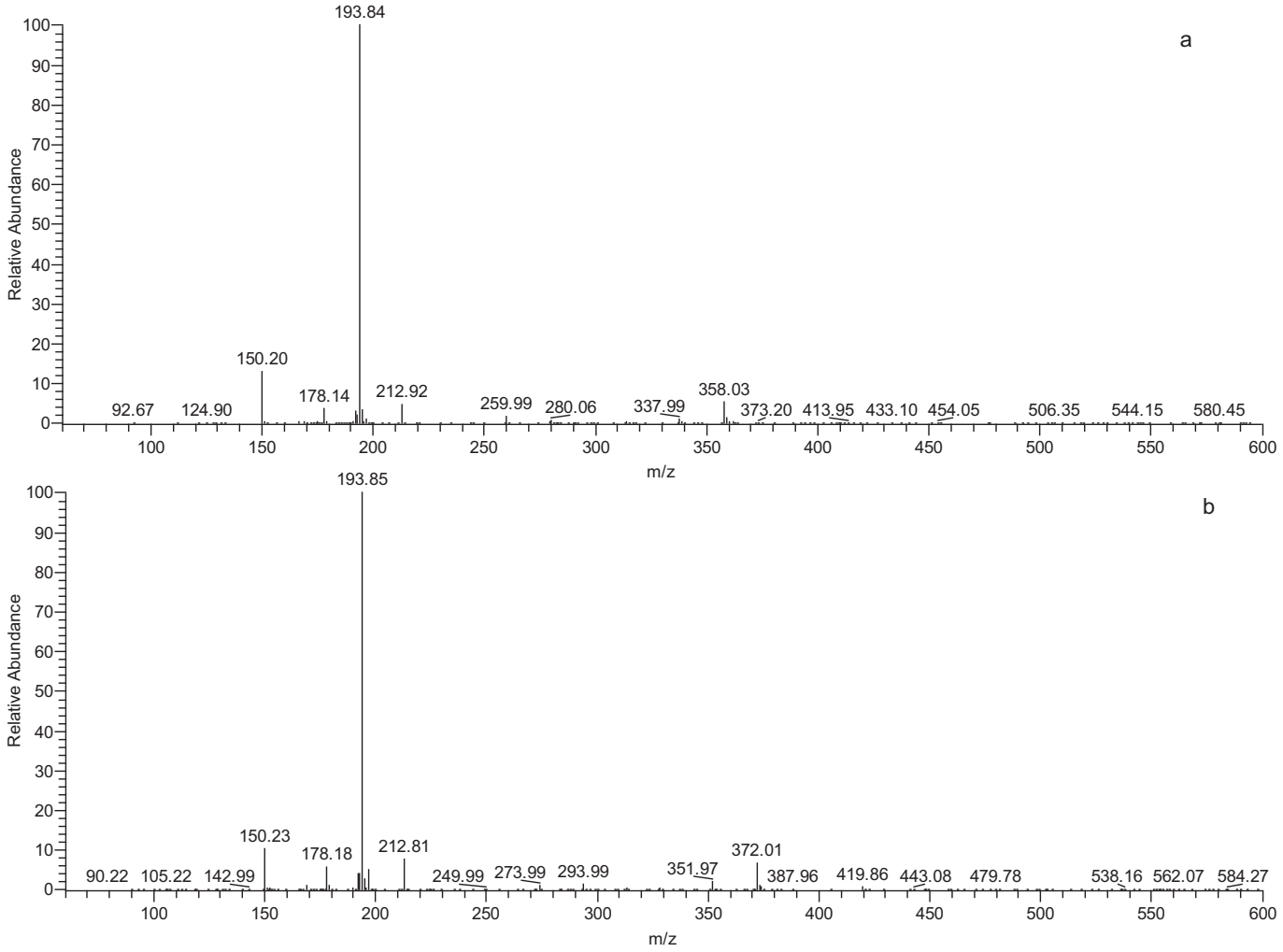
Fig. 1. Structure of the 3-HFB-*O*-10:0 methyl ester and the additional characteristic ions, *m*/*z* 213, 194, and 178, produced by fragmentation of the heptafluorobutyrate group.



To make electronegative derivatives of the methylated 3-OH oxylipins that would be suitable for NCI–MS analysis, a HFB group was added at the hydroxy functionality (Fig. 1). Stan and Scheutwinkel-Reich (1980) reported that in the NCI mode, HFB-derivatized hydroxy fatty acid methyl esters were characterized by $[M - 20]^{\bullet -}$ or $[M - 40]^{\bullet -}$ peaks that resulted from loss of 1 or 2 HF groups. Diagnostic ions of *m*/*z* 213, 194, and 178 that correspond to those ions shown in Fig. 1 were also evident. The derivatized 3-OH 10:0 analyte and 3-OH 11:0 internal standard exhibited the expected fragmentation pattern and were typified by *m*/*z* 358 and 372 $[M - 40]^{\bullet -}$ ions, respectively (Figs. 2*a* and 2*b*). Due to the lower limits of detection achievable during NCI–MS analysis, the chromatograms produced during full-scan mode may have spurious peaks arising from trace contaminants in the reagents. The specificity and sensitivity of the analysis was increased and contributions of the spurious peaks to analyte and internal standard area counts were eliminated when the SIM mode was used (Figs. 3*a* and 3*b*).

There was a lack of consensus in the literature concerning buffer usage following derivatization with HFBA. Several researchers chose to omit buffer use (Stan and Scheutwinkel-Reich 1980; Noonan and Sams 1985; Lee et al. 2011); however, Longo and Cavallaro (1996) advocated for buffer usage to remove residual heptafluorobutyric acid as they believed this acid caused progressive damage of the GC column. In this study it was found that without a buffer wash the *m*/*z* 194 peak (Fig. 1) persisted in the baseline even

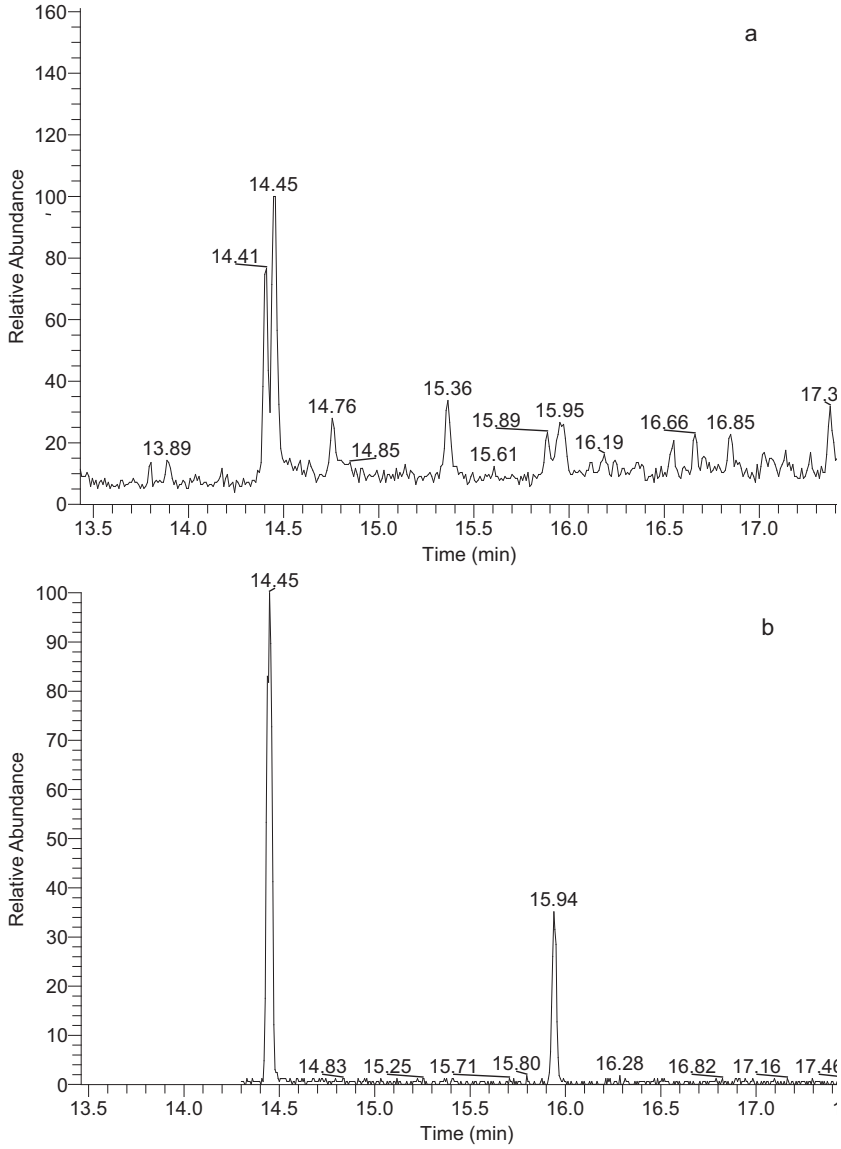
Fig. 2. Diagnostic spectra of 3-HFB-*O*-10:0 methyl ester (*a*) and 3-HFB-*O*-11:0 methyl ester (*b*) acquired using GC–NCI–MS in the full-scan mode.



during blank injections, and required a thorough cleaning of the GC–MS apparatus to be eradicated. The *m*/*z* 194 peak certainly contributed to background signal, and the best results were obtained during NCI–MS analysis when a buffer wash was used and when the system components contributing to background signal (column and septa bleed; injector cleanliness) were optimized. Pentafluorobenzoyl chloride, an alternative electronegative derivatizing reagent, may have been used, but Noonan and Sams (1985) found this reagent produced many large unidentifiable peaks in the chromatograms that were not mitigated by washing the products or altering the reaction conditions. Alternatively, Dalene et al. (1990) found that amines derivatized with HFBA gave better sensitivity and resolution relative to the matrix than those derivatized with each of trifluoroacetic anhydride, pentafluoropropionic anhydride, acetic anhydride, ethyl chloroformate, and isobutyl chloroformate. Thus, despite the persistent peaks from residual heptafluorobutyric acid in the sample, HFBA was likely the most appropriate derivatization reagent to make the 3-OH oxylipins suitable for NCI–MS detection.

Earlier researchers who have utilized GC–NCI–MS reported limits of detection (LOD) of 0.3–66.3 pg/injection in full-scan mode (Longo and Cavallaro 1996) and 0.01–0.57 pg/injection and 0.6 pg/injection in SIM mode (Longo and Cavallaro 1996; Mueller and Brodschelm 1994). In the current study, the LOD was approximately 16 pg/injection in full-scan mode when an analyte signal to noise ratio of 3 was used as a threshold. We found that an LOD of 16 pg/injection was possible when the ion trap and source were clean, when the buffer wash was used, and, in particular, when a properly conditioned low bleed column was installed on the instrument. After repeated sample injections, sensitivity and LOD were reduced. In part, this can be attributed to fouling of the MS ion trap and source by the reagent gas methane (Rosenfelder and Vetter 2009). However, approximately 850 pg of the 3-HFB-*O*-11:0 methyl ester internal standard was injected with each sample and there was never less

Fig. 3. GC–NCI–MS chromatographic analysis of 3-HFB-*O*-10:0 methyl ester (RT–14.45) and the 3-HFB-*O*-11:0 methyl ester (internal standard; RT–15.94) acquired using GC–NCI–MS in the full-scan (*a*) and SIM (*b*) modes for a sample from 36 h fermentation time.



than 174 pg/injection of the analyte (Table 1). Therefore, there is no concern that reduced sensitivity influenced the accuracy of oxylipin quantitation.

The coefficient of determination for the analytical curve (R^2 = 0.94) was comparable to or better than those produced in a similar study (R^2 = 0.94, 0.90, 0.85, and 0.96) by Schulze et al. (2006), who used NCI–MS to quantify 4 plant oxylipins. Similarly, the residuals of the analytical curve in this study were tested for normality (Anderson–Darling, Kolmogorov–Smirnov tests) and were examined by visual inspection for any pattern to evaluate the robustness of the curve fit further. According to both tests, the residuals were normally distributed ($p > 0.05$) and did not show any pattern. Furthermore, no heteroscedasticity was observed in the residuals plot of the analytical curve.

Fermentation dynamics and oxylipin formation

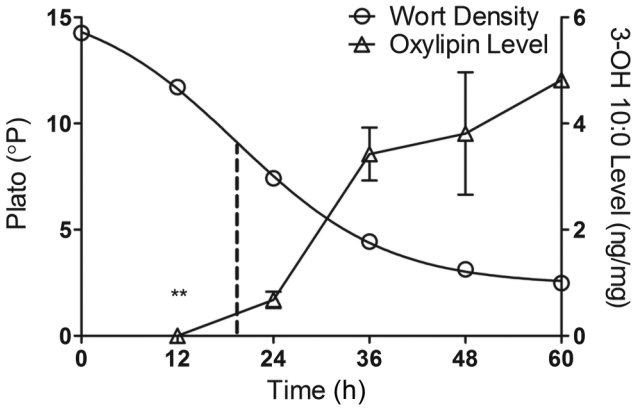
For all samples we were unable to detect 3-OH 10:0 at the first onset of collectable cell mass at 12 h fermentation. However, low levels of this oxylipin were present at 0.68 ± 0.22 ng/mg dry cell mass after 24 h and continued to increase thereafter to a maximum of 4.82 ± 0.18 ng/mg dry cell mass at 60 h fermentation time (Fig. 4; Table 1). This trend is consistent with our earlier investigation in which relative levels of 3-OH 10:0 were monitored during miniature fermentations (Potter et al. 2015). The % relative standard deviation (RSD) values were large, ranging from 3.73% to 42.8% (Table 1). However, these % RSD values were comparable to, and in some cases even lower than, those obtained in other NCI–MS studies of plant oxylipins by Mueller and Brodschelm (1994) (6.67%–

Table 1. Data used to derive the concentration of 3-OH 10:0 in the SMA strain of *Saccharomyces pastorianus* grown in the miniature fermentation assay.

Time (h)	Repeat	3-OH 10:0 oxylipin mass from analytical curve (ng)	Cell mass (mg)	Oxylipin concn. (ng 3-OH 10:0/ mg cell mass)	Mean oxylipin concn. ± SD (ng 3-OH 10:0/ mg cell mass)	% RSD
24	1	249	425	0.59	0.68±0.22	32.3
	2	393	401	0.98		
	3	174	369	0.47		
36	1	906	323	2.81	3.42±0.71	20.8
	2	2161	489	4.41		
	3	1395	456	3.05		
48	1	2894	473	6.11	3.81±1.63	42.8
	2	1546	612	2.52		
	3	1435	512	2.80		
60	1	2808	609	4.61	4.82±0.18	3.73
	2	3227	671	4.80		
	3	2715	538	5.04		

Note: % RSD, percent relative standard deviation.

Fig. 4. Fermentation performance of the SMA strain in the miniature fermentation assay with coincident formation of the 3-OH 10:0 oxylipin. Fermentation progress was determined on the Plato (°P) scale with readings conducted in triplicate and mean values for 3 miniature fermentations presented (SD bars for density measurements not visible). Density attenuation was modelled using with the 4 parameter logistic model, and the fitted value for the parameter *M* is indicated (dashed line). 3-OH 10:0 concentration was measured in triplicate and mean ± SD values are shown as ng 3-OH 10:0/mg dry cell mass. **, Peak area not detected by the Genesis™ peak finding algorithm.



51.2%) and Thoma et al. (2003) (17.2%–50.9%). This suggests an inherent variability in the NCI–MS technique.

The variation of GC–NCI–MS analysis undoubtedly contributed to the % RSD values, but the nature of the miniature fermentation assay did as well. In this bench-scale fermentation system, inoculated wort is distributed among 30 test tubes, which in effect become 30 individual fermenters. At each sampling interval, the yeasts from 6 test tubes were pooled to obtain sufficient cell mass for later lipid extractions. The considerable variation associated with the miniature fermentation assay is evident in the mean % coefficient of variation (CV) of replicate sample injections of 25% compared with the replicate injections of standards used to create the analytical curve that had a mean % CV of 18%. The variability associated with the miniature fermentation assay was further apparent in replicate proportions of 7 fatty acids identified in cells grown in the miniature fermentation using EI GC–MS (mean % CV = 30%) (Potter et al. 2015). Therefore, the greater contributor to the overall variance was likely the miniature fermentation assay and not the lack of fit of the analytical curve or the GC–NCI–MS technique. Nonetheless, examination of variability in the miniature fermentation assay demonstrated that measurements at the end of fermentation had low standard deviations (Macintosh et al. 2016), which was also observed in this work (Table 1).

Earlier studies of yeast oxylipins were unable to quantitatively assay these molecules (Kock et al. 2013; Madu et al. 2015). Therefore, comparable values do not exist in the literature, but jasmonic acid (JA), cyclopentenone A1, and cyclopentenone B1 have received extensive study in plant cell cultures and whole plant leaves (Mueller and Brodschelm 1994; Thoma et al. 2003). Using NCI–MS, Mueller and Brodschelm (1994) and Thoma et al. (2003) noted that basal levels of these plant oxylipins ranged from 0.90 to 131 ng/g dry mass (DM) and increased to 23–776 ng/g DM following tissue wounding and elicitation. Similarly, Kumari et al. (2014) found hydroxy-oxylipins in the macroalgae Chlorophyceae, Rhodophyceae, and Phaeophyceae ranged from 0.14 to 8161 ng/g fresh wet mass and surely would have been higher if determined on a DM basis. Thus, the concentration range of 3-OH 10:0 in the SMA strain at 0.68–4.82 ng/mg DM (680–4820 ng/g DM) was elevated but comparable with oxylipin concentrations in other biological systems.

In plants, JA and E_1 phytoprostanes can act as potent signalling molecules that help to coordinate physiological responses to a host of different stresses (Mueller and Brodschelm 1994; Thoma et al. 2003). The biological func-

tion of oxylipins in fermenting yeasts remains unknown, but we and other authors have speculated that these substances may also function as signalling molecules involved in quorum sensing (Strauss et al. 2005; Potter et al. 2015). This study has demonstrated that the maximal concentration of 3-OH 10:0 in the fermenting SMA strain was 35 times greater than the highest concentration of JA in elicited cell cultures of *Petroselinum hortense* (Mueller and Brodschelm 1994) and 24 times greater than the maximum level of E_1 phytoprostanes in elicited tobacco cell cultures (Thoma et al. 2003). This could imply that 3-OH 10:0 is too abundant to tightly mediate a physiological response; however, it is prudent to recognize the proximity of plant cells to each other in tissues compared with yeast cells. Plant cells may need to emit only small amounts of messenger molecules to mount a tissue-wide response. Yeast cells, however, may require higher concentrations of quorum-sensing mediators to coordinate a response in potentially dispersed unicellular populations.

The putative role of fermenting yeast oxylipins as bioactive signalling compounds is also supported by the specific localization of these molecules. In plant systems, temporal and spatial control of bioactive oxylipin production and release is crucial for normal development and a similar control of enzymes that synthesize oxylipins has been demonstrated in fungi such as *Aspergillus nidulans* (Tsitsigiannis and Keller 2007). In fungal systems, bioactive oxylipins are also known to function locally at low concentrations (nmol/L) via either autocrine or paracrine mechanisms in cell surface receptors linked to G-proteins (Tsitsigiannis and Keller 2007). Studies on 3-OH oxylipins in yeasts have found that these molecules are often localized at the cell exterior (Kock et al. 2000; Leeuw et al. 2006) and this could be, in part, due to 3-OH oxylipin-cell surface receptor binding. Nevertheless, further insights into the origin, cell location, and function of fermenting yeast oxylipins will be realized when the potential substrates for and presumed production mechanism of these molecules are considered.

The medium-chain free fatty acids 8:0 and 10:0 are well-established by-products of ethanolic fermentation (Taylor and Kirsop 1977; Lafon-Lafourcade et al. 1984) and the predominant oxylipins in fermenting yeasts are 3-OH 8:0 and 3-OH 10:0. It would seem then that 8:0 and 10:0 are potential substrates for 3-OH oxylipin production except that one cycle of β-oxidation, the presumed 3-OH oxylipin producing pathway in yeasts (Kock et al. 2007, 2013), reduces hydrocarbon chain length by 2 carbons. However, a link between associated production of 8:0, 10:0, 3-OH 8:0, and 3-OH 10:0 may yet exist. It is well-documented that *Saccharomyces cerevisiae* possesses a futile cycle whereby short- and medium-chain fatty acids from cytoplasmic fatty acid synthesis are shunted to the peroxisome for β-oxidation (Marchesini and Poirier 2003). Also, thioesterases with specificity for oxygenated acyl-CoA substrates have been identified, specifically thioesterase II for 3-hydroxy-acyl-CoA substrates in the bacteria *Rhodopseudomonas sphaeroides* (Seay and Lueking 1986) and a peroxisomal thioesterase for JA-acyl CoA in plants (Li et al. 2005). Thus, the 3-hydroxy-octanoyl-CoA and 3-hydroxy-decanoyl-CoA intermediates of 8:0 and 10:0 β-oxidation may be partially hydrolyzed by peroxisomal thioesterases to produce their respective 3-OH oxylipins.

It is also important to acknowledge that 8:0, 10:0 and other medium-chain fatty acids are toxic and inhibitory to *Saccharomyces cerevisiae* and exposure can result in a broad spectrum of detrimental physiological effects (Jarboe et al. 2013). In particular, exogenous concentrations of 1 mmol/L of 8:0 and 10:0 were shown to be completely inhibitory to *Saccharomyces cerevisiae* (Liu et al. 2013). In studying the response of *Saccharomyces cerevisiae* to 8:0 and 10:0 exposure using a transcriptomic approach, Legras et al. (2010) also noted an upregulation of genes involved in β-oxidation and ethyl ester synthesis. The elucidation of β-oxidation as a tactic to combat 10:0 toxicity was highlighted when deletion of the genes *OAF1* and *PIP2*, which are positive regulators of peroxisomal proteins, reduced resistance to 10:0 (Legras et al. 2010). Our previous study found a pronounced increase in the relative amount of 10:0 during growth of the SMA strain in the miniature fermentation assay (Potter et al. 2015). Given the increase in 10:0 over the course of the fermentation and an induction of genes involved in β-oxidation upon 10:0 exposure, it could be that the production of 3-OH oxylipins in fermenting yeasts is a detoxification response.

Alternatively, the development of 3-OH oxylipins in fermenting yeasts may reflect a change in catabolism of the cells. With the logistic model, the time of the maximal sugar consumption rate is indicated by the parameter *M*. In this study, according to the logistic model fit, the highest rate of sugar consumption occurred at 19.47 h and preceded the lowest detectable concentrations of 3-OH 10:0 by ~4.5 h and a pronounced increase in 3-OH 10:0 concentration by ~16.5 h (Fig. 4). The genes required for the catabolism of nonfermentable carbon sources and proliferation of peroxisomal compartments where β-oxidation occurs are also under the tight control of glucose repression and remain inactive until glucose levels are depleted (Gurvitz and Rottensteiner 2006). Studies of sugar consumption in near identical worts in the miniature fermentation assay have shown that glucose is almost completely consumed by 24 h fermentation time (Macintosh et al. 2016). In this study there was a sharp increase in 3-OH 10:0 concentration between 24 and 36 h (Fig. 4), which is well correlated with depleted glucose levels at 24 h and in increase in β-oxidation thereafter. Thus, the timing of these 2 phenomena is suggestive of the diauxic shift yeast cells undergo once glucose reaches sufficiently low levels.

Conclusions

In this work a GC–NCI–MS analysis of 3-OH oxylipins as HFB-derivatized methyl esters achieved the first quantitative analysis of 3-OH oxylipins in yeast. When the SMA strain of *Saccharomyces pastorianus* was grown in laboratory-scale beverage fermentations, 3-OH 10:0 ranged between 0.68 and 4.82 ng/mg DM from 0 to 60 h fermentation time. % RSD values were large (3.73%–42.8%) but were consistent with other studies that used GC–NCI–MS to assay low levels of oxylipins. The maximal sugar consumption rate during the miniature fermentations preceded the lowest concentration of 3-OH 10:0 by ~4.5 h and a distinct increase in 3-OH 10:0 concentration by ~16.5 h. To identify the exact role of 3-OH oxylipins in fermenting yeasts, the new analytical technique must be coupled with additional assays for β-oxidation activity and substrate utilization. It is also now important to study the effect of 3-OH oxylipins on fermenting cell ultrastructure considering the highly polar nature of these molecules and their abundance relative to oxylipins in other biological systems. The results from this study should facilitate dosage of defined amounts of 3-OH oxylipins in synchronous cell populations and further examination of their influence on cell structure.

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