ANALYTICAL AND NANOTECHNOLOGICAL METHODS FOR DETECTION OF 3-OH OXYLIPINS AND CELL ULTRASTRUCTURE IN FERMENTING YEASTS

by

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DEDICATION

I would like to dedicate this thesis to my parents who have always extoled the values of education and remained firm proponents of education for education's sake. I hope the consummation of this thesis and degree help to reinforce and justify everything you have done for your children. I am eternally greatful.

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ABSTRACT

While 3-hydroxy (OH) oxylipins, a class of hydroxy free fatty acids (FFA), have been previously presumed to play a role in the industrially important brewing yeast flocculation phenomenon, the exact biological function was not known. In earlier yeast 3-OH oxylipin investigations, these molecules were identified by diazomethane derivatization and then separation using gas chromatography-mass spectrometry (GC-MS). Unfortunately, this approach only allowed for qualitative analysis of 3-OH oxylipins in yeast. The difficulty associated with diazomethane use has also meant that lipid analysts must use alternate approaches that still target FFA specifically. A survey of the literature identified numerous approaches to detect FFA that did not require diazomethane. They were grouped as selective quantifications/extractions, purifications and alternate derivatizations.

Here it is shown that 3-OH oxylipins can be detected in the SMA strain of *Saccharomyces pastorianus* grown in lab-scale fermentations without diazomethane by extracting cellular lipids with ethyl acetate, trimethylsilylating the total lipid extract and GC-MS identification using the diagnostic m/z 233 fragment. A second detection strategy was also devised where 3-OH oxylipins were separated from the total lipid extract using thin layer chromatography. Thereafter, 3-OH oxylipins were methylated using BCl_3-MeOH and derivatized at the hydroxy group with heptaflouorobutyric anhydride. Detection of the heptafluorobuyrate methyl ester derivatives using negative chemical ionization-mass spectrometry allowed for the first quantitative analysis of 3-OH oxylipins in yeast. During growth of the SMA strain in lab scale fermentations, 3-OH decanoic acid ranged from 0.68 ± 0.22 - 4.82 ± 0.18 ng/mg dry cell mass.

The discovery of CO_2 bubbles inside fermentation yeasts using Auger-architechtomics has also necessitated more comprehensive studies of bubble formation and 3-OH oxylipin production. Using Nano scanning Auger microscopy, time-of-flight secondary ion mass spectrometry (TOF-SIMS) and immunofluorescence microscopy with 3-OH oxylipin specific antibodies, bubble formation and 3-OH oxylipin production were studied in fermenting and respiring SMA cells. Examinations showed networks of CO_2 in fermenting cells that increased in size with fermentation duration. TOF-SIMS analysis also showed a compositional difference at the interior and exterior of fermenting and respiring cells, while immunofluorescence results suggested contrasting 3-OH oxylipin profiles in fermenting vs. respiring SMA cells.

LIST OF ABBREVIATIONS AND SYMBOLS USED

°P	Degrees Plato
8:0	Octanoic acid
10:0	Decanoic acid
3-HETE	3-hydroxy-5,8,11,14-eicosatetranoic acid
3-OH	3-hydroxy
AES	Auger electron spectroscopy
Ar^+	Argon
ASBC	American Society of Brewing Chemists
B	Function of the slope at the inflection point in 4 parameter logistic model
BAME	Bacterial acid methyl ester
CLSM	Confocal laser scanning microscopy
CSH	Cell surface hydrophobicity
CV	Coefficient of variation
DMAP	4-Dimethylaminopyridine
DMP	2,2 Dimethoxypropane
DW	Dry weight
EI	Electron impact
ESI-MS	Electrospray ionization – mass spectrometry
eV	Electron volt
FAME	Fatty acid methyl ester
FFA	Free fatty acid
FOV	Field of view
GC	Gas chromatography

GC-MS	Gas chromatography – mass spectrometry
GPI	Glycophosphatidylinositol
HFB	Heptafluorobutyrate
HFBA	Heptafluorobutyric anhydride
HICF	Hydrophobic interaction chromatography for flocculation
HIP	Hexane isopropanol
Hi-Res	High resolution
HPLC	High performance liquid chromatography
JA	Jasmonic acid
LM	Light microscopy
LOD	Limits of detection
M	Time to reach the inflection point in 4 parameter logistic model
m/z	Mass-to-charge ratio
MS	Mass spectrometry
NanoSAM	Nano scanning Auger microscopy
NCI	Negative chemical ionization
NCI-MS	Negative chemical ionization – mass spectrometry
NEFA	Non-esterified fatty acid
NIST	National Institute of Standards and Technology
NMR	Nuclear magnetic resonance
PBS	Phosphate buffered saline
PCI	Positive chemical ionization
PCI-MS	Positive chemical ionization – mass spectrometry
P_e	Final asymptotic density in 4 parameter logistic model
P_i	Initial asymptotic density in 4 parameter logistic model

P_t	Extract density at time *t* in 4 parameter logistic model
PUFA	Polyunsaturated fatty acid
RSD	Relative standard deviation
SAM	Scanning Auger microscopy
SEM	Scanning electron microscopy
SIM	Selected ion monitoring
SPE	Solid phase extraction
TBDMS	*Tert*-butyldimethylsilyl
TEM	Transmission electron microscopy
TLC	Thin layer chromatography
TMA	Tetramethylammonium
TMAH	Tetramethylammonium hydroxide
TMPH	Trimethylphenylammonium hydroxide
TMS	Trimethylsilyl
TMSH	Trimethylsulfonium hydroxide
TMTFTH	Trimethyl (α,α,α-trifluoro-m-tolyl) ammonium hydroxide
TOF-SIMS	Time-of-flight secondary ion mass spectrometry
UFS	University of the Free State
UV	Ultra violet
YEPD	Yeast extract, peptone and dextrose
YM	Yeast malt
YPG	Yeast peptone glycerol

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I am also indebted to Professor Lodewyck Kock, formerly of the University of the Free State (UFS), who is really the "Godfather" of yeast 3-OH oxylipin research. Thank you for answering our initial questions around 3-OH oxylipin detection methods, for providing further clues in the literature and for inviting me to collaborate with you at your home institution. Of course, I am very appreciative of everything Dr. Chantel Swart did in taking over the reins from Prof. Kock and still making the collaborative project possible. All your hard work, organization and friendship have been greatly appreciated. Also, I must say thank you to all the kind people at the UFS and in "Bloem" who made me feel very welcome while there, especially Stephen Collett and family.

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CHAPTER 1 INTRODUCTION

1.1 DISSERTATION OVERVIEW

1.1.1 Oxylipins as a Class of Biomolecule

Oxylipins are oxygenated fatty acids with significant structural diversity that may be esterified, non-esterified or conjugated with different metabolites, such as amino acid conjugates of jasmonate (Gobel & Feussner, 2009) (Fig. 1.1). These biomolecules can occur as hydroxy, oxo or keto-fatty acids, divenyl ethers, volatile aldehydes or fatty acid hydroperoxides (Mosblech et al., 2009) (Fig. 1.1) and are ubiquitous in nature, occurring in animals, plants, fungi, bacteria, mosses and algae (Andreou et al., 2009; Tsitsigiannis & Keller, 2007). Typically, these oxygenated fatty acids are derived from lipoxygenase metabolism of polyunsaturated fatty acid precursors (C16-C22) (Barbosa et al., 2016) or free radical catalyzed, non-enzymatic lipid peroxidation by reactive oxygen species produced during normal aerobic metabolism (Gobel and Feusnner, 2009). Many oxylipins are bioactive and may either directly or indirectly mediate numerous biological processes. For example, in plants oxylipins play an important role in host microbe interactions and regulate growth and development (Barbosa et al., 2016; Pohl & Kock, 2014). In animals, this class of molecule, often found as leukotrienes and prostaglandins, is involved in inflammatory processes and allergic responses (Barbosa et al., 2016). Furthermore, oxylipins may function as anti-microbials and anti-fungals in a number of biological systems (Gobel & Feussner, 2009; Pohl et al., 2011).

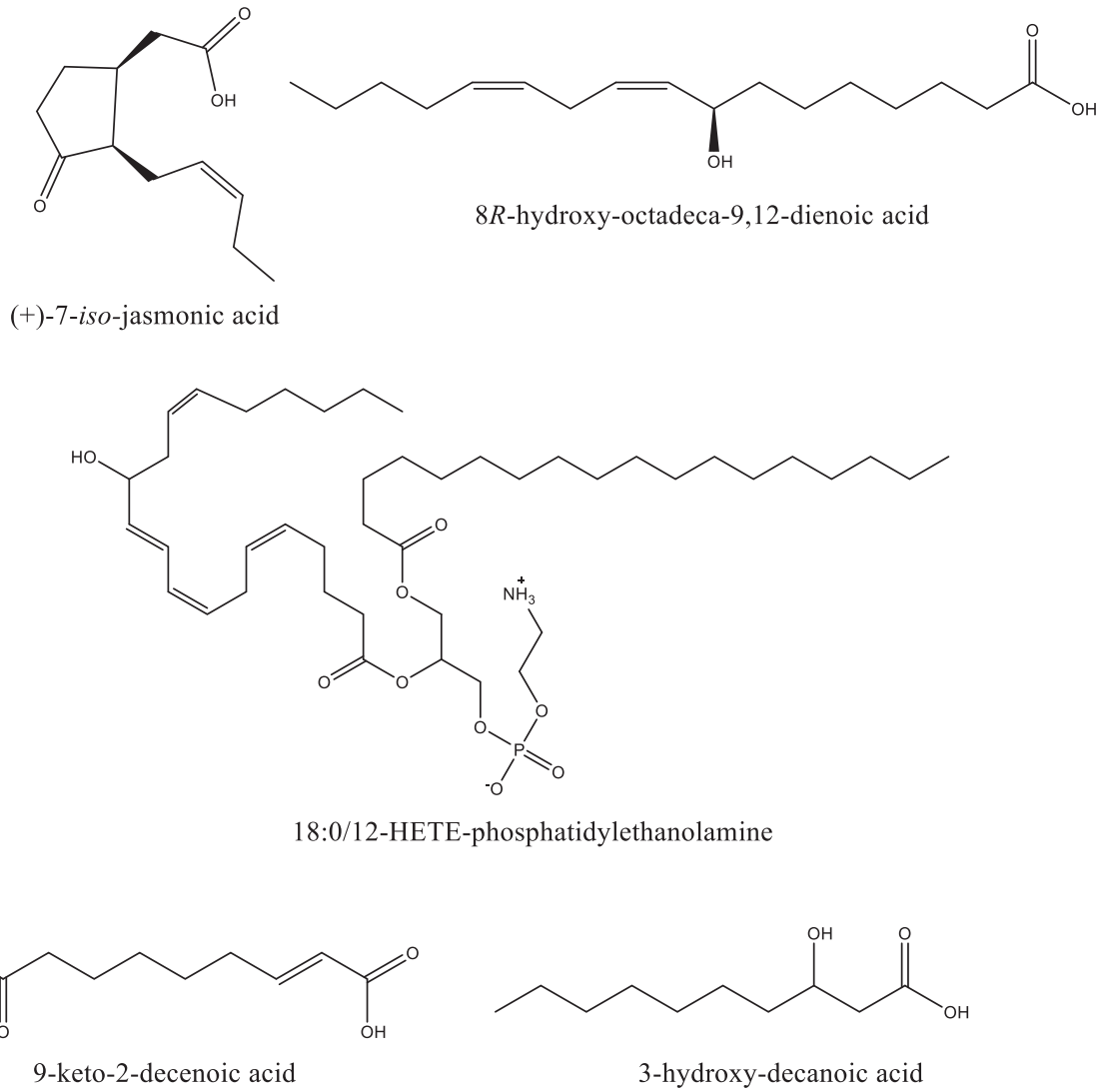

Figure 1.1. Depiction of the structural diversity of oxylipins where they may occur as free or esterified oxygenated fatty acids.

1.1.2 Oxylipin Detection Methods

Just as oxylipins encompass a wide variety of structures, there are numerous analytical approaches which may be followed when assaying these molecules. High performance liquid chromatography (HPLC) separation with ultra violet (UV) detection may be used for certain labile and highly polar oxylipins (Gobel & Feussner, 2009). Gas chromatography-mass spectrometry (GC-MS) methods may also be employed, and in the

case of hydroxy fatty acids they are often first converted to methyl esters at the carboxy functionality and then silylated at the hydroxyl group. For trace level analysis of oxylipins, derivatization with a suitable electronegative group (e.g., pentafluorobenzoyl esters) and detection using negative chemical ionization-mass spectrometry (NCI-MS) has been shown to achieve low limits of detection (LOD) and produce a greatly reduced background (Gobel and Feussner, 2009; Mueller et al., 2006). Electrospray ionization-mass spectrometry (ESI-MS), a gentler ionization approach which requires no derivatization of the analytes and produces an intact molecular ion, may also be employed for oxylipin detection. However, this approach is not capable of distinguishing between isobaric species without a sufficiently high resolution mass spectrometer (Gobel & Feussner, 2009).

MS/MS techniques may also be utilized for oxylipin identification such as the Ultra performance liquid chromatography-ESI-MS/MS method of Gouveia-Figuera & Nording (2015) for both oxylipins and endocannabinoids or the liquid chromatography-MS/MS method of Trap et al. (2015) for lineoleate oxylipins. It should be noted that enzyme-induced oxylipin formation leads to enantiomerically pure oxylipins, but non-enzymatic lipid oxidation produces a racemic oxylipin mixture (Gobel and Feussner, 2009). As such, specific methods may be employed to determine the chirality of oxylipins including nuclear magnetic resonance (NMR) spectroscopy or GC-MS analysis of methanoxycarbonyl derivatives after ozonolysis (Gobel & Feusnner, 2009).

1.1.3 Oxylipins in Yeast and Fungi

Oxylipin research in yeasts and other fungi has been conducted primarily by the Lipid Biotechnology group at the University of the Free State (UFS) where investigations focused on a particular class of nonesterified oxylipins, 3-hydroxy (OH) oxylipins. This work was initiated in 1988 when yeasts were considered as a cheaper source of pharmacologically active prostaglandins since many of these organisms produced the polyunsaturated fatty acids which serve as a prostaglandin precursors (Kock et al., 2011). Initial studies by this research group noted the production of 3-hydroxy-5,8,11,14-eicosaetetraenoic acid (3-HETE) when the yeast *Dipodascopsis uninucleata* was given exogenous arachidonic acid (van Dyk et al., 1991). The family Lipomycetaceae was then considered more broadly for production of 3-OH metabolites of arachidonic acid, but these molecules were only found in *Lipomyces anomalus* strains and again in the genus *Dipodascopsis* (Kock et al., 1992). The soil fungus, *Mucor genevensis*, was later found to produce 3-OH-5Z,8Z-tetradecadienoic acid from arachidonic acid which was in contrast to the 3-HETE produced by *Dipodascopsis uninucleata* (Pohl et al., 1998).

Candida albicans was also examined for 3-OH metabolites of arachidonic acid because this organism is a pathogen known to cause release of arachidonic acid and eicosanoids from human tissue. A unique 3-OH fatty acid, 3,18-dihydroxy-5, 8, 11, 14-eicosatetraenoic acid, was identified in this fungi (Deva et al., 2000). A shorter 3-OH oxylipin was also identified in the mucoralean fungus *Pilobolus* and was presumed to be 3-OH 9:1 based on GC-MS analysis (Kock et al., 2001). Meanwhile, *Saccharomycopsis malanga* was shown to produce 3-OH 16:0 that was predominantly associated with the cell wall surface of vegetative cells (Sebolai et al., 2001). GC-MS analysis also revealed

that a 3-OH oxylipin likely occurred in the yeast and mustard seed pathogen, *Eremothecium sinecaudam*, based on the presence of the diagnostic m/z 175 fragment arising from methylated and silylated 3-OH oxylipins following cleavage at the β-carbon (Bareetseng et al., 2004). A unique monounsaturated 3-OH oxylipin, 3-OH 10:1, was later identified on the surface of *Ascoidea africana* Batra and Francke-Grosmann ascopores (Bareetseng et al., 2005).

Brewing yeast were also studied for production of 3-OH oxylipins as these molecules seemed to localize at the cell exterior and were thought to play a role in cell-to-cell aggregation (Kock et al., 2000). Cell-to-cell aggregation or flocculation is of interest to both brewing scientists and brewing practitioners as this phenomenon aids in the natural clarification of the fermented beer. In their work Kock et al. (2000) identified two novel oxylipins, 3-OH octanoic acid (8:0) and 3-OH decanoic (10:0), in flocculating cells of *Saccharomyces cerevisiae* based on GC-MS identification. Using immunofluorescence microscopy methods, the same researchers noted that oxylipins formed in fluorescent protrubances on the cell wall and were present between flocculating cells. Further examination revealed that in the presence of 1 mM of aspirin, a cyclooxygenase inhibitor previously shown to have antifungal properties (Strauss, 2005), oxylipin production was completely inhibited and flocculation was substantially reduced. This correlation suggested the importance of 3-OH oxylipin production in brewing yeast flocculation (Strauss et al., 2005).

1.1.4 Auger-architectomics

Scientists at the UFS had a long-standing research program that screened potential new anti-mitochondrial drugs using yeasts cells in the sexual stage of growth (Kock et al., 2009). Auger-architectomics, or the application of Nano scanning Auger microscopy (NanoSAM) to the analysis of biological specimens, was developed to examine yeast cells that had been exposed to potential new anti-mitochondrial/anti-fungal drugs (Swart et al., 2010). It was presumed that this technology would be an ideal means to study the holistic effect of the potential drugs on yeast cells because of the multifunctional nature of the NanoSAM (Swart et al., 2014). NanoSAM instruments have the unique ability to concurrently examine the 3D architecture of cells using scanning electron microscopy (SEM), to progressively remove cell layers with an etching Argon gun and to perform elemental analysis using Auger electron spectroscopy (AES) (Kock et al., 2011). Specialized fixation and dehydration protocols were also devised so the biological samples could withstand the Argon etching process and the more energetic electron beam (25 kV) that occurs with the NanoSAM (Swart et al., 2014). The utility of Auger-architectomics was demonstrated by testing ascospores of *Nadsonia fulvescens* treated with the anti-mitochondrial compound fluconazole, and this revealed that exposed and unexposed cells had different elemental compositions and morphologies (Swart et al., 2010). Auger-architectomics was also applied to fermentation yeasts from the genus *Saccharomyces* which demonstrated the presence of CO_2 bubbles inside both strains of *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* (Swart et al., 2012; Swart et al., 2013).

1.2 THESIS GOALS AND OBJECTIVES

This research work has primarily focused on nonesterified 3-OH oxylipins (ie. free 3-OH fatty acids) in fermenting yeasts. Much of this research was, as mentioned, pioneered by the Lipid Biotechnology group at the UFS under the leadership of Professor Lodewyck Kock. Recently, the Lipid Biotechnology lab at the UFS moved away from chemical analysis of yeast oxlipins due to the lack of available diazomethane, a lipid methylating reagent. It is from here that this project began, in effect picking up where the Kock research group left off.

At the outset, the goal of this research was to examine cell surface hydrophobicity (CSH) as a governing force in brewing yeast flocculation, with a particular focus on 3-OH oxylipin detection. The initial hypothesis was that the production of 3-OH oxyipins over the course of fermentation drove an increase in CSH. However, it quickly became apparent that there were analytical challenges that had to be overcome concerning 3-OH oxylipin detection without diazomethane. Thus, this research project started with the design of experiments to address the hypothesis; however, the focus shifted to identifying and applying analytical protocols for 3-OH oxylipin detection that overcame the limitations of those methods previously employed by Kock and co-workers. Finally, this research project sought to create a comprehensive understading of fermenting cell structure and composition by using the nanotechniques developed by Kock and co-workers in conjunction with 3-OH oxylipin analysis. The contents of this thesis, therefore, i) document the analytical challenges involved in 3-OH oxylipin detection in fermenting yeast (Chapter 2) and then ii) demonstrate the alternative techniques that may

be employed (Chapter 4) or were employed (Chapter 3,5,6) to detect these compounds. The specific objectives of the study are as follows:

1.) To concurrently monitor flocculation, CSH and nonesterified 3-OH oxylipin levels during growth in the miniature fermentation assay (ASBC, 2013).

2.) To comprehensively review the available methods, other than diazomethane, for the detection of hydroxy and non-esterified fatty acids (NEFA).

3.) To identify, develop and apply an assay for the quantitative detection of nonesterified 3-OH oxylipins in fermentation yeast.

4.) To characterize the SMA strain of *Saccharomyces pastorianus* with Auger-architectomics and other nanotechnological techniques while simultaneously examining 3-OH oxylipin profiles.

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CHAPTER 2 TRANSESTERIFICATION OF LIPID EXTRACTS FROM WHOLE YEAST CELLS AND CELL WALL PREPARATIONS

2.1 PUBLICATION INFORMATION

This chapter is a modified version of two letters submitted to a journal. The first letter (2.2) was prepared by the thesis author and the second letter (2.4) was written by researchers at the UFS in response to the first letter.

Potter, G., Speers, A. and Budge, S.M. 2013. 3-OH oxylipins in *Saccharomyces cerevisiae*. Journal of the Institute of Brewing 119(3): 85.

Kock, L., Swart, C. and Pohl, C. 2013. Response: 3-OH oxylipins in *Saccharomyces cerevisiae*. Journal of the Institute of Brewing 119(4): 201.

2.2 3-OH OXYLIPINS IN *SACCHAROMYCES CEREVISIAE*

It has been determined that brewing yeast flocculation occurs due to three phenomena: zymolectin binding, hydrophobic interactions and to a lesser degree, surface charge neutralization (Speers, 2006). Changes in cell surface hydrophobic interactions, in particular, have been known to be involved in flocculation for some time and in fact our lab has previously determined that in two strains of brewing yeasts cells were shown to reach maximum flocculation potential with a concurrent increase in cell wall hydrophobicity (Speers et al., 2006; Speers, 2012). Furthermore, during an investigation of oxylipin distribution in brewing yeasts, it was shown that a class of hydrophobic hydroxy-FFA acids, 3-OH oxylipins, localized to the cell wall at flocculation onset (Kock et al., 2000). Thus, the level of unesterified 3-OH oxylipins showed strong potential as a putative predictor of flocculation.

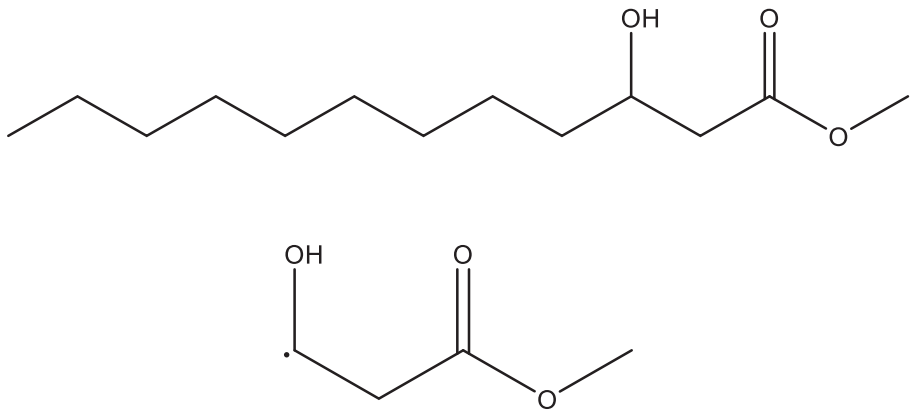
It was the general working hypothesis of our recent study that changes in cell wall hydrophobicity, which is an important contributing force to yeast cell flocculation, are due to oxylipin formation. While our lab has conducted extensive work to date on flocculation and measuring cell wall hydrophobicity, there has been no work which correlated flocculation, cell wall hydrophobicity and cell wall oxylipin concentration as a function of fermentation time. Other research groups had investigated 3-OH oxylipin levels in different species of brewer's yeast, notably the Kock Lab, and they were able to detect two 3-OH oxylipins in particular, 3-OH 8:0 and 3-OH 10:0 (Kock et al., 2000; Strauss, 2005). However, to our knowledge other labs had not investigated *Saccharomyces cerevisiae* 3-OH oxylipins nor monitored their concentration over the course of a fermentation.

Our research was conducted in two main phases. In the first phase, the SMA strain of *Saccharomyces cerevisiae* was grown in yeast extract, peptone and dextrose (YEPD) broth to validate the experimental techniques and to investigate the merits of whole-cell fatty acid analysis versus cell-wall associated fatty acid analysis. It was hypothesized that cell rupture and subsequent isolation of cell wall ghosts, which are cell wall/membrane complexes without the intracellular and cytoplasmic contents, would concentrate the cell wall-associated lipids. In the second phase, the yeast was grown by the American Society of Brewing Chemists (ASBC) miniature fermentation assay protocol (ASBC, 2013) to more closely mimic an industrial beer fermentation.

For all sample types, lipids were isolated using a hexane-isopropanol (HIP) extraction (Albrecht-von-Haller, 2005) and fatty acid methyl esters (FAME) were prepared using a standard acid-catalyzed transesterification protocol. FAME were analyzed by GC-MS with a FFAP column (nitroerephthalic acid-modified polyethylene glycol phase, 30 m,

id 0.32 mm). A mixed Bacterial Acid Methyl Ester (BAME) standard containing 3-OH 12:0 and 3-OH 14:0 was used to determine chromatographic properties of the oxylipins, the characteristic fragments of the 3-OH structure in GC-MS and to ultimately aid in detecting 3-OH 8:0 and 3-OH 10:0 specifically.

In both YEPD broth and the miniature fermentation assay (ASBC, 2013), flocculation and CSH increased over the course of the fermentation. It is notable that the SMA strain is the third industrial lager strain to show these behaviours. Examination of the BAME standards revealed an obvious fragment of *m/z* 103 in both 3-OH 12:0 and 3-OH 14:0 methyl esters, representing the CH3COOCH2CHOH fragment that was first reported by Vesonder et al. (1968) (Fig. 2.1). However, no peaks were identified in any yeast samples with that fragment in regular full-scan mode with GC-MS. When the more sensitive selected ion monitoring (SIM) for the m/z 103 diagnostic fragment was employed (Fig. 2.1), we still failed to identify any fatty acids with the 3-OH functionality.



Chemical Formula: $C_4H_7O_3^{\bullet}$
Exact Mass: 103.04

Figure 2.1. Structure of the 3-OH 12:0 methyl ester and the diagnostic m/z 103 fragment arising from cleavage at the β-carbon.

Thus, it is the purpose of this letter to advise future researchers to avoid 3-OH oxylipin identification and/or quantification in *Saccharomyces cerevisiae* using the HIP extraction and acid-catalyzed transesterification approach we employed. We chose the HIP extraction protocol described by Göbel and Feussner (2009) because it was used to successfully isolate oxylipins from plant material. It may be that a more a traditional chloroform and methanol extraction would give better yields. In addition, it is possible that the SMA strain has very low levels or no oxylipins at all. In future studies of 3-OH oxylipins in brewer's yeast it would be advisable to use those strains reported by Kock et al. (2000) and Strauss (2005).

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2.4 RESPONSE: 3-OH OXYLIPINS IN *SACCHAROMYCES CEREVISIAE*

As we understand from this publication, Potter and co-workers prepared FAME of the extracted yeast lipids using a standard transesterification protocol. Transesterification is the chemical process of converting one ester, in this case the glycerol esters of the yeast lipids (such as triglycerides), into other esters, such as FAME, which can be used in gas chromatography (GC) analysis (Gunstone & Herslof, 1993).

In our experience, free unesterified 3-OH oxylipins (i.e. free 3-OH fatty acids) are present in only very small amounts in yeasts. We could only qualitatively establish their presence in most yeasts (Kock et al., 2011). It was found that when we transesterified the total yeast lipid fraction, the free oxylipins that were derivatized were dwarfed by the other esterified fatty acids (previously linked to triglycerides, phospholipids, etc.). This happened to such an extent that it is impossible to obtain sufficient separation and mass spectrometry (MS) identification. Therefore, in order to analyse free yeast oxylipins, it is important that a strategy is followed where the free fatty acid (FFA) portion is selectively targeted for analysis, while the esterified fatty acids, which usually forms the bulk of cellular lipids (part of triglycerides, phospholipids, etc.), are not included in the analysis. In the case of the free unesterified 3-OH oxylipins, we first methylated with diazomethane, which only attacks acid functional groups while hydroxyl groups are normally unaffected. This was followed by trimethylsilylation of the hydroxyl group with Bis-(trimethylsilyl) trifluoroacetamide. This sequence of derivatization was followed to minimize side reactions (Barrow & Taylor, 1987).

Previously we isolated a novel unesterified 3-OH oxylipin from the yeast *Dipodascopsis uninucleata* which was successfully analysed after the methyl and methyl-

trimethyl-silyl derivatives of the samples were prepared as above (Van Dyk et al., 1991). From this work this novel free 3-OH oxylipin was chemically synthesised by various researchers for successful bioactivity studies (Kock et al., 2011).

We suggest that the authors adapt their method so that they selectively target free unesterified oxylipins in the yeast lipid fractions of interest. Here the methods disclosed in Barrow and Taylor (1987) may be followed. Our experience shows that most yeasts contain these oxylipins that are known to be produced via beta oxidation – a basic function of cells (Gunstone & Herslof, 1993). Such research is of utmost importance to elucidate the role of 3-OH oxylipins in flocculation.

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2.6 LETTER COMMENTARY

The contents of the initial letter co-written by the thesis author (2.2) and the response letter prepared by Kock and co-workers (2.4) were included in the thesis to show the development of the 3-OH oxylipin research project. Furthermore, the response letter (2.2), in particular, contains information which shaped the research and analytical guides presented in later chapters of this work. When the 3-OH oxylipin research began, the intention was to measure flocculation level, CSH and 3-OH oxylipin levels in the fermenting SMA yeast strain. However, as the "3-OH oxylipins in *Saccharomyces cerevisiae*" (2.2) letter mentions, the HIP extraction and acid-catalyzed derivatization proved to be unsuccessful. Kock and co-workers (2.4) then suggested we employ an approach that targeted the FFA specifically.

In Chapter 3, the identification of 3-OH oxylipins in the SMA strain is detailed using an analytical approach that targeted the FFA fraction of the total lipid extract. The response letter of Kock et al. (2.4) also describes the methods they previously used where 3-OH oxylipins were first methylated with diazomethane. Because of safety concerns, alternative methods for 3-OH oxylipin analysis that did not require diazomethane were considered during this research project. There seemed to be a need in the lipid science literature for a compendium of methods for FFA detection that did not require diazomethane, and such a review/analysis guide is presented in Chapter 4. Kock et al. (2.4) also mentioned they had only been able to qualitatively analyze 3-OH oxylipins in most yeasts. Chapter 5 then details an assay that achieved a quantitative analysis of 3-OH oxylipins in the SMA strain. Finally, in Chapter 6, the research project culminates with the first coincident examination of cell ultrastructure and 3-OH oxylipin profile in

fermenting yeast. The dialogue in the literature (2.2, 2.4) and the manuscript detailed in Chapter 3 were part of the application used to obtain funding for the collaborative study described in Chapter 6.

CHAPTER 3 FLOCCULATION, CELL SURFACE HYDROPHOBICITY AND 3-OH OXYLIPINS IN THE SMA STRAIN OF *SACCHAROMYCES PASTORIANUS*

3.1 PUBLICATION INFORMATION

This chapter is a modified version of a manuscript, reworked here for consistency. Originally published as:

Potter, G., Budge, S.M. and Speers, R.A. 2015. Flocculation, cell surface hydrophobicity and 3-OH oxylipins in the SMA strain of *Saccharomyces pastorianus*. Journal of the Institute of Brewing 121(1): 31-37.

Suzanne Budge and Alex Speers contributed to the study design and manuscript preparation. Greg Potter was the principal investigator and lead author.

3.2 ABSTRACT

3-OH oxylipins have been previously detected in brewing yeast production strains at flocculation onset. In this work, the SMA strain of *Saccharomyces pastorianus* was characterized during growth in a miniature fermentation assay by measuring flocculation and CSH. Proportions of 3-OH oxylipin were also measured concurrently during growth in the miniature fermentation assay and a defined 3-OH oxylipin extraction protocol using ethyl acetate is presented along with a novel derivatization and GC-MS detection approach. When the SMA strain was grown in the assay, near maximal CSH and flocculation levels were achieved by 36 hours fermentation time. Under the same culture conditions, the oxylipin 3-OH 10:0 was identified. This oxylipin could not be detected early in the fermentation, but elevated relative levels of 3-OH 10:0 were reached by 36

hours, coinciding with increased CSH levels. It was previously presumed that the formation of 3-OH oxylipins at flocculation onset might increase the CSH. However, results from this study suggest that 3-OH 10:0 may not contribute to cell wall hydrophobicity. The flocculation behaviour of the SMA strain was also monitored in the presence of 3-OH 10:0, but exposure to this oxylipin did not impact the sedimentation of this yeast, suggesting 3-OH oxylipins may not act as mediators of quorum sensing in this strain.

3.3 INTRODUCTION

The current brewing yeast flocculation paradigm has identified three predominant factors that collectively control this phenomenon: zymolectin binding, hydrophobic interactions and surface charge neutralization (Speers, 2012). The impact of hydrophobic interactions on yeast flocculation has been studied by numerous early researchers (Iimura et al., 1980; Kamada & Murata, 1984; Amory & Rouxhet, 1988; Mozes et al., 1989; Mestdagh et al., 1980*;* Straver et al., 1980*;* Straver & Kijne, 1996). While past investigators have used different methods to quantify CSH with varying degrees of success, one method that was developed by Jibiki et al. (1997) has been shown to give reproducible results (Jin et al., 2001; Speers et al., 2006). This method is an adaptation of hydrophobic interaction chromatography and is called hydrophobic interaction chromatography for flocculation (HICF).

With HICF, it is assumed that components on the exterior of the cell are attracted to phenyl sepharose beads by hydrophobic interactions because the beads carry no charged groups and thus, exhibit no electrostatic effect (Jin, 1999). Yet, there remains a

lack of understanding as to what specific components on the surface of the yeast cell wall confer the hydrophobic effect. One would think that lipids in the cell and cell wall are drawn to the phenyl sepharose beads, but literature reports of cell wall lipid content, and thus hydrophobic wall constituents, vary. In an in depth review of *Sacccharomyces cerevisiae* cell wall architecture by Lipke and Ovalle (1998), lipids are not mentioned at all as significant structural components. Despite that, Kock et al. (2000) observed 3-OH oxylipin containing osmiophilic layers that migrated through the cell to the cell wall at flocculation onset. Klis et. al. (2006) noted that glycophosphatidylinositol (GPI) anchored proteins destined for the *Saccharomyces cerevisiae* cell wall arrived at the cell exterior with an intact GPI anchor, but that mature GPI anchored cell wall proteins were without the lipid moiety following cleavage between the first manose residue and glucosamine of the glycophospholipid-protein aggregate (Fig. 3.1). The deposition of the GPI anchor lipid moiety in the plasma membrane, in turn, may increase CSH. Alternatively, other studies have demonstrated that there are proteins at the cell surface of *Saccharomyces cerevisiae* that impart a hydrophobic character to the cell exterior (Smit et al., 1992; Guo et al., 2000; Kang & Choi, 2005).

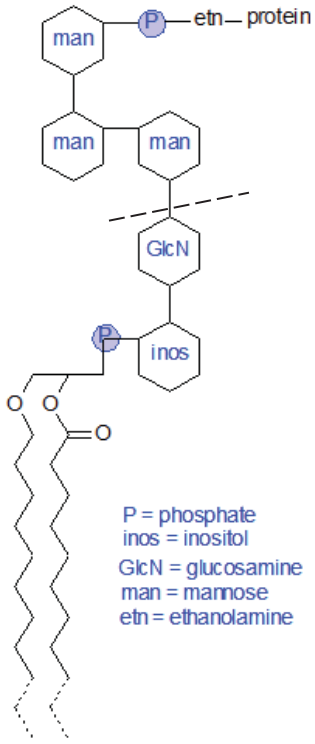


Figure 3.1. Schematic of a GPI anchored protein complex. Nascent peptides destined for the *Saccharomyces cerevisiae* cell wall contain an intact GPI anchor, but mature *Saccharomyces cerevisiae* proteins typically undergo cleavage between glucosamine and the first mannose residue (hashed-line). (Used with permission from AOCS Lipid Library, 2016).

While the main factor for increasing CSH remains undetermined, the "flocculation paradigm" is further confounded by the myriad of strain genotypes routinely used in brewing science research (Speers et al., 1992). In addition, some strains mutate and become less flocculent over successive generations (Sato et al., 2001). Furthermore, production strains used in research are not necessarily "pure" strains (Speers, 1991; Speers et al., 1993) and single populations of brewing yeast can have non-homogenous flocculation characteristics (Speers et al., 1992). Due to the lack of consistency and standardization, brewing scientists have often suggested that the field would benefit from selecting a small number of "research" strains (Speers et al., 1992). Recently, the SMA strain of *Saccharomyces pastorianus* has become a *de facto* research

strain (ASBC, 2013). Even though trials have documented the flocculation characteristics of this strain by tracking changes in suspended yeast with turbidity measurements (600 nm) (Lake et al., 2008), this strain has not been examined using more conventional brewing yeast assays.

This communication, therefore, has three primary purposes: 1) to detail work that characterized the SMA test strain with standard tests for brewing yeast flocculation (ASBC, 2013) and CSH (Jibiki et al., 1997) during growth in the new miniature fermentation assay (ASBC, 2013); 2) to investigate the potential role of fatty acids and 3-OH oxylipins in cell hydrophobic interactions; and 3) to present an improved approach for 3-OH oxylipin extraction and detection based on several previously published works.

3.4 MATERIALS AND METHODS

3.4.1 Determination of Flocculation Level and Cell Surface Hydrophobicity of the SMA Strain During Growth in the Miniature Fermentation Assay

3.4.1.1 Culture in Miniature Fermentation Assay

The SMA yeast was cultured in the miniature fermentation assay and the initial inoculum was prepared according to the standard protocol (ASBC, 2013). However, a pre-made Pale Ale wort (Festa Brew, Vaughn, ON) with the following specifications was used in place of the congress wort: Original Gravity of 12.39-12.58 °P (measured as 12.5 °P), International Bittering Units=25 and Real Degree of Fermentation=66.4 %. The pre-made wort had been previously portioned into 473 mL cups that were frozen in a -30 °C blast freezer. Prior to use, the contents of two 473 mL cups of microwave-defrosted wort were

autoclaved for 20 min at 121 °C, and the sterile wort was cooled overnight at 4 °C for no more than 12 hours.

Following the cold break, the wort was centrifuged at 1950 x g for 15 minutes, and the supernatant was saved. Then 16.2 g of D-glucose was dissolved in 410 mL of wort supernatant instead of the 18 g specified in the protocol (ASBC, 2013). The mixture was aerated by bubbling it with medical-grade, compressed oxygen for 5 minutes to achieve a dissolved oxygen content of approximately 8.2 mg/mL[1]. The wort was then pitched with the SMA yeast at a rate of 1.5 x 10^7 cells/mL based on a final volume of 450 mL and adjusted to this volume with sterile distilled H_2O if required. When the pitched wort had been thoroughly mixed, 15.0 mL aliquots were transferred to each of 30 sterile 20 mL test tubes that contained a sterile polytetrafluoroethylene boiling stone. All tubes were plugged with a sterile sponge bung and the rack containing the tubes was placed in a 21 °C water bath until sampling.

3.4.1.2 Density Determination

Density was determined on a Plato scale (°P) using a handheld densitometer (Anton Parr DMA 35, Graz, Austria) at 12, 24, 36, 48 and 60 hours fermentation time and before each reading the sample was filtered as suggested in the standard method (ASBC, 2013).

3.4.1.3 Determination of Cell Surface Hydrophobicity and Flocculation Level

At each sampling time (12, 24, 36, 48 and 60 hours) flocculation level was determined by the Absorbance Method of the ASBC Flocculation Test (ASBC, 2013) where flocculation is measured by absorbance (600 nm) following a settling reaction in a test tube containing $CaSO_4$ solution buffered at pH 4.5 (B). The absorbance in the $CaSO_4$ solution buffered

[1] Approximated from United States Environmental Protection Agency (2012) data.

solution is then compared to that in a second tube containing 0.5M ethylenediaminetetraacetic acid which chelates any available Ca^{2+} ions, and thus prevents yeast flocculation (A). Flocculence is, therefore, determined as [($Abs_{600}A$ – $Abs_{600}B$)/$Abs_{600}B$] x 100. In this work, the Absorbance Method (ASBC, 2013) was employed with several notable adaptations. In particular, the SMA strain was grown in the miniature fermentation assay protocol and initial 10 mL aliquots of fermenting wort were collected from two separate vortexed 20 mL test tubes. Further, cell pellets were always re-suspended in buffer by withdrawing and expelling through a Pasteur pipette approximately 10 times in addition to the recommended vortexing.

CSH was determined using the method of Jibiki et al. (1997) which is an adaptation of hydrophobic interaction chromatography. In this method miniature disposable chromatographic columns (1.5-3.0 mL) are packed with phenyl sepharose CL-4B and the columns are then equilibrated with a salt-containing acetate buffer. A standardized (5 % w/v) sample of yeast cells is subsequently applied to the column, a volume of the same salt-containing acetate buffer is added and the eluent is collected. Those cells which are hydrophobic will adhere to the phenyl sepharose CL-4B. The hydrophobicity of the sample is determined based on the % differential absorbance (660 nm) between a 5 % w/v sample of the yeast in the salt-containing sodium acetate buffer (A) and the eluent collected after the same sample and buffer volume were applied to the column (E). CSH, therefore, is determined as [($Abs_{660}A$ – $Abs_{660}E$)/$Abs_{660}A$] x 100. In this study, the HICF method (Jibiki et al., 1997) was adapted to test yeast grown in the miniature fermentation assay where samples were collected from 20 mL test tubes that had been vortexed to re-suspend any settled yeast.

3.4.2 Hydrophobicity Studies

3.4.2.1 Culture in Miniature Fermentation Assay and Whole Yeast Cell Collection

The SMA yeast was grown using the ASBC miniature fermentation assay protocol (ASBC, 2013) as described above. To collect yeast pellets at each sampling point (12, 24, 36, 48 and 60 hours), four to six test tubes containing 15 mL each of fermenting wort were vortexed to re-suspend any settled yeast and the contents were transferred evenly to two sterile 50 mL centrifuge tubes. The 50 mL tubes were centrifuged for 2.5 minutes at 630 x g and the supernatant was discarded. To each tube, 5 mL of sterile distilled water was then added and the pellets were re-suspended by vortexing. The tubes were then pooled into one sterile 50 mL centrifuge tube and again this tube was centrifuged for 2.5 minutes at 630 x g. The supernatant was discarded and the yeast pellet was stored at -30 °C until later use. Samples for the ethyl acetate extraction were partially dried by temporary storage in a freeze-drying unit.

3.4.2.2 Hexane-isopropanol Extraction

Lipids were extracted from the yeast pellets following a modified version of a Goettingen Center for Molecular Biosciences HIP extraction (Albrecht-von-Haller Institute, 2005). To do this, 0.5 g of wet yeast that had been allowed to just thaw was aseptically added to a 40 mL durable, solvent-cleaned glass tube. 20 mL of n-hexane/2-propanol (3:2 v/v) was added to each glass tube. The tubes were then sonicated for 4 minutes, shaken by hand for 15 seconds and centrifuged for 4 minutes at 160 x g. Following centrifugation, 12.5 mL of 6.7 % (w/v) K_2SO_4 that had been prepared with triple chloroform-washed water was added to the glass tube and the tube was shaken vigorously by hand for two minutes. After the mixture had settled, the resultant upper hexane layer was then transferred to a

10 mL solvent-cleaned test tube and was thoroughly dried by adding ~1.3 g of anhydrous Na_2SO_4 to each tube. The dried hexane-rich layer was subsequently transferred to a second clean 10 ml tube and the solvent was evaporated under streaming nitrogen. Between all aforementioned tube manipulations, the tube atmospheres were evacuated with streaming nitrogen.

3.4.2.3 Ethyl Acetate Extraction

Partially freeze-dried yeast (0.5 g) that had been allowed to just thaw was aseptically added to a 40 mL durable, solvent-cleaned glass tube. Following this 10 mL of triple chloroform-washed, distilled water was added to each tube along with a saturating amount of 4 g NaCl to help mitigate emulsion formation, as suggested by Salmon and Flower (1982). Each sample was then acidified with 5 µL of 0.25 M citric acid prepared from chloroform-washed distilled water and 10 mL of ethyl acetate was added to every tube. All tubes were sonicated for 4 minutes.

After sonication, the tubes were shaken for 10 minutes on a Burrell "Wrist-action" bench top shaker (Pittsburgh, PA) and subsequently centrifuged for 10 minutes at 160 x g. Despite the addition of NaCl, after centrifugation an emulsion formed in some samples at the interface and extended into the ethyl acetate layer. This was dispersed by adding ~ 1.5 mL cold acetone to the tube to precipitate protein and other macromolecular components of the emulsion (Salmon & Flower, 1982), swirling the tube and centrifuging for a further 4 minutes at the same speed. Once adequate phase separation occurred, the upper ethyl acetate layer was transferred to a new, clean 30 mL test tube. A second 10 mL aliquot of ethyl acetate was then added to each 40 mL sample tube, and another extraction and centrifugation was carried out as before. The pooled lipid extracts in ethyl

acetate were then dried using anhydrous sodium sulphate (~1.3 g) and the solvent removed under streaming nitrogen.

3.4.2.4 Production of Trimethylsilyl Esters and Ethers for Gas Chromatography

To prepare trimethylsilyl (TMS) esters and ethers for GC, 1 mL of Tri-Sil/BSA Pyridine (Thermo Scientific Pierce, Rockford, IL) reagent was added to the dried products of the HIP and EA extracts. The tube atmospheres were evacuated with streaming nitrogen and the closed tube was placed on a heat block at 68 °C for 20 minutes. A 3-OH 10:0 standard (Matreya LLC, Pleasant Gap, PA) was also trimethylsilylated to determine the retention time and characteristic fragments of this oxylipin previously identified in production strains of brewing yeast by Strauss et al. (2005).

3.4.2.5 Analysis and Identification of Trimethylsilyl Esters and Ethers using Gas Chromatography-Mass Spectrometry

GC-MS analysis was adapted from a gas chromatographic protocol described by Molee et al. (2005) to separate and quantify neutral lipids, including FFA. This analysis was performed using a Trace GC Ultra fitted with a Varian FactorFour VF-1ms column (100 % dimethylpolysiloxane, 15 m x 0.25 mm ID, 0.25 μm film thickness), a Polaris Q mass spectrometer and a Thermo Triplus AS autosampler. Programmed temperature vapourizing splitless injection was employed to minimize degradation of thermolabile TMS esters and ethers (Sparkman et al., 2011). A 1.0 μl injection volume was used at an initial temperature of 130 °C for 0 minutes, followed by a ramp at 13 °C/sec to 280 °C with a splitless time of 2 minutes. GC oven temperature began at 100 °C and was held for 3 minutes. This was followed by an increase to 170 °C at 30 C°/min, then a ramp at 20 C°/min to a temperature of 250 °C and a final ramp at 7.5 C°/min to 320 °C, which was

held for 9 minutes. Helium flowing at 1.0 mL/min was used as the carrier gas. The MS was operated in electron impact (EI) mode (70 eV) and the mass spectrum was recorded from *m/z* 60 to 400. Compounds were identified using library matches with the NIST MS 2.0 Search Database and by retention time; peak areas were integrated with the Thermo Xcalibur™ Qual Browser (Waltham, MA, USA) and are expressed as percent relative area of those compounds identified. Fatty acids were named following standard convention in the format A:Bn-y; where A denotes the total carbon chain length, B the number of double bonds and y the location of the double bond counted from the methyl end.

3.4.2.6 Statistical Analysis

The difference between the percent relative proportions of 16:1n-7 and 16:0 in the HIP extracted samples and 10:0 and 12:0 in the EA extracted samples were tested for signfigance by a matched pairs t test (α=0.05; one-tailed). The statistical analysis was performed with the Minitab 17 (Minitab Inc., State College, USA) software package.

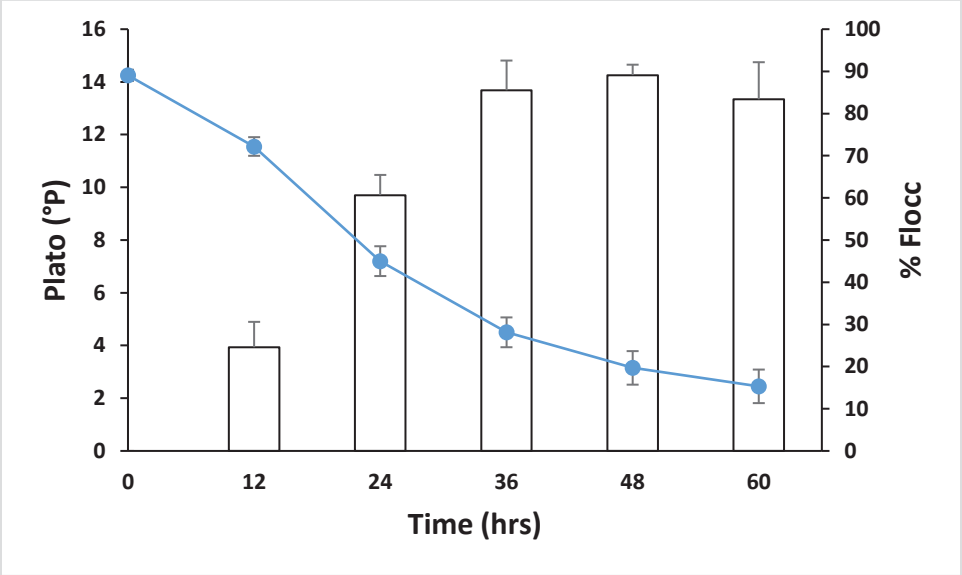
3.5 RESULTS AND DISCUSSIONS

3.5.1 Determination of Cell Surface Hydrophobicity and Flocculation Level of the SMA Strain During Growth in the Miniature Fermentation Assay

The SMA yeast strain exhibited a similar flocculation behaviour (Fig. 3.2A) to another NewFlo strain (LCC125) tested with the ASBC Flocculation Test (ASBC, 2013) in our laboratory, where a maximal flocculation level was not achieved until substantial sugar consumption had occurred (Speers et al., 2006; Jin, 1999). However, the SMA yeast, characterized as a medium flocculent strain, was less flocculent early in the fermentation

than the LCC125 strain previously tested (Speers et al., 2006; Jin, 1999). Similarly, the change in CSH during the fermentation was analogous to the LCC125 strain, but again, CSH values early in the fermentation were comparitively lower (Speers et al., 2006; Jin, 1999). It should be noted that in the earlier studies (Speers et al., 2006; Jin, 1999) the yeast were grown in YEPD broth (200 mL) shaker flasks at 30 °C and 100 rpm and then in tall tube fermenters (1.22 m tall; 18.5 mm inner diameter) filled with 250 mL of wort, and not in the miniature fermentation assay. Despite this discrepancy in fermenter geometry, both NewFlo strains (SMA and LCC125) exhibited overall similar changes in flocculation and CSH behaviours during the fermentations.

A



B

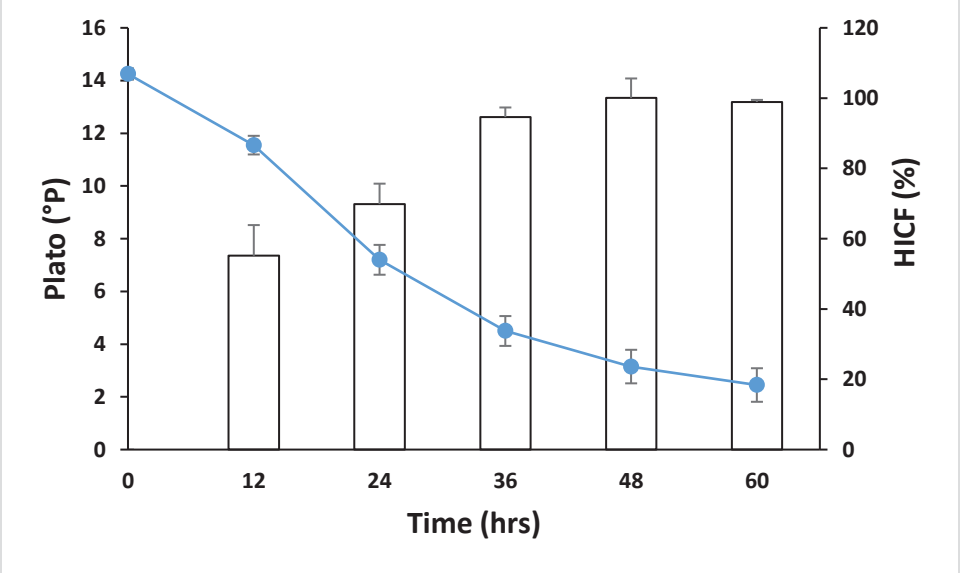


Figure 3.2. Fermentation properties of the SMA yeast strain grown in the miniature fermentation assay (ASBC, 2013). Fermentations were conducted in duplicate and error bars represent one standard deviation from the mean.
3.2A. Flocculation level measured with the ASBC Flocculation Test (ASBC, 2013) (bar graph) and fermentation progress measured on the Plato (°P) scale (line).
3.2B. CSH determined by the method of Akiyama-Jibiki et al. (1997) (bar graph) and fermentation progress measured on the Plato (°P) scale (line).

3.5.2 Hydrophobicity Studies

3.5.2.1 Free Fatty Acid Content Over the Course of the Miniature Fermentation with a Hexane-isopropanol Extraction and Trimethylsilyl Derivatization

In the GC method of Molee et al. (2005), neutral lipids, including monoglycerides, diglycerides, triglycerides, FFA and cholesterol were separated and quantified. In this study, when the trimethylsilylated products of the HIP extract were analyzed 10:0, dodecanoic (12:0), palmitoleic (16:1), palmitic (16:0), oleic (18:1n-9) and stearic (18:0) acids were identified (Fig. 3.3). Ergosterol and squalene were also tentatively identified based solely on spectral matches with the NIST library (Fig. 3.3) but are excluded from the data portrayed in Figure 3.4. These results differ slightly from those of Strauss et al. (2004) who did not detect 10:0 and 12:0 in a NewFlo strain; however, those authors employed a transesterfication approach which does not derivatize FFA. Note that while traces of 3-OH 10:0 were evident (Fig. 3.3; RT 6.03) based on the presence of the diagnostic m/z 233 fragment, a retention time match to the authentic 3-OH 10:0 standard and a positive match with the NIST library search, the peak areas in the total ion chromatogram trace were too small to accurately integrate and/or quantify in relative terms (Appendix A).

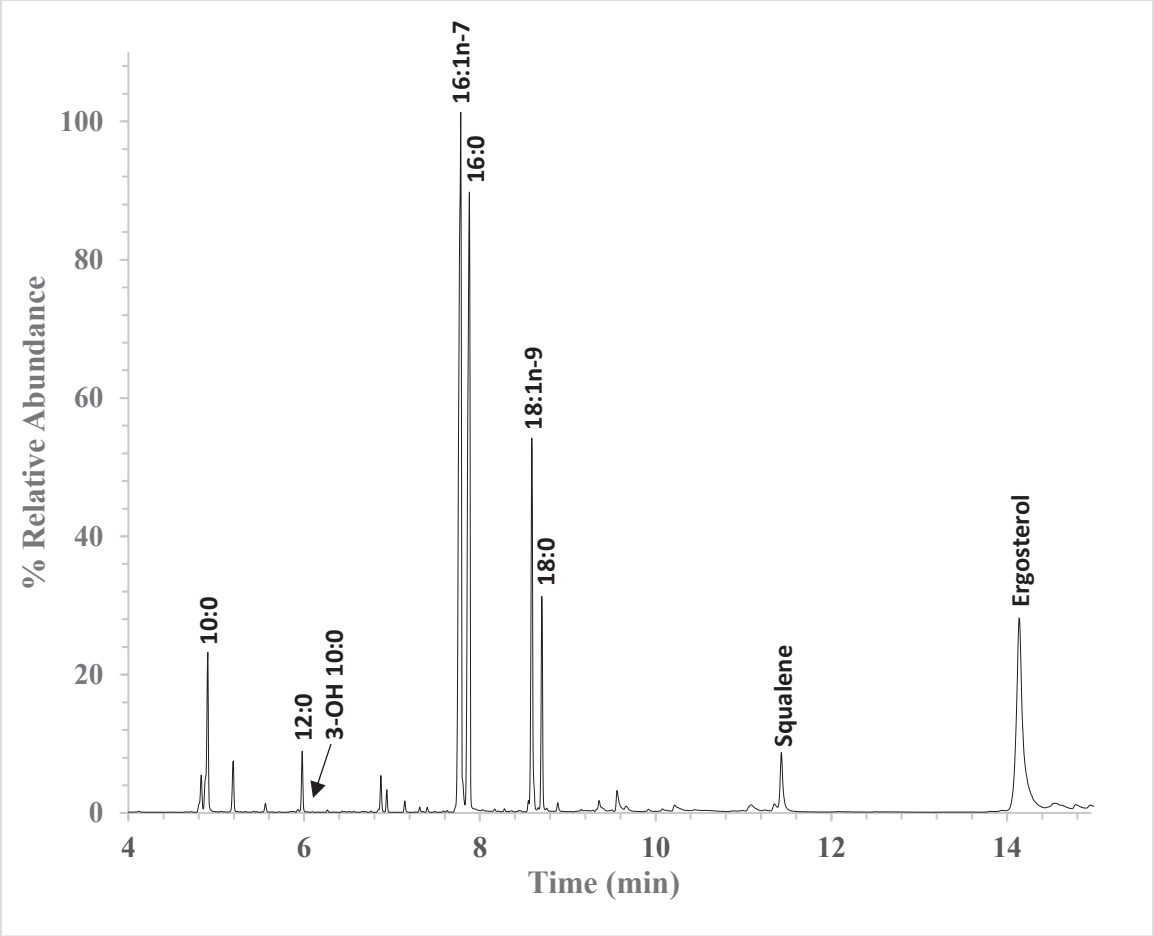


Figure 3.3. A chromatogram showing separation of neutral lipids extracted from the SMA strain with a HIP extraction and derivatized as TMS esters, except for squalene. Neutral lipids detected (and their retention time) included 10:0 (4.91), 12:0 (5.98), 16:1 (7.78), 16:0 (7.88), 18:1n-9 (8.59), 18:0 (8.70), squalene (11.43) and ergosterol (14.14). The elution time of 3-OH 10:0 (6.03) is indicated by a black arrow.

At all times, 16:1 and 16:0 were the predominant fatty acids; however, their relative proportions changed over the fermentation in a noticeable, but non-signifigant ($p>0.05$) manner (Fig. 3.4). Early in the fermentation 16:1 and 16:0 were present in similar relative amounts, while at 24 hours 16:1 was less abundant and from 36 hours onwards 16:1 was present in greater relative amounts. Interestingly, this flux corresponds to the onset of flocculation where flocculation of the SMA strain reached near maximal levels by 36 hours fermentation time (Fig. 3.2A) and could implicate the proportion of

16:1/16:0 as a marker of flocculence. Strauss et al. (2004) also noted that the level of 16:1 decreased to a minimum as the cells approached stationary phase and increased thereafter but also could not offer any explanation for this phenomenon.

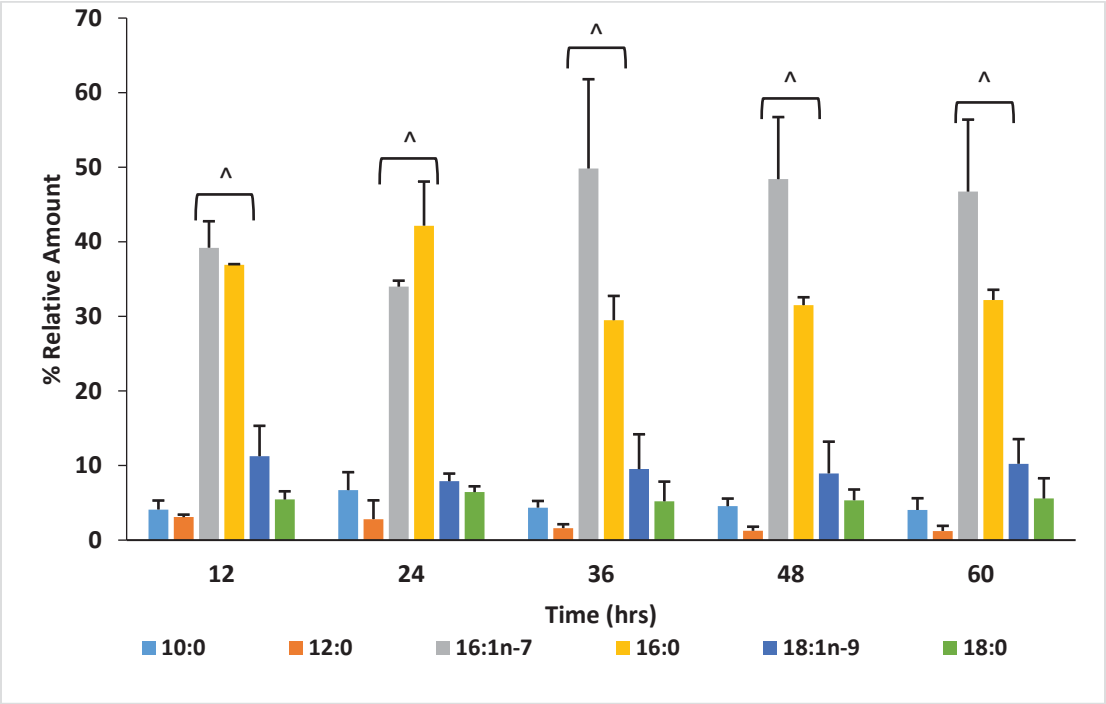


Figure 3.4. Relative amounts of FFA in the SMA yeast strain during growth in the miniature fermentation at 12, 24, 36, 48 and 60 hours growth time. Lipids were extracted from the yeast pellets with a HIP extraction. Fermentations and measurements were conducted in duplicate and error bars represent one standard deviation from the mean. ^ mean differences not statistically signifigant ($p>0.05$)

3.5.2.2 Fatty Acid and 3-OH Oxylipin Content Over the Course of the Miniature Fermentation with an Ethyl Acetate Extraction and Trimethylsilyl Derivatization

In a previous communication (Potter et al., 2013) we reported that we were unable to detect 3-OH oxylipins in the SMA yeast strain using a HIP extraction and an acid-catalyzed transesterification. This method results in the formation of methyl esters of both free and esterified fatty acids. Kock et al. (2013) subsequently suggested we employ an approach that targeted FFA specifically to find 3-OH oxylipins. In earlier works by

these authors and coworkers (Kock et al., 2000; Strauss et al., 2005), they utilized a two-step derivatization where 3-OH oxylipins were first methylated at the carboxylic acid functional group with diazomethane and then trimethylsilylated at the β-hydroxyl group with an appropriate reagent. While this technique has been used in numerous earlier applications, diazomethane has increasingly fallen into disuse for safety reasons. Interestingly, there is also a suggestion it may result in partial transesterification of glycerol esters (Ord and Bamford, 1967). Instead, we elected to trimethylsilylate the total lipid extract which would add a TMS group to the carboxylic acid of FFA and to β-hydroxyl groups of any 3-OH oxylipins present. This derivatization technique has the advantage of being unreactive with esterified fatty acids; thus, only FFA and other structures bearing a hydroxyl group will undergo reaction. Trimethylsilylation has been used by previous researchers to resolve FFA from other lipid species (Myher & Kuksis, 1984), with the drawbacks that TMS esters and ethers do not store well and are prone to hydrolysis (Kuksis et al., 1976).

When lipids were extracted from the SMA yeast strain at 12, 24, 36, 48 and 60 hours growth with an ethyl acetate extraction and trimethylsilylated as described, 3-OH 10:0 was detected. In other studies investigating 3-OH oxylipins in yeasts, methylation and methylation plus trimethylsilylation produced characteristic spectral peaks in GC-MS upon fragmentation at the β-carbon (Table 3.1). This fragmentation pattern was also observed in this study and we identified a spectral peak at m/z 233 as indicative of 3-OH oxylipins with two TMS moieties. The structure of the fragments arising from cleavage at the β-carbon and other general fragments from yeast 3-OH oxylipin GC-MS studies shown in Table 3.1 are illustrated in Figure 3.5. At 12 hours fermentation there were

chromatographic peaks that eluted at the retention time of 3-OH 10:0, but the peak area was too small to be accurately integrated (Fig. 3.6). The onset then of detectable relative amounts (as integratable chromatographic peaks) of 3-OH 10:0 at 24 hours fermentation was 12 hours before elevated flocculation levels were achieved (Fig. 3.2A). This observation could imply these compounds play some role in brewing yeast flocculation, as has been suggested by both Kock et al. (2000) and Strauss et al. (2005).

Table 3.1. Past research on 3-OH oxylipins in yeasts and the diagnostic peaks produced in GC-MS by fragmentation at the β-carbon.

Structure	Authors	Derivatization at Carboxyl End	Derivatization at β-hydroxyl group	m/z of Characteristic Fragment	m/z of Base Peak
3-OH 16:0	Vesonder et al. (1968)	Methylation	None	103	103
3-HETE	van Dyk et al. (1991)	Methylation	None	103	103
3-HETE	Van Dyk et al. (1991)	Methylation	Trimethylsilylation	175	175
3-OH 8:0	Strauss et al. (2005)	Methylation	Trimethylsilylation	175	231
3-OH 10:0	Straus et al. (2005)	Methylation	Trimethylsilylation	175	73
3-OH 10:0	Current Study	Trimethylsilylation	Trimethylsilylation	233	147

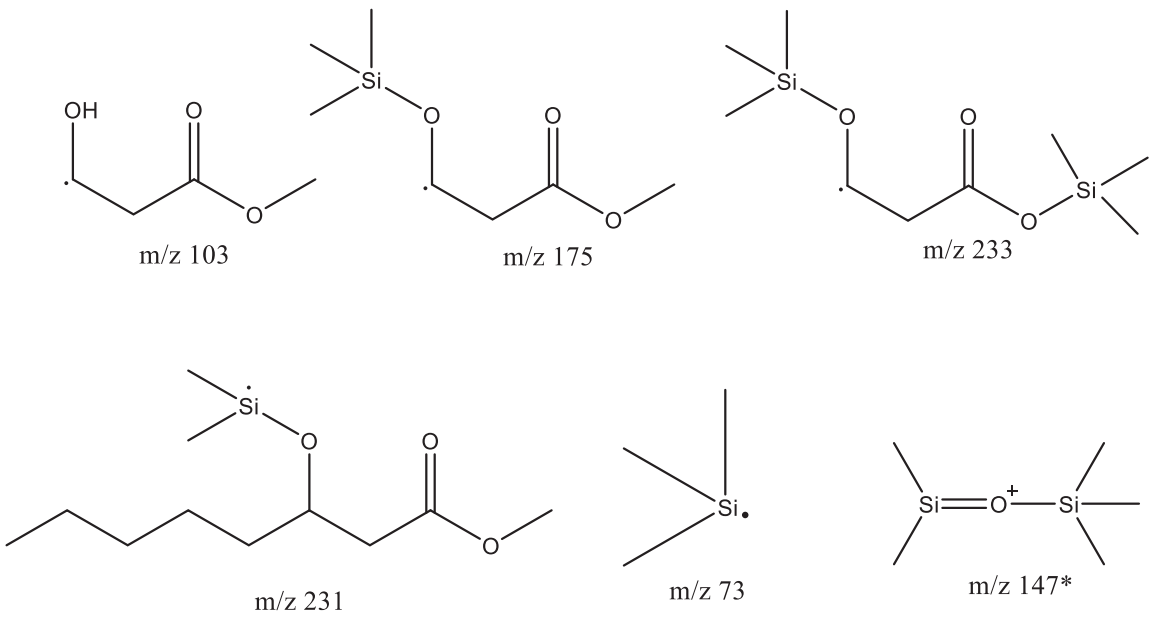


Figure 3.5. Structure of the characteristic fragments produced by cleavage at the β-carbon and other notable fragments that have occurred in the current and previous yeast GC-MS 3-OH oxylipin studies. *As predicted by McCloskey et al. (1968).

In our previous letter (Potter et al., 2013) we had hypothesized that 3-OH oxylipins that formed at the cell exterior might have increased the CSH leading to increased flocculence. Indeed, other authors have suggested that these short-chained hydroxylated fatty acids on the cell surface could impart a hydrophobic effect (Leeuw et al., 2006). Yet, parts of this work would indicate that these short-chained 3-OH oxylipins, such as 3-OH 10:0, may be more amphiphilic than hydrophobic, particularly as free molecules not associated with the cell. In this study, the 3-OH 10:0 standard was not soluble in non-polar hexane, and required a solvent of more intermediate polarity, such as chloroform, ethanol or methanol, for dissolution. Similarly, we noted poor recovery of 50 µg 3-OH 10:0 in the HIP extraction (0.27 ± 0.18 %) when recovery experiments were performed. Furthermore, with HIP extracted SMA cells we could obtain positive mass spectral identifications for 3-OH 10:0 (m/z 233) but the chromatographic peaks were so minimal they could not be accurately integrated.

Other researchers who have used a HIP extraction to detect oxylipins in plant material reported that jasmonic acid (JA), a 12 carbon fatty acid containing a pentane ring and carbonyl functionality, partially partitioned into the lower isopropanol layer in addition to the upper hexane-rich layer with the majority of other lipids (Personal communication, Dr. Ivo Feussner, University of Goettingen), preventing their complete extraction. Thus, the extraction efficiency of these compounds was reduced. Chappell et al. (2004) also reported that the use of n-hexane as the extraction solvent gave low recoveries of *gamma*-hydroxy butyric acid during a liquid-liquid extraction, further supporting the idea of polar oxylipins and their minimal role in determining CSH.

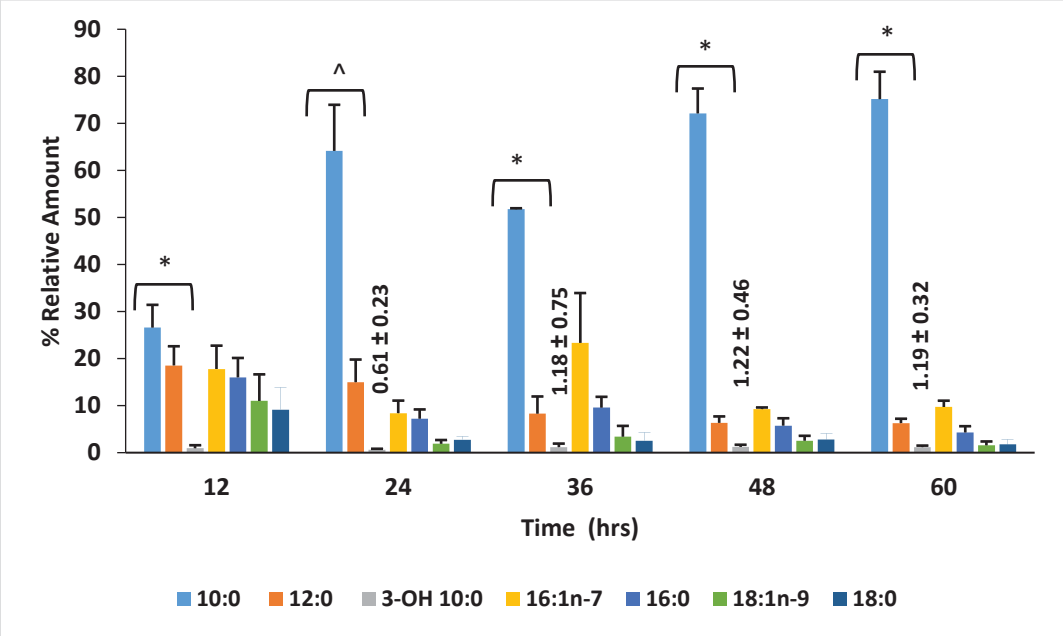


Figure 3.6. Relative amounts of FFA in the SMA yeast strain during growth in the miniature fermentation at 12, 24, 36, 48 and 60 hours growth time. Lipids were removed from the yeast pellets with an ethyl acetate extraction. Fermentations and measurements were conducted in duplicate and error bars represent one standard deviation from the mean. ^mean differences not stastically signifigant ($p>0.05$) *mean differences statistically signifigant ($p<0.05$)

In an effort to demonstrate the validity of the approach employed in this study, it is important to emphasize that the methods described are an amalgam of previously published methods. In the Kock et al. (2013) response to our initial 3-OH oxylipin communication (Potter et al., 2013), they suggested we specifically target free unesterified oxylipins using any of the methods described by Barrow and Taylor (1987). In the work of Barrow and Taylor (1987) they highlight ethyl acetate as the solvent of choice for conventional organic extractions of prostaglandins. Indeed, Kock and co-workers have used a double ethyl acetate extraction to detect oxylipins in yeast in earlier studies (Strauss et al. 2005; Leeuw et al., 2006). We modelled our extraction on the work of Kock and co-workers except for several deviations.

Leeuw et al. (2006) used large volumes of ethyl acetate. However, we had previously found that lipids were suitably extracted from 0.5 g yeast by 20 mL of hexane-isopropanol, which is in fact the ratio of sample-to-solvent suggested in the seminal Folch et al. (1957) paper. Therefore, we elected to use 0.5 g of yeast, 10 mL of water and two 10 mL volumes of ethyl acetate. Additionally, Strauss et al. (2005) cited a work that references the prostaglandin extraction method of Salmon and Flower (1982). In this method, these authors precipitated proteins and other molecules by the addition of cold acetone before removing the neutral lipids from the sample with petroleum ether. We were not concerned with removing neutral lipids from our samples, but did have some emulsions form in our sample interfaces so we used the cold acetone to disperse the protein and other molecules in the emulsion. As well, Salmon and Flower (1982) wrote that emulsion formation could be mitigated by the addition of a saturating amount of NaCl, which is why we incorporated this step in our procedure.

As we mentioned, earlier authors had derivatized yeast oxylipins with diazomethane or with a combination of diazomethane and a reagent that adds a trimethylsilyl moiety to the OH group. However, we elected to use a trimethylsilyating reagent only. The only potential problem with the trimethylsilylation method is hydrolysis due to the presence of water in the extract (Barrow & Taylor, 1987). With this in mind, we thoroughly dried our ethyl acetate extracts with exposure to anhydrous Na_2SO_4 for 20 minutes. The dried lipid extract in solvent was then transferred to a new tube and the solvent removed with streaming nitrogen before carrying out the TMS derivatization.

Our GC run conditions were also not novel. They were adapted from a protocol described by Molee et al. (2005) to resolve silylated neutral lipids, including free fatty acids, on a non-polar column. The method of Molee et al. (2005) was itself also developed from the published method of Myher and Kuksis (1984). GC-MS analysis also offers the advantage of establishing peak purity through analysis of the mass spectra. Thus, we are confident that co-elution of 3-OH 10:0 with other lipids did not occur and our data reliable.

Given that the short-chained 3-OH oxylipins may not be particularly hydrophobic, there are several other reports in the literature which could provide insight as to their function in *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* flocculation. Specifically, oxylipins have been implicated as potent mediators of quorum sensing in the fungi *Candia albicans* (Nigam et al., 2011) and *Aspergillus nidulans* (Affeldt et al., 2012). With this in mind we also monitored the level of flocculation in a 15 mL test tube of a 24 hour shaker flask culture of the SMA strain in the presence of 25 µg/mL 3-OH

10:0, a concentration in excess of those used by Nigam et al. (2011) and Affeldt et al. (2012). It was notable that the rate of sedimentation in the tube with 3-OH 10:0 did not differ from a control between 0 and 60 minutes, monitored at 5 minute intervals. However, in future work the expression of flocculation controlling FLO genes should be monitored during exposure to 3-OH 10:0 as Nigam et al. (2011) noted an up-regulation of gene transcripts in *Candida albicans* within 30 minutes subjection to a 3-OH oxylipin.

In an earlier study, Lafon-Lafourcade et al. (1984) noted the production of 8:0 and 10:0 during wine must fermentation. These authors demonstrated that cell counts were reduced by the addition of these acids to the fermenting medium. Interestingly, they showed that the addition of cell ghosts to the ferment reduced the concentration of these short-chained fatty acids and increased the cell count. This suggests that accumulation of 8:0 and 10:0 (and their 3-OH oxylipins) in the extracellular environment in threshold concentrations could have an inhibitory effect. It is notable that in this study relative proportions of 10:0 increased and accumulated at signifigant levels ($p<0.05$) over the fermentation course (Fig. 3.6). Whether this is an inhibitory effect or some unknown function, in this work we were unable to conclusively demonstrate that oxylipins play a particular role in brewing yeast flocculation. However, this study does present a defined ethyl acetate extraction and GC-MS detection protocol for 3-OH oxylipins in *Saccharomyces pastorianus*. We believe that these methods, in conjunction with approaches to more specifically probe the cell wall architecture, will help to better understand the specific role of 3-OH oxylipins in brewing yeast flocculation.

3.6 CONCLUSIONS

Earlier studies of 3-OH oxylipins in *Saccharomyces cerevisiae* successfully detected these compounds by methylation with diazomethane or by diazomethane methylation followed by trimethylsiylation. Due to the danger and scarcity of diazomethane, other derivatization approaches were considered. In this work, 3-OH oxylipins were detected in the SMA strain of *Saccharomyces pastorianus* with an ethyl acetate extraction, trimethylsiylation of the total lipid extract and with attention to a diagnostic m/z 232 fragment. Previous reports by our lab hypothesized that 3-OH oxylipin formation at the onset of flocculation may increase CSH. However, this study suggested free 3-OH 10:0 in particular is not that hydrophobic. To understand the precise role of 3-OH oxylipins in brewing yeast flocculation, the methods we present must be combined with novel techniques to target the cell wall architecture.

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CHAPTER 4 BEYOND DIAZOMETHANE: ALTERNATIVE APPROACHES TO ANALYZING NON-ESTERIFIED FATTY ACIDS

4.1 PUBLICATION INFORMATION

This chapter is a modified version of a manuscript, reworked here for consistency. Originally published as:

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Greg Potter devised the review concept and was the lead author. Suzanne Budge and Alex Speers contributed to manuscript preparation.

4.2 ABSTRACT

In many branches of lipid science, researchers are interested in NEFA content and composition. For many years, diazomethane was the reagent of choice to selectively derivatize and then detect NEFA due to its highly specific methylation of the carboxylic acid functional group. While the activity of this derivatizing reagent is very defined, it is dangerous and can be difficult to obtain. In this brief review, we have compiled a collection of methods which allow for the detection of NEFA and hydroxy NEFA without the use of diazomethane. We have concentrated on methods that employ three distinct approaches of selective quantification/extraction, purification from total lipids and derivatization techniques.

4.3 INTRODUCTION

Diazomethane has been utilized by lipid analysts for many years as a highly effective and selective means of derivatizing NEFA to their corresponding methyl esters for subsequent analysis by GC (Christie, 1982). However, the use of this reagent poses several serious risks to the analyst, primarily due to its toxic and explosive nature. Researchers have been aware of these risks for some time and the first report of diazomethane poisoning dates back to 1938 (Sunderman et al., 1938). During recent research efforts we had hoped to identify short-chained hydroxylated NEFA in *Saccharomyces pastorianus* which earlier studies had achieved with diazomethane (Vesonder et al., 1968; Van Dyk et al., 1991). In light of the danger and scarcity of diazomethane, we sought out and evaluated alternative means to analyze these hydroxylated NEFA. It quickly became apparent that while other methods existed within the literature, there was not a body of work which aggregated these methods into one succinct review. Thus, the goal of this short review is to explore and report on methods that provide an alternative to diazomethane and allow for selective quantification/extraction, purification and derivatization of NEFA, with a particular focus on short-chained hydroxylated NEFA. In particular, this review focuses on traditional methods for lipid extraction, separation and GC/GC-MS analysis, but the techniques highlighted are not exhaustive. Nonetheless, is our hope that this report and the methods and approaches we highlight herein will aid future researchers and analysts in their detection of NEFA and hydroxy-NEFA.

4.4 SELECTIVE QUANTIFICATION AND EXTRACTION

4.4.1 Summary

Diazomethane is particularly useful for FFA detection largely because this compound will only react with the free carboxyl end of NEFA, leaving esterified fatty acids intact. An alternative approach to diazomethane use is to employ a selective quantification or selective extraction scheme. In the literature we found numerous such methods and have grouped them as general selective quantification/extraction methods involving a base (e.g., Dole, 1956; Schotz et al., 1970, Hušek et al., 2002), a reportedly true selective extraction (Höckel et al., 1980) and selective extractions involving quaternary ammonium salts (e.g., MacGee & Allen, 1974; Kishiro & Yasuda, 1988; Williams & MacGee, 1983). We then go on to discuss extraction of short-chained hydroxylated NEFA, and describe the method we used in our work (Potter et al., 2015). Other extraction methods outside the scope of this review are described elsewhere (Wu et al., 2016; Pati et al., 2016).

The carboxyl functional group at the head of NEFA is a convenient target to selectively quantify and extract these molecules. Due to the acidic nature of the carboxyl group, NEFA can be quantified by titration with alkali. Similarly, NEFA may be selectively extracted by harnessing their altered solubility in organic or aqueous solvents upon exposure to acid or base. A number of methods have achieved selective quantification and extraction with serum samples (Dole, 1956; Schotz et al., 1970, Hušek et al., 2002) and each follows the same series of preliminary steps. NEFA are first acidified to 1) ensure protonation of the carboxyl functional group and they are then 2) extracted into a suitable organic solvent. Thereafter, the NEFA can be quantified in the organic layer by titration with alkali or can be further extracted and concentrated from the

solvent using base deprotonation to yield salts of the NEFA in an aqueous layer. For instance, Dole et. al. (1956) presented an early alkali titration option for selective quantification of NEFA in serum where the sulphuric acid acidified samples were first extracted using heptane, and were then titrated with NaOH and a thymol blue indicator. This quantification scheme was found to be quite selective for NEFA with less than 2% co-extraction of other organic acids commonly found in biological samples.

Another selective extraction approach that used hexane as the solvent and KOH to deprotonate NEFA also added spikes of radioactively-labelled triolein (Schotz et al., 1970). By adding the triolein, Schotz et al. (1970) demonstrated that there was less than 0.1 % carryover of the labelled triacylglycerol into the NEFA concentrate, which implied quantitative extraction of long chain NEFA. Hušek et al. (2002) described a second selective extraction method using oxalic acid, isooctane and KOH and reported that some polar phospholipids were co-extracted in their system. However, they were able to confirm that none of the NEFA later detected resulted from hydrolysis of the co-extracted phospholipids. Any similar method where salts of NEFA are recovered in aqueous base with minimal interference from other lipid classes may employ a final acidification step to reform NEFA. This pure fraction of NEFA can then be analyzed by GC, in the derivatized or underivatized form, to determine their structures and concentrations.

There is a single report of a purportedly true NEFA-specific extraction described by Höckel et al. (1980). This method used a 1:1 mixture of n-heptane:chloroform with 2 % methanol to extract NEFA from serum or plasma in a phosphate buffer at pH 6.4. The resulting extractant contained principally NEFA; cholesterol esters, triacylglycerols and phospholipids were co-extracted but only in trace amounts. Quantitative recovery of

NEFA ranging in lengths from lauric (12:0) to lignoceric (24:0) was demonstrated, indicating that the method is suitable for both medium and long-chain NEFA. While applicable to serum samples, this NEFA-specific extraction method has not been validated with other sample types that may contain more complex lipid mixtures.

Basic solutions of quaternary ammonium salts can also be used to selectively extract NEFA. As with the aforementioned protocols involving basic aqueous solutions, basic quaternary ammonium salts contain hydroxide ions and are able to deprotonate the carboxyl group of NEFA (Fig. 4.1a.), thus concentrating NEFA from the organic extraction solvent. These quaternary ammonium salts are particularly useful because they allow for direct pyrolytic derivatization of NEFA in a hot injector of a GC (Fig. 4.1b). A number of basic quaternary ammonium salt preparations have been employed including trimethyl (α,α,α-trifluoro-m-tolyl) ammonium hydroxide (TMTFTH) and trimethylphenylammonium hydroxide (TMPH). These salts have facilitated selective extraction of NEFA from a variety of starting materials including serum, tissue samples and edible oils, demonstrating their versatility and utility in numerous different applications (Table 4.1).

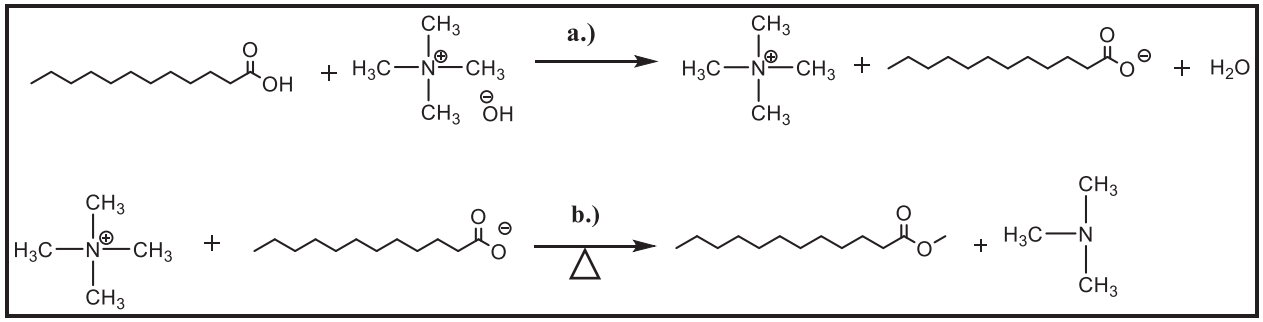

Figure 4.1. Chemical reactions that occur during selective extraction of NEFA using a quaternary ammonium salt (a) and the later pyrolytic derivatization when the mixture is placed in the hot injection port of a GC (b).

MacGee and Allen (1974) reported on such a selective extraction approach to isolate NEFA from serum samples with TMTFTH. These authors found that their method was rapid and suggested that food samples may also be analyzed for NEFA so long as appropriately small initial samples were used. Kishiro and Yasuda (1988) also used TMTFTH to detect free arachidonic acid (20:4) from Folch-extracted brain tissue with little co-extraction of triacylgylcerols, phospholipids and cholesterol esters. Furthermore, these authors noted that the method was highly sensitive and was likely useful with other applications where NEFA must be removed from complex tissue matrices. Williams and MacGee (1983) later developed a similar protocol to remove NEFA from vegetable oil containing no more than 5 % FFA with TMPH. These authors reported that the method was quantitative and could extract saturated and unsaturated NEFA ranging in length from dodecanoic (12:0) to linoleic (18:2) acids.

While the previously described methods were undoubtedly suitable ones for NEFA, our recent study (Potter et al., 2015) found that a hexane-based extraction was inappropriate for hydroxylated short-chained NEFA. When a HIP extraction solvent was applied to yeast cells, we noted minimal chromatographic peaks that could not be accurately integrated at the 3-OH 10:0 retention time. Furthermore, we achieved very poor extraction efficiency (0.26 ± 0.18 %) when recovery studies were performed with 50 µg of an authentic 3-OH 10:0 standard in the HIP extraction. To achieve better recovery of 3-OH 10:0 from our material we utilized an extraction solvent of intermediate polarity, ethyl acetate. This extraction approach does not necessarily exclusively remove short-chained hydroxylated NEFA from the material; however, it is an important preliminary step in their quantitative recovery.

In our ethyl acetate extraction procedure, the material was acidified to pH 3.5-4.5 to protonate the NEFA and ensure their recovery in the organic extraction solution (Salmon & Flower, 1982). A saturating amount of NaCl was also added to the mixture to reduce emulsion formation (Salmon & Flower, 1982). The 3-OH 10:0 and other lipids were then double extracted with successive aliquots of ethyl acetate. When recovery studies were performed using 50 μg of authentic 3-OH 10:0 and 15:0 standards in this extraction, extraction efficiencies were 106 % and 77 %, respectively. In another protocol designed to extract similarly polar compounds from an aqueous matrix (Rompa et al., 2005), alkanocarboxylic herbicides were best extracted with ethyl acetate as the extractant and 4 % sodium sulphate as a salting agent. Thus, the addition of NaCl to our extraction medium also likely improved recovery of 3-OH 10:0 by a "salting out" effect. A summary of our extraction procedure, and all those previously described, are presented in Table 4.1.

The work of Chappell et al. (2004) also revealed pertinent information regarding extraction of short-chained hydroxy-fatty acids, particularly hydroxy-butyric acid (hydroxy 4:0). After testing the recovery of γ-hydroxy butyric acid (γ-hydroxy 4:0) in various organic solvents during a liquid-liquid extraction, they found that those with an oxygen-containing functional group, including methyl acetate and ethyl acetate, were most effective. They attributed this effectiveness to the hydrogen-bonding potential of the hydroxyl and the carbonyl groups. Interestingly, these authors also found that *n*-hexane achieved very little extraction of hydroxy-butyric acid (hydroxy 4:0) and that extraction efficiency was improved considerably when a saturating amount of salt was added to the extraction medium (Chappell et al., 2004).

Table 4.1. A summary of the selective quantification and extraction methods described.

Type	Applications	Advantages	Disadvantages	References
a	-Serum containing all chain lengths of NEFA	-Ease of removal of lipid-containing organic phase -No evaporation step	-Tedious titration required	(Dole, 1956)
b	-Serum with long-chain NEFA	-Quantitative for long-chain NEFA	-Less ideal for short-chain NEFA	(Schotz et al., 1970)
b	-Serum with NEFA prone to breakdown	-Rapid	-Co-extraction of phospholipids	(Hušek et al., 2002)
c	-Serum with medium and long-chain NEFA	-Supposedly, truly selective for NEFA	-Timely concentration step required -Other lipid species co-extracted	(Höckel et al., 1980)
d	-Serum and food with NEFA prone to breakdown	-Rapid	-TMTFTH solution laborious to prepare	(MacGee & Allen, 1974)
d	-Tissue samples with long-chain PUFA	-Sensitive and quantitative	-N/A	(Kishiro & Yasuda, 1988)
d	-Edible oils with medium and long-chain PUFA	-Rapid and quantitative	-Only validated with ≤ 5 concentrations % NEFA	(Williams & MacGee, 1983)
e	-Yeast and other microbiological samples containing hydroxy lipids	-Simple	-Slow evaporation of ethyl acetate	(Potter et al., 2015)

a-Selective quantification involving a base; b-Selective extractions involving a base; c-True NEFA selective extraction; d-Selective extractions with quaternary ammonium salts; e-Extraction for hydroxy-NEFA

4.5 PURIFICATION OF NEFA FROM TOTAL LIPID EXTRACTS

4.5.1 Summary

NEFA can also be isolated by purifying total lipid extracts. Here, we present two approaches to purify NEFA by each of thin-layer chromatography (Dudzinkski, 1967; Rehncrona et al., 1982) and solid-phase extraction (Notter et al., 2008; Paik et al., 2008). In the solid-phase extractions, we describe one conventional and one static method. Other purification approaches are described in the literature (Wu et al., 2016; Pati et al., 2016).

One common and extensively used means to separate NEFA from total lipid extracts is thin layer chromatography (TLC) and many early and recent authors have described protocols to achieve this separation (Schlierf & Wood, 1965; Louis-Ferdinand et al., 1967; Blank, 1970; Nedelchova et al., 2007; Massart et al., 2014). Herein, we describe two representative methods where NEFA separation was accomplished with slightly different proportions of petroleum ether, diethyl ether and acetic acid (Table 4.2). These techniques should allow analysts to purify NEFA from a variety of sample types. In the first report, Dudzinski (1967) noted, unexpectedly, that FFA on a TLC plate could be selectively detected as a “rose-violet spot” on a “pale yellow background” when the spray reagents 2’,7’-dichlorofluoroscein, alcoholic aluminium chloride and acid ferric chloride were used together. Additionally, Dudzinski (1967) reported this method was widely applicable as it could purify short, medium and long-chain as well as saturated, monounsaturated and polyunsaturated NEFA. However, one drawback of this method is that alkaline reagents in the developing solvent interfered with colour development of the spray reagents.

Rehncrona et al. (1982) describe a TLC method to detect NEFA from Folch-extracted brain tissues. These authors noted that their method was able to sufficiently separate saturated (palmitic (16:0), stearic (18:0)), monoenoic (oleic (18:1)) and polyenoic (arachidonic (20:4), docohexaenoic (22:6)) NEFA, making this protocol better suited to the analysis of long-chain NEFA than that of Dudzinski (1967). However, the visualization reagents used in this method lack the specificity of the Dudzinski (1967) approach, and genuine NEFA standards must be employed when identifying the NEFA band on the TLC plate.

Although frequently used, TLC does have drawbacks in that sizeable samples may be required, prep times can be long and recoveries are often low (Allen et al., 1984). As an alternative, other chromatography approaches have been developed, with solid-phase extraction (SPE) among the most simple and convenient. In many SPE applications for purification of lipid extracts, the samples are run through pre-packed cartridges that can be eluted under gravity or on a vacuum manifold. Notter et al. (2008) describe such an approach to isolate NEFA from lipids recovered from samples of wet porcine adipose tissue. These authors described their approach as rapid, simple and reproducible; however, they reported that in some instances there was co-elution of non-NEFA molecules in the NEFA fraction. Therefore, this purification technique must be used with caution if a sample free of contaminants is required. Summaries of each purification approach are presented in Table 4.2.

An alternative approach to the use of pre-packed cartridges is to carry out static SPE; this has the distinct the advantage of being more affordable when a large number of samples must be processed (Paik et al., 2008). For instance, Paik et al. (2008) have

developed such a simple and rapid static SPE method for purification of NEFA from triacylglycerol in lyophilized algal samples. In their approach, lipids recovered with a modified Bligh and Dyer (1959) extraction were added to sodium carbonate that had been pre-wetted with 0.1 M KOH to promote deprotonation of the NEFA and strong adsorption to the sodium carbonate. Following elution of neutral lipids, NEFA that were still adsorbed to the sodium carbonate were recovered by 1) acidification to protonate NEFA; and then 2) extraction with solvent. During method validation, Paik et al. (2008) found that they could obtain at least 85% recovery, and usually more, of fatty acids ranging in length from caprylic (8:0) to behenic (22:0) acids. Furthermore, phospholipids in their initial sample did not interfere with NEFA recovery. However, these authors did not confirm the selectivity of their method for NEFA in the presence of lipid species other than triacylglycerols and phospholipids.

Table 4.2. A summary of the purification methods described.

Type	Development/Elution Solvent	Applications	Advantages	Disadvantages	References
TLC	-Petroleum ether:diethyl ether:acetic acid (80:20:1 v/v/v)	-Short to long-chain saturated and unsaturated NEFA purification	-Visualization reagent completely selective for NEFA	-Alkaline development reagents interfere with specificity	(Dudzinski, 1967)
TLC	-Light petroleum:diethyl ether:acetic acid (55:45:2 v/v/v)	-Purification of NEFA from tissue matrices	-Better suited to long-chain PUFA then Dudzinksi (1967) method	-Standards needed for NEFA identification	(Rehncrona et al., 1982)
SPE	-Diethyl ether containing 2 % acetic acid	-Applicable to wet tissue samples	-Rapid and simple	-Co-elution of non-NEFA during elution	(Notter et al., 2008)
SPE	-Dichloromethane:n-hexane (1:4 v/v) following selective adsorption to alkalized sodium carbonate	-Purification of NEFA from triacylglycerols and phospholipids	-Rapid and simple	-Purification not validated for lipid species other than triacylglycerols and phospholipids	(Paik et al., 2008)

4.6 DERIVATIZATION

4.6.1 Summary

Here, we first describe several methods in the literature where derivatization[2] was achieved by placing quaternary ammonium salts of the fatty acids in a hot GC injector. The salts we describe include TMA (Robb & Westbrook, 1963; MacGee & Allen, 1974), TMTFTH (Oakes & Willis, 1972; MacGee & Allen, 1974) and TMPH (Brochmann-Hansen & Oke, 1969; Williams & MacGee, 1982). Then, we mention pyrolytic derivatization techniques that did not use quaternary ammonium salts, but instead used dry salts of the fatty acids and ethyl potassium sulfate (Hunter, 1962). We then highlight several other techniques which used methyl iodide (Dünges,1973, Gehrke & Goerlitz, 1963; Grunert & Bassler, 1973; Allen et al., 1984; Höckel et al., 1980). Later, we describe organic compounds, alkyl chloroformates (Hušek et al., 1990; Hušek et al., 2002) and dimethoxypropane (Tserng et al., 1981), used to derivatize NEFA and how similar compounds have been utilized with hydroxy-NEFA (Vosmann et al., 1996; Hušek, 1993). Then, we comment on several protocols that identified NEFA amongst other lipid species by trimethylsilylating the lipid extract (Kuksis et al., 1969; Kuksis et al., 1976; D'Alonzo et al., 1981) and describe how the diazomethane derivative, trimethylsilyldiazomethane, can be used to esterify NEFA (Presser & Hüfner, 2004). Finally, we explain how we used trimethylsilylation for identification of 3-OH fatty acids (Potter et al., 2015) and then suggest alternative approaches to derivatize these analytes with TBDMS (Mamer et al., 2013) and heptafluorobutyrates (Stan & Schuitwinkel-

[2] Here derivatization is concerned with alteration of the carboxylic acid functionality of non-hydroxy NEFA. When the derivatizaion of 3-OH fatty acids is discussed, this refers to the alteration of both the carboxylic acid and the hydroxyl functionality.

Reich, 1980). It should be noted NEFA and hydroxy-NEFA may be analyzed with methods that do not require derivatization, such as atmospheric pressure chemical ionization LC-MS, and these techniques have been detailed by other authors (Wu et al., 2016; Pati et al., 2016).

Diazomethane remains a powerful reagent for selective derivatization of NEFA, but there are other derivatization tools at the disposal of lipid researchers and analysts. When salts of NEFA are heated with an appropriate reagent, the aforementioned pyrolytic derivatization can occur. This was first reported by Prelog and Picentanida (1936) who observed that when tetramethylammonium (TMA) salts of acids were heated, trimethylamine was driven off and nearly pure methyl esters remained. Robb and Westbrook (1963) later described the methylation of NEFA in a hot injection port of a gas chromatograph with TMA salts and noted that this happened in a near quantitative manner. Overall, this work (Robb & Westbrook, 1963) revealed that TMA can be used to derivatize each of short, medium and long-chain NEFA and short-chain hydroxy NEFA without conversion of other organic acids. However, MacGee and Allen (1974) detailed an important disadvantage of the hydroxide salt of TMA: tetramethylammonium hydroxide (TMAH) caused complete destruction of polyunsaturated fatty acids (PUFA). Recovery of PUFA was improved by adding methyl propionate to the quaternary ammonium hydroxide extracts and the authors postulated that the hydrolysis of the propionate ester in the hot injector neutralized additional alkalinity.

While simple quaternary ammonium salts such as TMA may be used, bulkier salts which pyrolize more readily seem to be favoured. Accordingly, early researchers noted TMTFTH and TMPH were more suitable than TMA because dimethyltrifluorotoluidine

and dimethylaniline were more stable leaving groups than trimethylamine (Oakes & Willis, 1972; Brochmann-Hansen & Oke, 1969). However, like TMAH, these other basic salt preparations require addition of a suitable neutralizing agent to prevent degradation of PUFA. For example, MacGee and Allen (1979) did not detect any degradation of the PUFA linoleic (18:2), linolenic (18:3) and arachidonic acid (20:4) with a small addition of methyl propionate to their TMTFTH mixture. Similarly, methyl acetate was found to be an effective neutralizing reagent to prevent PUFA breakdown during TMPH use (Williams & MacGee, 1982). Despite TMFTH's utility, several authors have opted in favour of TMPH due to its low cost, easy preparation and more uniform pyrolytic derivatization of fatty acids (Williams & MacGee, 1982; Rompa et al., 2004).

We initially considered a selective extraction and pyrolytic derivatization technique similar to that described by Williams and MacGee (1983) to detect 3-OH oxylipins. We assumed that the gas-liquid chromatography conditions of the Williams and MacGee (1983) protocol could be adapted for GC-MS analysis as quaternary ammonium salts have been reported in GC-MS applications (Rompa et al., 2005). However, upon further consultation with experts in the field, we were urged not to inject these non-volatile salts into our system as they would likely be damaging. In fact, we confirmed that Sigma Aldrich, who manufactures a TMPH solution in methanol for GC derivatization, had not validated the use of this reagent with GC-MS. Therefore, strong consideration should be taken before choosing certain quaternary ammonium salts for analysis by GC-MS.

Pyrolytic derivatization has been widely achieved using quaternary ammonium salts of NEFA but pyrolytic derivatization can also been produced with a potassium

salt/NEFA approach (Hunter, 1962). In Hunter's (1962) protocol, dry potassium salts of fatty acids mixed with ethyl potassium sulfate placed in a hot injection port of a GC produced ethyl esters that eluted as sharp, distinct peaks. However, this procedure required a complicated setup where an argon gas source was connected to the injection needle. Summaries of each of the pyrolytic derivatization approaches are presented in Table 4.3.

Table 4.3. A summary of each of the pyrolytic derivatization approaches described.

Reagents	Applications and Advantages	Disadvantages	References
TMA	-Applicable to NEFA of different chain lengths -Can be used for hydroxy-NEFA	-Alkalinity of TMAH completely destroys PUFA	(Robb & Westbrook, 1963)
TMTFTH	-Applicable for long-chain PUFA when methyl propionate co-injected	-Ghost peaks can occur if insufficient TMTFTH used	(MacGee & Allen, 1974)
TMPH	-Easily prepared -Low cost -More uniform derivatization than other salts	-Degradation of PUFA without co-injection of methyl acetate	(Williams & MacGee, 1982; Rompa et al., 2004)
TMSH	-High conversion efficiency to methyl esters -Useful in screening for hydroxy-NEFA in GC-MS	-Low conversion to *O*-methyl ether for hydroxy-NEFA	(Vosmann et al., 1996)[a]
Ethyl potassium sulphate	-Sharp, distinct peaks produced by GC	-Specialized injection technique required	(Hunter, 1962)

[a]-Used in hydroxy-NEFA derivatization

Iodides have also been reported to efficiently derivatize NEFA as these compounds undergo a nucleophilic substitution reaction in the presence of fatty acids, but methyl iodide in particular has been widely used (Dünges, 1973; Kishimoto & Radin, 1959; Kishimoto et al., 1968). Furthermore, methyl iodide represents a true alternative to the specificity of diazomethane when the reaction occurs in a polar aprotic solvent (Hušek et al., 1990) and derivatization of NEFA with this reagent can be quite rapid when conditions are optimized. Some reports have suggested methyl iodide methylation could take upwards of 8 hours (Gehrke & Goerlitz, 1963; Grunert & Bassler, 1973), but Allen et al. (1984) obtained complete methylation of fatty acids in 10 minutes in aqueous KOH at 65 °C with N, N-dimethylacetamide as a catalyst. Hockel et al. (1980) also describe a quick methyl iodide approach where methylation was complete 10 minutes after reflux of a mixture of fatty acids with crown ether (dibenzo-18-crown-6), dried potassium carbonate and methyl iodide. However, considerable care should be taken with the use of this chemical because it is toxic and can be damaging to human health (Gan et al., 2011).

Chloroformates, a class of compounds used in organic chemistry to derivatize amino and hydroxy groups, can also be used to derivatize NEFA. Hušek et al. (1990) developed an approach to prepare methyl esters of NEFA using methyl chloroformate. This method was quantitative and almost instantaneous at room temperature when methyl chloroformate was added to a reaction medium that contained the fatty acids, a pyridine catalyst and the solvents acetonitrile (for non-aqueous systems) or acetonitrile-water-methanol (for aqueous systems). Several of the same authors (Hušek et al., 2002) developed a three-step procedure for selective extraction and subsequent methylation of

NEFA in serum, using 4-dimethylaminopyridine (DMAP) as a catalyst in place of pyridine. With this method, >95% of NEFA in the sample were methylated, and carryover of the DMAP into the sample did not have an impact on quantitation

Provided the proper reaction conditions are used, dimethoxypropane (DMP), like methyl iodide, offers an alternative to the selectivity of diazomethane. Research has verified that this compound will derivatize NEFA while intact, esterified acyl lipids are unaffected. For instance, Tserng et al. (1981) developed a NEFA-specific derivitzation technique by adding 2,2-DMP and hydrogen chloride to serum samples. In this system the DMP was multi-functional and acted as a water-scavenger, a protein precipitant and a source of methyl groups for NEFA methylation. .

Other researchers have attempted to methylate hydroxy NEFA at the carboxyl and hydroxyl functional groups with mixed success. Vosmann et al. (1996) pyrolytically derivatized these compounds with trimethylsulfonium hydroxide (TMSH) in a hot injector. The group noted complete conversion to the corresponding methyl esters but only around 3-5 % conversion to *O*-methyl ethers. Hušek (1993) also assessed whether methyl chloroformate reagents could be applied to hydroxy fatty acids. Similarly, Hušek (1993) noted *O*-methoxycarbonyl methyl esters were the main products with certain hydroxy fatty acid standards, but also reported side-chain reaction products that included lactones and inter-ester oligomers. Overall, derivatization with TMSH and methyl chloroformate are likely suitable derivatizing reagents to screen for hydroxy-NEFA in GC-MS applications, but they are not ideal for quantitative analysis. The key features of methyl chlorformates and other non-pyrolytic derivatization compounds are described in Table 4.4.

Table 4.4. A summary of the non-pyrolytic derivatization reagents described.

Reagents	Applications and Advantages	Disadvantages	References
Methyl iodide	-Fast with appropriate catalyst -True alternative to diazomethane -Simpler than other NEFA derivatization approaches	-Toxic, must be used with care -Potential degradation of modern capillary columns	(Dünges, 1973) (Gehrke & Goerlitz, 1963) (Grunert & Bassler, 1973) (Allen et al., 1984) (Höckel et al., 1980)
Methyl chloroformate	-Methylation is instantaneous at room temperature -Useful in screening for hydroxy-NEFA in GC-MS	-NEFA unreacted with certain catalysts -Carryover of catalyst into sample -Side chain reactions when used with hydroxy-NEFA	(Hušek et al., 1990) (Hušek et al., 2002) (Hušek, 1992)[a]
Dimethoxypropane	-Helps to precipitate proteins in sample -True alternative to diazomethane	-N/A	(Tsergn et al., 1981)

[a]-Used in hydroxy-NEFA derivatization

Methyl esters are undoubtedly the most common derivatives of fatty acids formed prior to chromatographic analysis but other approaches can be equally effective. One such popular derivatization technique which allows for NEFA detection is silylation, which adds a TMS moiety to the acid carboxyl head of fatty acids to produce TMS esters. Preparation of these derivatives has facilitated resolution of NEFA without a selective extraction by silylation of total lipid extracts from plasma and serum samples (Kuksis et al., 1969; Kuksis et al., 1976). Additionally, silyl esters offer lipid analysts several advantages in that their elution order is similar to methyl esters and they are easily and quickly made at room temperature (Kuksis et al., 1969; Kuksis et al., 1976). Unfortunately, TMS esters and ethers are hydrolytically unstable (Middleditch, 1989) and may only be stored for 24-72 hours before noticeable degradation occurs (Kuksis et al., 1976; D'Alonzo et al., 1981). Nonetheless, silylation is a simple and versatile approach with broad applications which D'Alonzo et al. (1981) demonstrated by separation of NEFA from intact glycerides following silylation of soy bean oil lipid extracts.

Trimethylsilylation is an attractive derivatizing reagent, but trimethylsilyldiazomethane, a safer and more stable derivative of diazomethane, can also be used to esterify NEFA (Presser & Hüfner, 2004). For instance, Presser and Hüfner (2004) demonstrated that methyl esters of long-chain NEFA and hydroxy long-chain NEFA were prepared almost instantaneously by dropwise addition of trimethylsilyldiazomethane. Furthermore, the yields of the methyl esters were nearly 100 %. However, the same authors found partial *O*-methylation of tertiary alcohols, implying trimethylsilyldiazomethane lacks the specificity of diazomethane. Not surprisingly, other research has found use of trimethylsilyldiazomethane can lead to artifact and byproduct

formation (Park et al., 2001; Shahin et al., 2003), so some consideration should be taken when using this reagent to methylate NEFA.

Our recent study (Potter et al., 2015) identified trimethylsilylation of hydroxy-fatty acids, in our case 3-OH oxylipins, as an effective derivatization approach. In this procedure TMS moieties were added to both the carboxyl and hydroxyl functional groups on the molecule. In earlier works which analyzed 3-OH FAME by GC-MS (Vesonder et al., 1968; Van Dyk et al., 1991), a base peak at m/z 103 was indicative of a hydroxyl group on the β-carbon. When we double silylated the 3-OH 10:0 molecule at the carboxyl and β-hydoxyl functionalities, we observed that the same fragmentation at the β-carbon produced a m/z 233 fragment, although the base peak was now at m/z 147. The advantage then of methylating 3-OH fatty acids is that the LOD in GC-MS SIM mode can be very low since the diagnostic fragment is also the base beak, which is the preferred scenario for GC-MS quantitative studies (Raina & Hall, 2008). However, some care is required as selectivity may be reduced by mass interferences which are more common in biological samples at low mass intervals (Raina & Hall, 2008). Despite the fact that the diagnostic m/z 233 peak is not the base peak, trimethylsilylation of 3-OH oxylipins still represents a safer and easy to use substitute to diazomethane. Mass interference may also be mitigated because the diagnostic fragment is of higher mass.

Another alternative approach to still achieve low LOD with 3-OH and other short-chained hydroxy fatty acids is to prepare *tert*-butyldimethylsilyl (TBDMS) derivatives. For example, Mamer et al. (2013) have developed a protocol to quantify all the intermediates of the Kreb's cycle, including α-hydroxyglutaric acid, by preparing TBDMS derivatives. These authors noted that the TBDMS derivatives of the acids were

characterized by an intense [M^+-57] fragment, which should allow for lower LOD then the identical TMS derivatives. Of course, preparation of these heavier derivatives would require higher oven temperatures and may require other GC adaptations such as narrow bore columns. Therefore, preparation of TBDMS derivatives should be carefully considered for long-chained hydroxylated NEFA.

Heptafluorobutyrate (HFB) derivatives of hydroxy methyl esters offer a third option to achieve low detection limits, on the order of 1 femtogram (10^{-15} g). The key to this technique is the use of NCI-MS (Stan & Schuitwinkel-Reich, 1980) which showed 20 X greater response than positive chemical ionization (PCI)-MS of the same analytes. Furthermore, Stan and Schuitwinkel-Reich (1980) noted hydroxy methyl ester standards with varying numbers of HFB derivatized OH groups had conserved fragmentation patterns in both positive chemical ionization-mass spectrometry (PCI-MS) and NCI-MS. A pseudomolecular ion (M+H) as the base peak, M+29 and M+41 were reported in PCI-MS from heptafluorbutyrate derivatized mono-hydroxy, di-hydroxy and tri-hydroxy fatty acid methyl esters. Meanwhile, in NCI-MS, large spectral peaks of m/z 213, 194 and 178 were noted as diagnostic. Comparison of trimethylsilylating reagents, trimethylsilyldiazomethane, TBDMS and heptafluorobutyrates are summarized in Table 4.5.

Table 4.5. Comparison of techniques utilizing trimethylsilylating reagents, trimethylsilyldiazomethane, TBDMS and heptafluorobutyrates.

Reagents	Applications and Advantages	Disadvantages	References
Trimethylsilylating reagents	-Derivatives can be produced at room temperature -Silyl esters elute like methyl esters but give better response in FID -Good for hydroxy-NEFA detection	-Very prone to hydrolysis -Limited storage time	(Kuksis et al., 1969) (Kuksis et al., 1976) (D'Alonzo et al., 1981) (Potter et al. 2015)[a]
Trimethylsilyl-diazomethane	-Methyl esters formed instantly -Near 100 % yield -Can be used for hydroxy-NEFA	-Produces artifacts and byproducts that obscure chromatograms	(Presser & Hüfner, 2004)[a] (Park et al., 2001) (Shahin et al., 2003)
TBDMS	-Intense [M^+-57] peak produced; can achieve low LOD	-Require longer elution times -May not be good for long-chain hydroxy-NEFA	(Mamer et al., 2013)[a]
Heptafluorobutyrates	-Produce diagnostic spectra in PCI-MS and NCI-MS -Very low LOD (femtogram) in NCI-MS	-Specialized instrument calibration required	(Stan & Schuitwinkel-Reich, 1980)[a]

[a]-Used in hydroxy-NEFA derivatization

4.7 CONCLUSION

Despite diazomethane's danger and toxicity, current research efforts still focus on more efficiently producing and more effectively utilizing this reagent (Barkawi & Cohen, 2010; Rossi et al., 2011). We elected not to use this derivatizing chemical largely because we were concerned for the general safety of our laboratory personnel. Once we delved into the literature, there were clearly numerous replacement methods for the specificity of diazomethane derivatization which still allowed for detection of both NEFA and hydroxylated NEFA. We found that by selectively quantifying/extracting, purifying from total lipids and applying alternative derivatization approaches, NEFA and hydroxylated NEFA could be resolved from other lipids species. For our own purposes, we chose to trimethylsilyate our lipid extract to detect 3-OH 10:0 in yeast cells (Potter et al., 2015), but this detection could have been achieved with several of the other protocols we described.

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CHAPTER 5 QUANTITATIVE ANALYSIS OF 3-OH OXYLIPINS IN FERMENTING YEASTS

5.1 PUBLICATION INFORMATION

This chapter is modified version of a manuscript accepted for publication in the Canadian Journal of Microbiology on August 31, 2016. It has been reworked here for formatting and consistency. Wei Xia, Suzanne Budge and R. Alex Speers contributed to the study design and manuscript preparation. Greg Potter was the principal investigator and lead author.

5.2 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 HFB methyl ester derivatives and subsequent analysis with GC-NCI-MS allowed for the first quantitation of yeast 3-OH oxylipins. The concentration of 3-OH 10:0 (0.68-4.82 ng/mg dry cell weight) in the SMA strain of *Saccharomyces pastorianus* grown in lab-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 lab 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.

5.3 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 inter-kingdom 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 GC-MS, while van Dyk et al. (1991) were successful when employing silica TLC, radio TLC, NMR 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 (TEM) analysis (Smith et al. 2000) and Ultra HPLC-MS (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 which still specifically target the unesterified FFA 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 TMS 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 due 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, two 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 have analyzed yeast 3-OH oxylipins with NCI-MS analysis of HFB methyl ester derivatives, which has been previously used to assay low levels of hydroxy fatty acids from other biological samples (Stan and Scheutwinkel-Reich 1980). Using this method it was possible for the first time to i) quantify the level of the 3-OH 10:0 oxylipin in the SMA strain of *Saccharomyces pastorianus* during growth in lab-scale fermentations

and to ii) correlate the quantitative analysis of 3-OH oxylipins with accepted brewery fermentation kinetic modelling results

5.4 MATERIALS AND METHODS

5.4.1 Double Grow Up and Culture

The SMA strain of *Saccharomyces pastorianus* that had been stored on a YEPD 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 x 450 mL) to conduct three coincident miniature fermentations. In a deviation from the standard protocol, 30 taller test tubes (15 cm) per fermentation were utilized so settled yeast could be collected by vortexing the tube contents.

5.4.2 Sample Harvesting and Preparation

At each of 12, 24, 36, 48 and 60 h of the fermentation, six test tubes were selected for sampling from each of the three coincident miniature fermentations. Within each group of six tubes, the contents of the first three 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 three tubes were filtered through 7 cm Whatman # 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 spun 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 were 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, USA) for 55 h. Once the freeze drying process was complete, the samples were returned to -30 °C cold storage until analysis.

5.4.3 Lipid Extraction

All glassware used in the lipid extractions were 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 425-600 µm diameter acid washed glass beads (Sigma, St. Louis, 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 M citric acid. Ethyl acetate (10 mL) and 3-OH 11:0 (200 ng) (Matreya LLC, Pleasant Gap, USA) as an internal standard were also added to each tube. All tubes were vortexed for 4 min to ensure cell breakage and a thorough solvent penetration (Personal communication, Dr George Carman, Rutgers University). The tubes were then shaken for 10 min on a bench-top shaker (Burrell Wrist-Action, Pittsburgh, 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 re-dissolved in 100 µL of chloroform.

5.4.4 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, USA) along with authentic 3-OH 9:0 and 3-OH 11:0 standards (Matreya LLC, Pleasant Gap, USA). The TLC plates were developed with 80:20:2 (v/v/v) hexane: diethyl ether: acetic acid. 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 2:1 methanol:chloroform for 1 min, centrifuging the slurry for a further min and filtering the supernatant through Whatman 4 filter paper. Thereafter, the solvent was removed under streaming nitrogen.

5.4.5 Preparation of Heptafluorobutyrated Methyl Ester Derivatives

To prepare methyl ester derivatives of oxylipins, 1.5 mL of BCl_3-methanol solution (Supelco, Bellefonte, USA) was added to each tube and all were heated at 100 °C for 1 hr. After heating, the tubes were allowed to cool to room temperature and 1.5 mL water and 1 mL 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 three 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 isooctane and 100 µL heptafluorobutyric anhydride (HFBA) (Sigma Aldrich, St. Louis, USA) 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 HFBA was eliminated by addition of 2 mL pH=8 phosphate buffer solution (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 re-dissolved in 200 µL of hexane and were transferred to GC vials.

5.4.6 Gas Chromatography-Negative Chemical Ionization-Mass Spectrometry 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 x 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 at 250 °C and a splitless time of 2 min. The GC oven temperature began at 60 °C and was held for 2 min. This was followed by an increase to 250 °C at 7.5 C°/min which was subsequently held for 5 min. Helium flowing at 1.0 mL/min was used as the carrier gas and the transfer line was set at 280 °C.

The MS was optimized for negative chemical ionization (NCI) analysis by first tuning in EI ionization mode and then in the 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 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 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 $[M-40]^{\cdot-}$ fragment measured from 14.30 to 15.38 min and m/z 372 for the 3-HFB-*O*-11:0 methyl ester internal standard $[M-40]^{\cdot-}$ 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, Pleasant Gap, USA). Peak areas were integrated with the Thermo Xcalibur™ Qual Browser Genesis™ peak finding algorithm (Waltham, USA).

5.4.7 Analytical Curve

To account for differential 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 four 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.

5.4.8 Fermentation Modelling

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

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

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, USA) software package.

5.5 RESULTS AND DISCUSSIONS

5.5.1 Analytical Methodology

The distribution of yeast cell 3-OH 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. 2013; Kock et al. 2007). 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 typically 8 distinct bands on the TLC plates compared to 3 bands in the bead free ethyl acetate extracts. 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 to BCl_3. Thus, BF_3 was avoided and BCl_3 was utilized in this experiment.

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. 5.1). Stan and Scheutwinkel-Reich (1980) reported that, in the NCI mode, HFB-derivatized hydroxy FAME were characterized by $[M-20]^{\cdot -}$ or $[M-40]^{\cdot -}$ peaks that resulted from loss of 1 or 2 HF groups. Diagnostic fragments of m/z 213, 194 and 178 that correspond to those

ions shown in Figure 5.1 were also evident. The derivatized 3-OH 10:0 analyte (MW=398) and 3-OH 11:0 internal standard (MW=412) exhibited the expected fragmentation pattern and were typified by m/z 358 and 372 $[M-40]^-$ ions, respectively (Figs. 5.2a and 5.2b). It is notable that the $[M-40]^-$ peaks were small relative to the m/z 194 peak (Figs. 5.2a and 5.2b), but this is consistent with the mass spectra for the 9-HFB-*O*-18:0 methyl ester published by Stan and Scheutwinkel-Reich (1980).

Interestingly, Stan and Scheutwinkel-Reich (1980) did note the presence of a M^- fragment in the 9-HFB-*O*-18:0 methyl ester spectra, but no such peak was apparent with any of the 3-HFB-*O*-9:0, 3-HFB-*O*-10:0, 3-HFB-*O*-11:0 or 12-HFB-*O*-12:0 standards analyzed in this work. Kostianien and Rizzo (1988) found that the parent ion peaks (M^-, $[M-20]^-$ and $[M-40]^-$) of HFB esters of trichothecenes in NCI-MS were more intense at lower source temperatures. However, we encountered problems with elevated baselines at low source temperatures (200 °C vs. 230 °C), and could not operate the instrument at a temperature that was likely more conducive to M^- peak formation. Due to the lower LOD achievable during NCI-MS analysis, the chromatograms produced during full-scan 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. 5.3a and 5.3b).

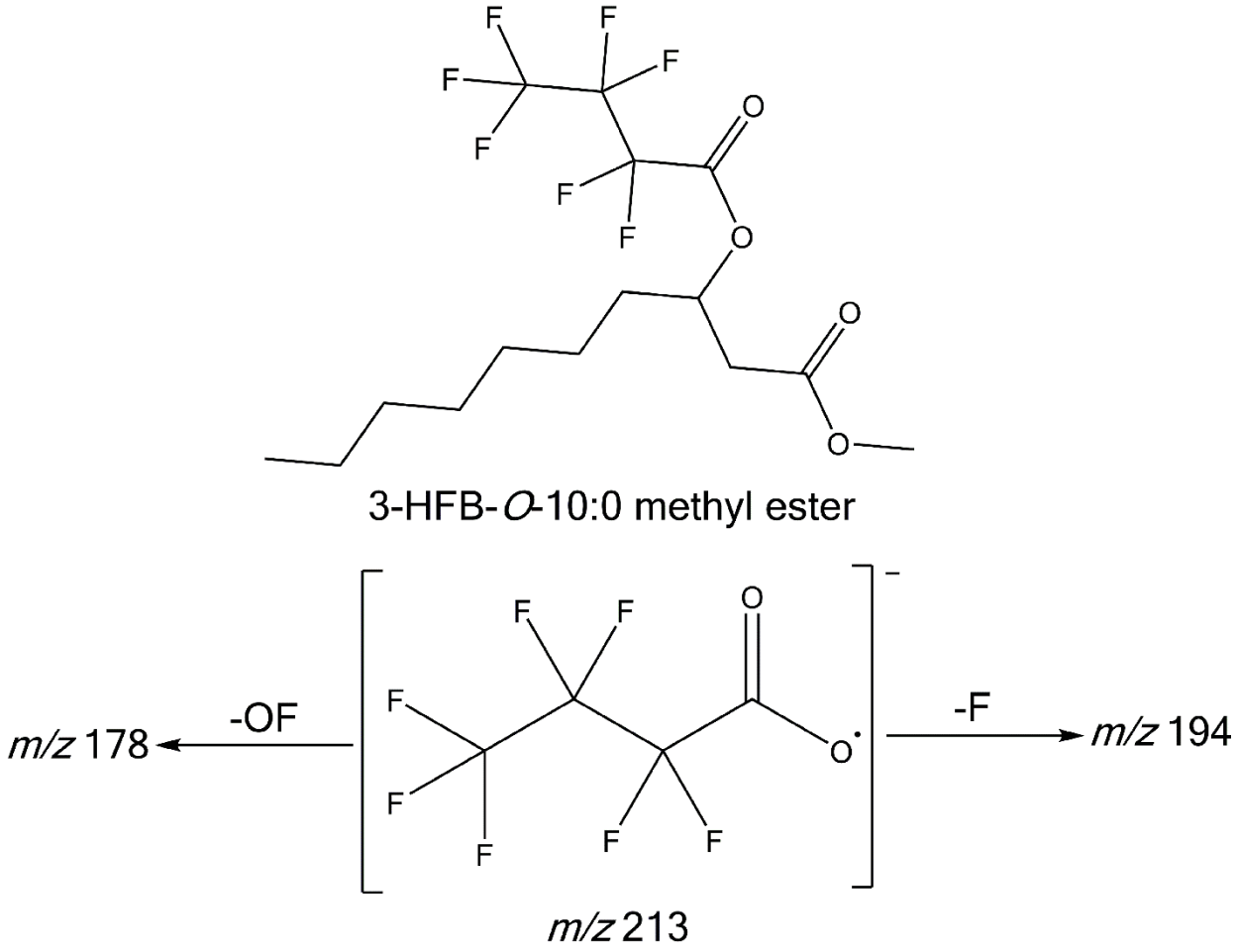

Figure 5.1. Structure of the 3-HFB-*O*-10:0 methyl ester and the additional characteristic ions, *m/z* 213, 194 and 178, produced by fragmentation of the HFB group.

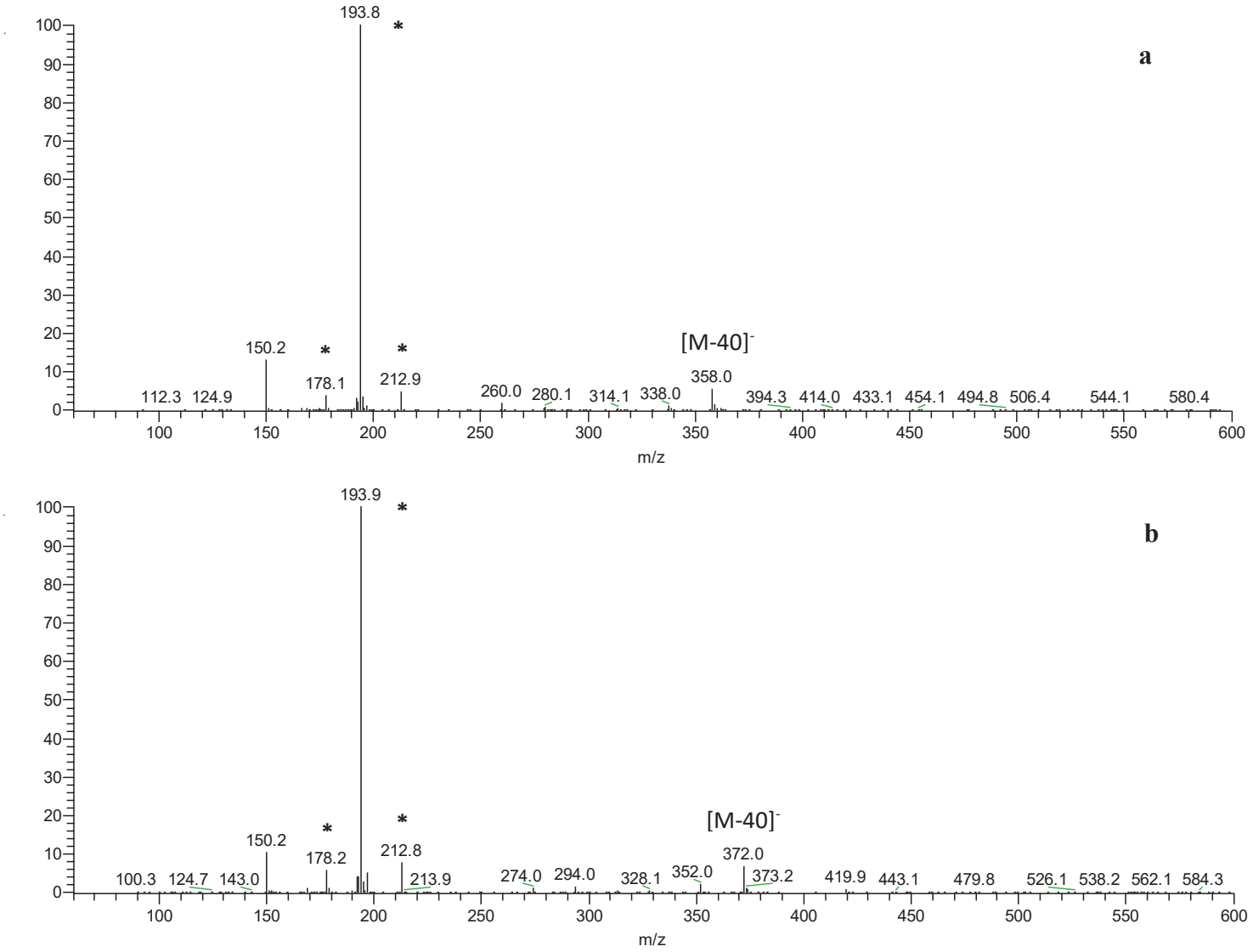

Figure 5.2. Diagnostic HFB spectra (*) and the pseudomolecular ions ([M-40]$^-$) of the 3-HFB-*O*-10:0 methyl ester (a) and 3-HFB-*O*-11:0 methyl ester (b) acquired using GC-NCI-MS in the full scan mode.

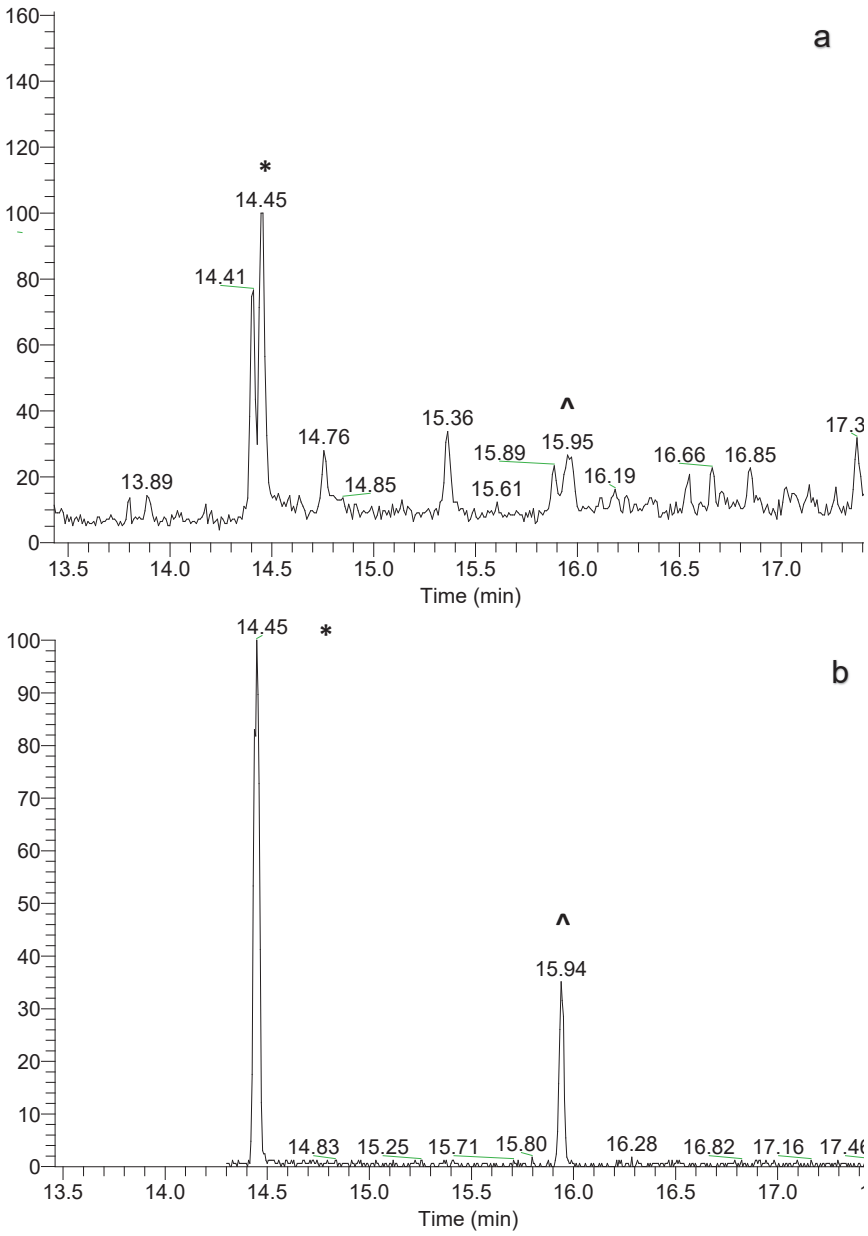

Figure 5.3. GC-NCI-MS chromatographic analysis of 3-HFB-*O*-10:0 methyl ester (*) (RT-14.45) and the 3-HFB-*O*-11:0 methyl ester (^) (internal standard; RT-15.94) acquired using GC-NCI-MS in the full scan (a) and SIM (b) modes for a sample from 36 h fermentation time.

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 HFBA 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. 5.1; Fig. 5.2) persisted in the baseline even 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 LOD of 0.3-66.3 pg/injection in full scan (Longo and Cavallaro 1996) and 0.01-0.57 pg/injection and 0.6 pg/injection in SIM (Longo and Cavallaro 1996; Mueller and Brodschelm 1994). In the

current study the LOD was approximately 16 pg/injection in full scan when an analyte signal to noise ratio of three was used as a threshold. We found that the 16 pg/injection LOD 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 than 174 pg/injection of the analyte (Table 5.1). Therefore, there is no concern that reduced sensitivity influenced the accuracy of oxylipin quantitation.

Table 5.1. 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 (hr)	Rep	3-OH 10:0 Oxylipin Mass from Cal. Curve (ng)	Cell Mass (mg)	Oylipin Concentration (ng 3-OH 10:0/mg cell mass)	Mean Oxylipin Concentration ± 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		

The coefficient of determination for the analytical curve (R^2=0.94) was comparable 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) that used NCI-MS to quantify four 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 further evaluate the robustness of the curve fit. 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.

5.5.2 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. 5.4; Table 5.1). This trend is

consistent with our earlier investigation where 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 3.73-42.8 % (Table 5.1). However, these % RSD values were comparable, and in some cases even lower, to those obtained in other NCI-MS studies of plant oxylipins by Mueller and Brodschelm (1994) (6.67-51.2 %) and Thoma et al. (2003) (17.2-50.9 %). This suggests an inherent variability in the NCI-MS technique.

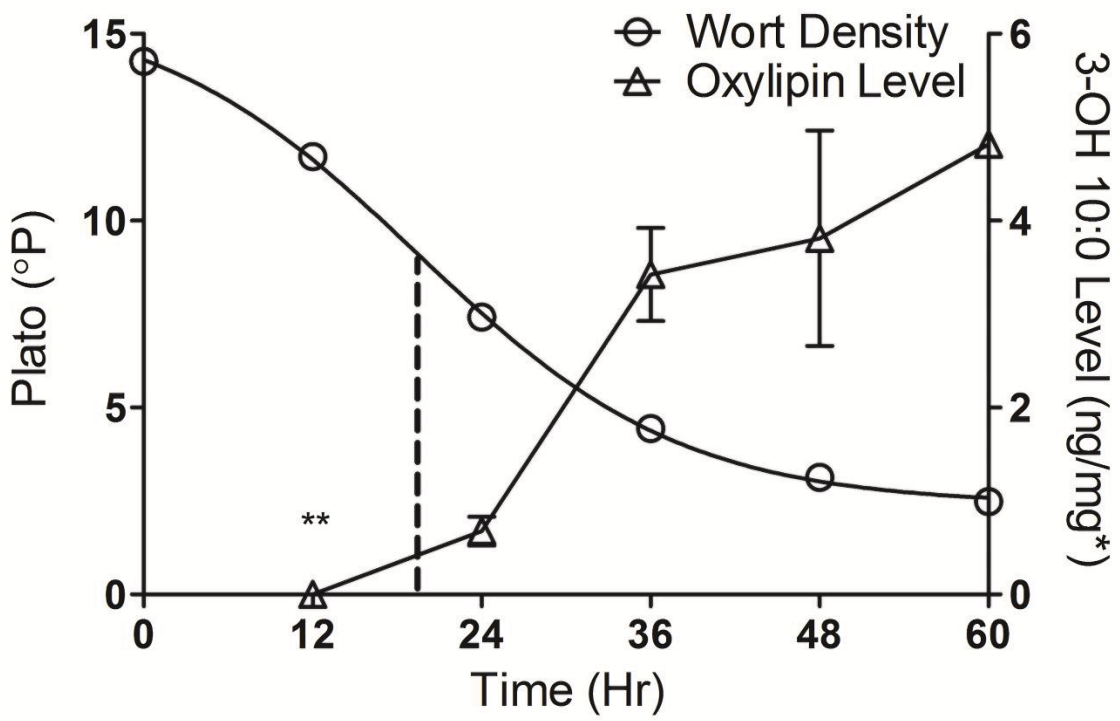

Figure 5.4. Fermentation performance of the SMA strain in the miniature fermentation assay with coincident formation of the 3-OH 10:0 oxylipin. Fermentation progress was determined on the Plato (°P) scale with readings conducted in triplicate and mean values for three miniature fermentations presented (SD bars for density measurements not visible). Density attenuation was modelled using with the four 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.

*mg dry cell weight. ** Peak area not detected by the Genesis™ peak finding algorithm.

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 amongst 30 test tubes which in effect become 30 individual fermenters. At each sampling interval, the yeast from six 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 to 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 seven fatty acids identified in cells grown in the miniature fermentation using EI GC-MS (mean % CV = 30 %) (Potter et al. 2015). Therefore, the miniature fermentation assay was likely the greater contributor to the overall variance 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 2013), which was also observed in this work (Table 5.1).

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, 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 basal levels of these plant oxylipins ranged from 0.90-131 ng/g dry weight (DW) and increased to 23-776 ng/g DW following tissue wounding and elicitation. Similarly, Kumari

et al. (2014) found hydroxy-oxylipins in the macroalgae Chlorophyceae, Rhodophyceae and Phaeophyceae ranged from 0.14-8161 ng/g fresh wet weight and surely would have been higher if determined on a DW basis. Thus, the concentration range of 3-OH 10:0 in the SMA strain at 0.68-4.82 ng/mg DW (680-4820 ng/g DW) 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 function 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 (Potter et al. 2015; Strauss et al. 2005). 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 to 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 (nM) 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 FFA 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. 2013; Kock et al. 2007), reduces hydrocarbon chain length by two 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-OH-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-OH-octanoyl-CoA and 3-OH-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 mM 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 transcriptiomic 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 four parameter logistic model, the time of the maximal sugar consumption rate is indicated by the parameter *M.* In this study, according to the four parameter 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. 5.4). The genes required for the catabolism of non-fermentable 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 2013). In this study there was a sharp increase in 3-OH 10:0 concentration between 24 and 36 h (Fig. 5.4), which is well correlated with depleted glucose levels at 24 h and in increase in β-oxidation thereafter. Thus, the timing of these two phenomena are suggestive of the diauxic shift yeast cells undergo once glucose reaches sufficiently low levels.

5.6 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 lab-scale beverage fermentations, 3-OH 10:0 ranged between 0.68-4.82 ng/mg DW from 0-60 h fermentation time. % RSD 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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CHAPTER 6 COMPOSITIONAL AND ULTRASTRUCTURAL CHARACTERIZATION OF THE SMA STRAIN OF *SACCHAROMYCES PASTORIANUS*

6.1 CHAPTER INFORMATION

The experimental work in this chapter was conducted at the UFS in Bloemfontein, South Africa upon invitation to engage in collaborative study with the researchers at this institution.

6.2 ABSTRACT

NanoSAM (or Auger-architectomics) and time-of-flight secondary ion mass spectrometry (TOF-SIMS) have been used in materials science research for some time, but NanoSAM, in particular, has only recently been applied to biological specimens. Examination of the standard SMA strain using Auger-architechtomics, TOF-SIMS and microscopic techniques uncovered the presence of intracellular networks of CO_2 in fermenting cells. Respiring cells produced few bubbles and instead had large vacuolar structures inside the cells. TEM analysis also showed osmiophilic layers at the cell exterior of fermenting cells that became more prevalent with fermentation duration. Osmiophilic layers were largely absent in respiring cells. TOF-SIMS trials showed a compositional difference at the exterior and interior of SMA cells and between fermenting and respiring cells. Fermenting cells also appeared to have different 3-OH oxylipin profiles compared to respiring cells upon examination with immunofluorescence microscopy.

6.3 INTRODUCTION

A key tenet of materials science is that a comprehensive compositional survey of a substance is only possible when surface analysis is combined with depth profiling. Fortunately, the field has developed several nanotechnological instruments to achieve such a complete approach including both NanoSAM and TOF-SIMS. NanoSAM is a relatively new technique which combines the imaging capacity of SEM, the elemental analysis capability of AES and depth profiling conferred by the etching functionality of an Argon (Ar^+) gun. Recently, NanoSAM was applied to biological specimens for the first time (Swart et al., 2010) in a new application called Auger-architectomics[3]. Auger-architectomics, while still a relatively novel technique in biological sciences, has proven immensely useful in diverse fields such as translational medicine, cancer biology and fermentation science (Swart et al., 2012; 2013; 2014). In yeast and fungal biology, several different strains, including *Nadsonia fulvescens* (Swart et al., 2010), *Cryptococcus curvatus* (Leeuw et al., 2010) and the fermentation strains *Saccharomyces cerevisiae* CBS 1171 NT and *Saccharomyces pastorianus* WS 34-70 (Swart et al., 2012; 2013), have already been characterized using this nanotechnology.

Even though it is well-known that CO_2 and ethanol are released as by-products during yeast fermentations, CO_2 was never observed in yeast cells until the application of Auger-architectomics indicated the presence of intracellular CO_2 gas bubbles (Swart et al., 2012; 2013). This discovery represented a paradigm shift in current models of intracellular gas generation, transport and cellular metabolism. Interestingly, during Auger-architectomal analysis, it was also shown that the presence of gas bubbles inside cells

[3] (https://en.wikipedia.org/wiki/Auger_architectomics)

deformed and compressed internal organelles (Swart et al., 2013). With this finding, it has now become paramount to examine how gas bubble formation effects the metabolism, performance and vitality of fermenting yeasts. Since 3-OH oxylipins are presumed to play a significant role in flocculation during fermentation (Kock et al., 2000; Strauss et al., 2005; Potter et al., 2015), the effect of gas bubble formation and thus, fermentation, on 3-OH oxylipin production is also of interest. Furthermore, additional fermenting yeasts, including standard test strains, must be studied with Auger-architectomics and TOF-SIMS to understand the variability between strains in terms of bubble generation, deformation of cell ultrastructure and cellular composition.

Currently, the SMA strain of *Saccharomyces pastorianus* has been adopted as a standard test strain and it is the strain of choice in the ASBC miniature fermentation assay (ASBC, 2013). The SMA strain has been characterized in terms of its flocculation behaviour and growth kinetics (Lake et al., 2008; ASBC, 2013), and has proven to be useful in studies on wort fermentability (Macintosh et al., 2012) and premature yeast flocculation (Macintosh et al., 2014). Coincident changes in fatty acid profile, flocculation and CSH have also been studied in the SMA strain during growth in the miniature fermentation assay (Potter et al., 2015). Furthermore, the SMA strain of *Saccharomyces pastorianus* has also been shown to produce the potentially bioactive oxylipins, 3-OH 8:0 and 3-OH 10:0, when grown in lab-scale fermentations (Potter et al., 2015). Thus, the SMA strain is well characterized using classical methods and an appropriate candidate for further analysis of its cellular structure and composition using nanotechniques.

In this study we have analyzed the standard SMA strain of *Saccharomyces pastorianus* with Auger-architectomics and TOF-SIMS (i) to further demonstrate the

nascent biological applications of these techniques, (ii) to investigate the influence of bubble formation on cellular composition in fermenting yeasts and (iii) to examine the effects of fermentative growth and bubble formation on 3-OH oxylipin production. To achieve this, we have coupled nanotechnological analyses of the SMA strain using Auger-architectomics (or NanoSAM) and TOF-SIMS with microscopic examination via light microscopy (LM), TEM, high resolution (Hi-Res) SEM and immunofluorescence with confocal laser scanning microscopy (CLSM). For the purposes of comparison, all analyses were jointly conducted on fermenting and respiring yeast cells.

6.4 MATERIALS AND METHODS

6.4.1 Cultivation and Analysis

The SMA strain of *Saccharomyces pastorianus* (obtained from VLB Berlin, Biological Laboratory, Seestraße 13, D-13353 Berlin) was streaked from an agar slant onto a yeast malt (YM) agar plate and cultivated for 48 hours (h) at 29 °C. A pre-inoculum was then prepared by inoculating a loopful of the fresh cells into 250 mL Erlenmeyer flasks containing 100 mL of fermentable and non-fermentable media, respectively and incubating the flasks at 29 °C for 24 h (160 rpm). The fermentable media was glucose YM broth consisting of 10 g/L glucose, 3 g/L yeast extract, 3 g/L malt extract and 5 g/L peptone while the non-fermentable media was yeast peptone glycerol (YPG) broth containing 30 mL/L glycerol, 10 g/L yeast extract and 20 g/L peptone. After 24 h, 1 mL of the respective pre-inoculums were transferred to additional 250 mL flasks containing the same fermentable and non-fermentable media and these were incubated using the same conditions as the

previous flasks. At each of 24, 48 and 72 h growth time, cells were collected in triplicate from both growth mediums and were analyzed using LM, NanoSAM, Hi-Res SEM, TEM, TOF-SIMS and CLSM.

6.4.2 Light Microscopy

The cells were viewed using LM (Axioplan, Zeiss, Germany) with coupling to a Colourview Soft Digital Imaging System (Münster, Germany) at 1000 X magnification under oil immersion to check the cells for granularity and also to ensure the purity of the cultures. A granular appearance was expected in the fermenting cells since intracellular gas bubbles appear as light scattering granules.

6.4.3 Nano Scanning Auger Microscopy

Cells were prepared according to Swart et al. (2010) and Kock et al. (2011). At each sample collection time, 5 mL culture volumes of the fermenting cells (YM broth) were collected. Since cells grown in YPG broth (respiring) grow at a decreased rate, 15 mL volumes were collected from this media in order to obtain a pellet of sufficient mass for further analyses. Thereafter, the culture volumes were centrifuged at 1450 g for 5 min and the pellets were double-washed with sterile distilled water. The pellets were then fixed for at least 2 h at room temperature in 0.1 M (pH 7) sodium phosphate-buffered 3 % glutaraldehyde and subsequently for 1 h in 1 % osmium tetroxide buffered in the same solution. Between each fixation step the cells were rinsed with the same buffer solution (once after the glutaraldehyde step and twice after the osmium tetraoxide step).

The cells were then dehydrated by sequential exposure to 50, 75 and 95 % ethanol for 20 minutes at each concentration and were subjected to two final 100 % ethanol steps, each lasting for 1 h. Cells were dried into powder form using a critical point drier, after

which the cells were mounted on stubs and sputter coated with gold to make them electron conductive. Samples were then examined with the NanoSAM which consisted of a PHI 700 Nanoprobe (Physical Electronics, Inc. Japan) equipped with SEM and scanning Auger microscopy (SAM) capabilities. For SEM and SAM analyses the field emission electron gun was set as follows: 2.29 A filament current, 3.58 kV extractor voltage and 226 µA extractor current. At these settings, a 25 kV and 10 nA electron beam with a diameter of 12 nm was produced to facilitate the Auger analyses and SEM imaging. The upper pressure of the electron gun unit was 9.9E-10 Torr and the pressure of the main chamber was 3.47E-10 Torr. Aperture A was used for all measurements. The field of view (FOV) for SEM was 10 µm with 4 frames, 6 nm SE image resolution at analysis position, 8 nm Auger resolution, 1 – 10 nm information depth and a 1 – 0.1 % detection limit (Evans Analytical Group A, 2015). To obtain the Auger point analyses, 10 cycles per survey, 1 eV per step and 50 ms per step were used. The Ar^+ ion sputtering gun was set as follows: 2 kV beam voltage, 1.5 µA ion beam current with a 1 mm x 1 mm raster area, which produced a sputter rate of 15 nm/min and the ion emission current was set at 15 mA. An alternating sputter mode with sputter intervals of 1 min and sputter time of 2 min was used without any rotation. The % atomic concentrations of C, Au, Os, N, O, P and S were monitored during AES analysis. Relative sensitivity factors were used to calculate the concentration of elements above atomic number 3.

6.4.4 High-Resolution Scanning Electron Microscopy

The cells that were harvested, prepared, analyzed and Ar^+ etched in the NanoSAM were also examined with a Hi-Res SEM in order to obtain high resolution images of intracellular ultrastructure. This instrument was a JEOL JSM-7800F Extreme-resolution Analytical

Field Emission microscope (USA) fitted with a lower electron detector at the following settings: 5 kV and 9.634E-5 Torr.

6.4.5 Transmission Electron Microscopy

Cells were prepared according to Swart et al. (2010). Cells for TEM analysis were harvested, washed and fixed in the same manner as the NanoSAM analyzed cells. Thereafter, the cells were dried by sequential exposure to 50, 75 and 95 % acetone for 20 minutes at each concentration and were subjected to two final 100 % acetone steps, each lasting for 1 h. Once dehydrated, the cells were embedded in epoxy resin which was polymerized at 70 °C for 1 h. The embedded cells were then cut into ultra-thin 60 nm sections using a Leica Ultracut UM7 microtome that was fitted with a glass knife. A double staining was then applied to all sections, first with uranyl acetate for 3 minutes and then with lead citrate for 10 minutes. All sections were viewed using TEM [FEI (Phillips) CM 100, Netherlands].

6.4.6 Time-of-Flight Secondary Ion Mass Spectromety

Cells for TOF-SIMS analysis were harvested and prepared in the same manner as cells examined with the NanoSAM, except they were not coated with gold and they were mounted on copper stubs with double-sided tape. TOF-SIMS mass spectra and ion maps were obtained with a secondary ion mass spectrometer TOF-SIMS V (ION-TOF GmbH, Münster, Germany) fitted with a high mass resolution (>10 000) time-of-flight analyzer. TOF-SIMS has a lateral resolution of 0.5 – 2 μm and a depth resolution of 1-5 nm (Bexell, 2003). The TOF-SIMS detection limits for most trace elements are between 1E12 and 1E16 atoms/cc (Evans Analytical Group B, 2015). Secondary ions mass spectra were recorded from an approximately 20 μm × 20 μm area of the sample surface. During measurement

the analyzed area was irradiated with pulses of 30 keV Bi_1^+ ions from a primary ion gun at a 10 kHz repetition rate and a flood gun (low-energy electrons) was used to compensate for any surface charging. A 2.5 kV argon cluster gun was also used to sputter the samples in a 100 µm x 100 µm region to allow for depth profiling analysis. Mass spectra were recorded from m/z 0-200 and intensities of the negative ions C^-, NH^-, O^-, OH^-, P^-, and S^- were monitored. Intensities were normalized based on total intensity at each sputtering time to allow for comparison between samples and to account for any operational variability between runs. Relative sensitivity factors can be used to calculate the concentration of different ions, but a reference sample with known concentrations of each ion is required (Evans Analytical Group C, 2015). Quantitative analysis with TOF-SIMS is therefore difficult to conduct, but semi-quantitative analysis is still possible.

6.4.7 Confocal Laser Scanning Microscopy

Cells for CLSM were cultivated in the same manner as cells for NanoSAM analysis. At each harvesting time, 400 µL of cell culture from YM media and a pellet from a concentrated 10 mL aliquot of cell culture in YPG media were transferred to 2 mL microcentrifuge tubes and washed in 400 µL pH 6.5 phosphate buffered saline (PBS). Thereafter, the cells were incubated with 30 µL rabbit-raised 3-OH oxylipin-specific antibodies (1:10 dilution v/v) for 1 h in the dark (Kock et al., 1998). Unbound primary antibody was then removed by centrifugation and a PBS wash. A 30 µL aliquot of the FITC-conjugated secondary antibody (1:10 dilution v/v) was subsequently added to the pellet and a second 1 h dark incubation followed. The secondary antibody was then removed by washing and slide-mounted cells were viewed with Nikon TE2000-E inverted

Confocal C1 microscope (Tokyo, Japan), equipped with a 200 mW Argon-ion 454-676 nm excitation laser (Spectra Physics, Santa Clara, CA 95054, USA).

6.5 RESULTS

6.5.1 Light Microscopy

The SMA strain showed gas bubbles as light scattering granules inside the cells when grown in YM (fermentable) media (Fig. 6.1a). There was little to no light scattering granules inside the cells grown in YPG (non-fermentable) media (Fig. 6.1b). Cells grown in YPG media also tended to have large vacuolar structures when compared to cells grown in YM media. In YM-grown cells, across the population, a granular appearance (suggesting gas bubble presence) was observed in both mother and daughter cells. The population-wide granularity also increased successively over the course of the fermentation (24 h – 72 h). Cells grown in YM were often misshapen, whereas cells grown in YPG maintained a more ovoid shape.

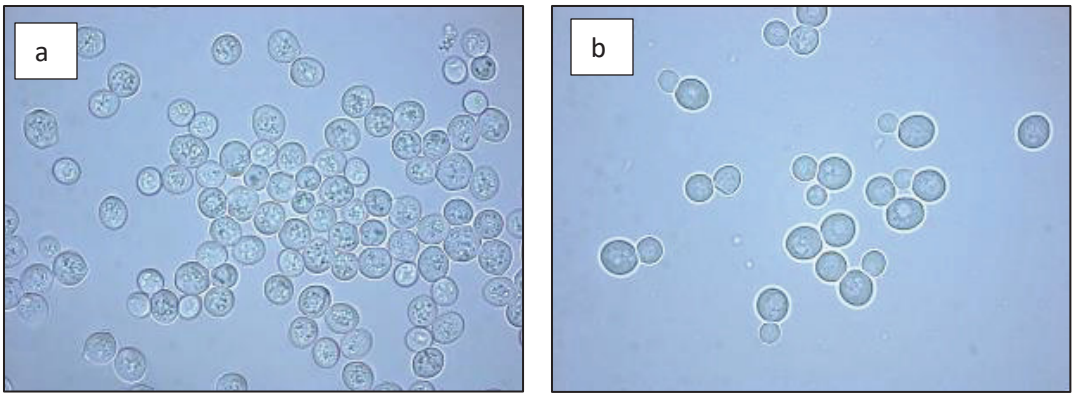

Figure 6.1. LM analysis of the SMA strain of *Saccharomyces pastorianus* grown in YM and YPG media for 72 h. (a) LM analysis (1000 X) of cells grown in YM media for 72 h. A distinct granular appearance of the cells is evident. (b) LM analysis (1000 X) of cells grown in YPG for 72 h. The cells were less granular and seemed to have distinct vacuolar structures.

6.5.2 Nano Scanning Auger Microscopy and Hi-Res Scanning Electron Microscopy

Targeted spot analysis (Fig. 6.2a,c) in all samples from both YM and YPG media revealed the cells were composed primarily of carbon, ranging from 82-96 %, while Au, Os, N and O were present in all samples in smaller but similar amounts (Fig. 6.2b,d); % relative atomic concentrations of C, N, O, Au and Os varied with sputtering time (Fig. 6.2b,d) from the same defined location in the cell. P and S were also monitored in an attempt to differentiate between protein and lipid-rich regions in the cell as most proteins contain sulfur but not phosphorus and because phosphorus analysis may be used for lipid quantification (Hershey & Chase, 1952; Choi et al., 2016). Unfortunately, neither P nor S were detectable.

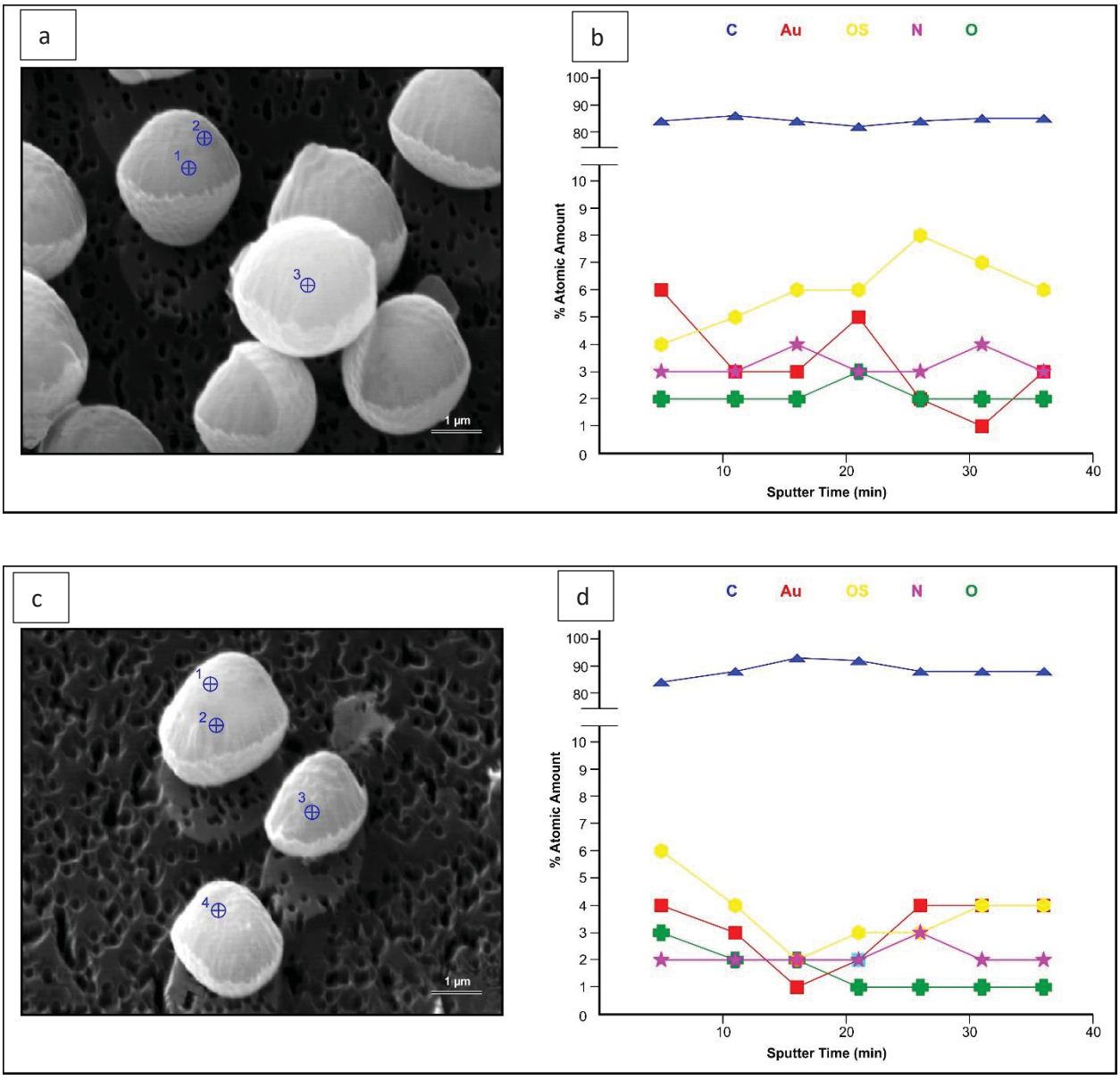

Figure 6.2. NanoSAM SEM and AES analysis of the SMA strain of *Saccharomyces pastorianus* grown in YM and YPG media for 24 h. SEM micrograph of (a) YM 24 h cells and (c) YPG 24 h cells. The targets for elemental analysis are shown by the circles and cross circles. A graph of elemental analysis as % atomic concentration relative to sputtering for (b) target 3 from YM 24 h cells and (d) target 2 from YPG 24 h cells.

After NanoSAM etching, cells were viewed with Hi-Res SEM to obtain high quality images of the gas bubbles and larger vacuolar structures inside the cells grown in YM and YPG media, respectively. A similar trend was observed in the YM-grown cells with an increase in the number of gas bubbles with fermentation time (24 h – 72 h) (Fig. 6.3a,c,e). This corresponds well with that observed with LM. The size and complexity of the bubble networks within the YM-grown cells also seemed to increase with fermentation duration; however, there was a non-homogenous distribution of bubble networks between individual cells within a FOV. Cells from the 72 h culture cultivated in YM media appeared to have a more wrinkled cell exterior (Fig. 6.3e). Consistent with the LM results obtained, the YPG-grown cells contained larger vacuolar structures and very few bubbles could be observed at any sampling time (Fig. 6.3b,d,f).

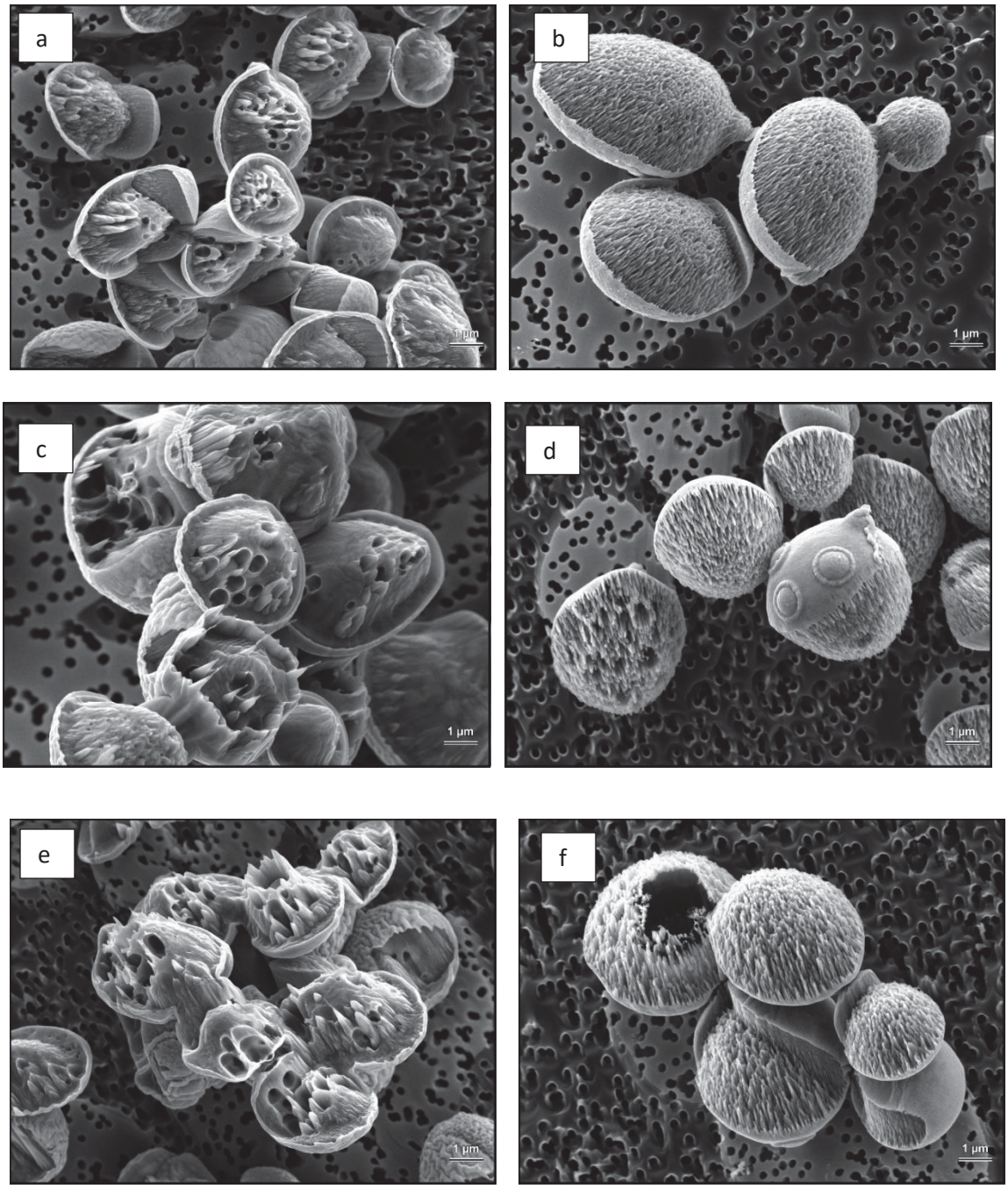

Figure 6.3. Post-Ar^{+} etching (36 mins) Hi-Res SEM analysis of the SMA strain of *Saccharomyces pastorianus* grown in YM and YPG media. For the 24 h (a), 48 h (c) and 72 h (e) cultures grown in YM media, the network of bubbles became more complex as the fermentation progressed. The 24 h (b), 48 h (d) and 72 h (f) cultures grown in YPG media lacked a network of bubbles, but certain cells contained large holes left by vacuolar structures.

6.5.3 Transmission Electron Microscopy

To verify the results obtained with LM and NanoSAM (including Hi-Res SEM imaging), cells grown in both fermentable (YM) and non-fermentable (YPG) media were subjected to TEM analysis. Results obtained indicate a number of electron-transparent gas bubbles inside the fermenting cells that increase with fermentation time to a point where the 72 h aged cells were almost completely filled (Fig. 6.4a,c,e). The intracellular structures were not enveloped by a membrane suggesting that they were indeed bubbles and not cell organelles. Cells grown in non-fermentable media contained few electron-transparent gas bubbles (Fig. 6.4b,d,f). These results coincide with observations using LM, NanoSAM and Hi-Res SEM. Cells grown in YM media also developed more pronounced dark-staining, osmiophilic layers at the cell exterior as the fermentation progressed (Fig. 6.4a,c,e). Osmium textroxide, in addition to functioning as a fixative, also serves as a lipid specific stain that turns tissues rich in unsaturated lipid, in particular, dark in colour (Belazi et al., 2009). No such osmiophilic layers could be observed at the exterior of any of the YPG-grown cells (Fig. 6.4b,d,f). Cells grown in YM media also developed more wrinkles at the cell exterior over the fermentation course (Fig. 6.4a,c,e).

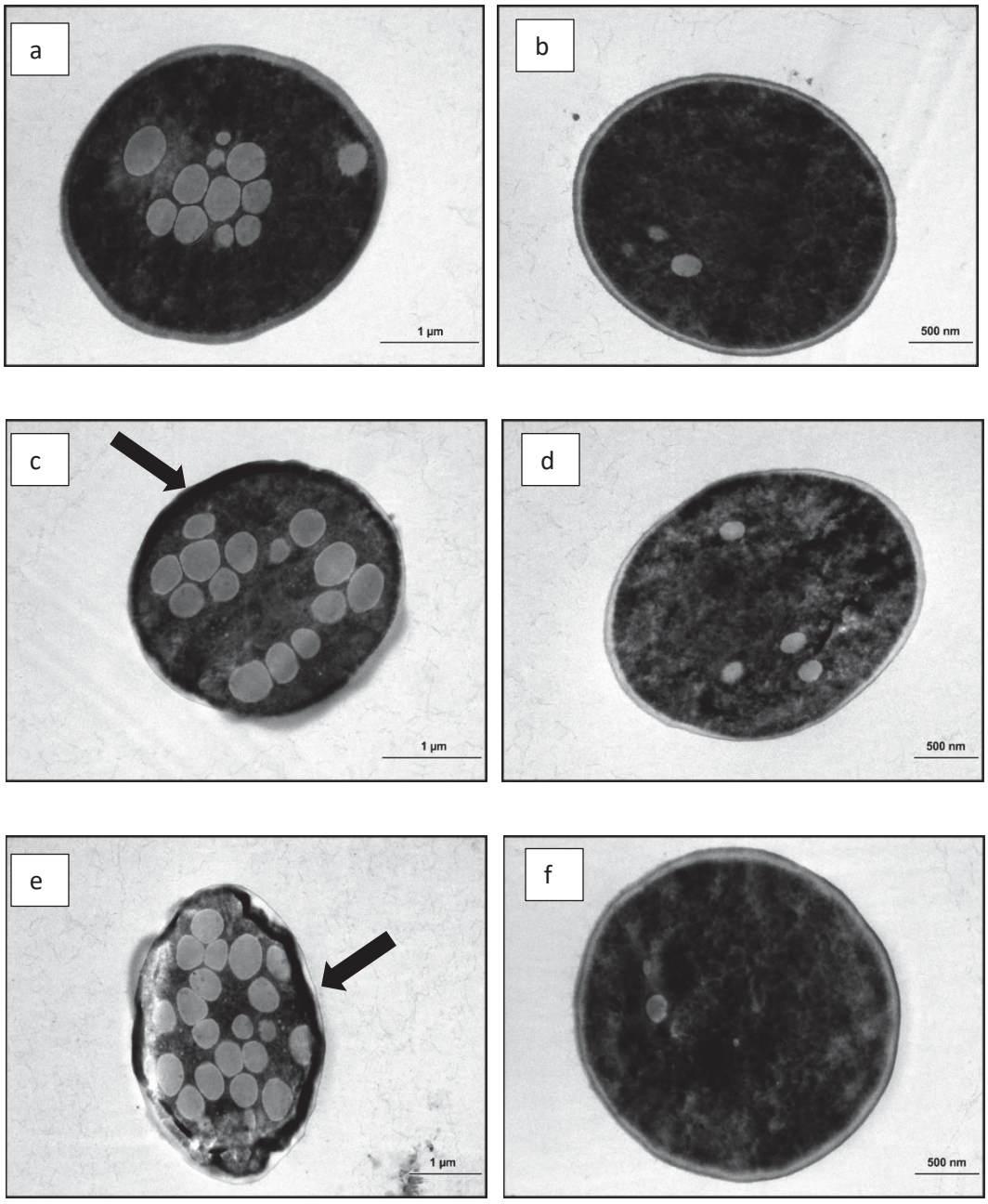

Figure 6.4. TEM analysis of the SMA strain of *Saccharomyces pastorianus* grown in YM and YPG media. Cultures grown for 24 h (a), 48 h (c) and 72 h (e) in YM media demonstrated the increase in number of bubbles and change in cell morphology that occurred as fermentation progressed. Cultures grown for 24 h (b), 48 h (e) and 72 h (f) in YPG media showed diminished bubble formation. Dark staining osmiophilic layers are indicated by the black arrow.

6.5.4 Time-of-Flight Secondary Ion Mass Spectrometry and Confocal Laser Scanning Microscopy

TOF-SIMS depth profiling analysis showed that the cell compositions changed with sputtering time (Fig. 6.5; Fig 6.6), and thus, differed at the exterior and interior of the cell. In cell clumps from both YM and YPG-grown cultures, C^- and O^- were the most abundant ions and these converged as sputtering time increased. In particular, C^- levels were highest and O^- were lowest in 48 h fermenting cells. Respiring cells had maximal relative levels of C^- at 24 h that decreased over time, while O^- increased with growth time to elevated levels at 72 h. TOF-SIMS analysis also indicated O^- was much more abundant than NH^- and that P^- and S^- could be detected in both cell types, with greater amounts of S^- than P^- present.

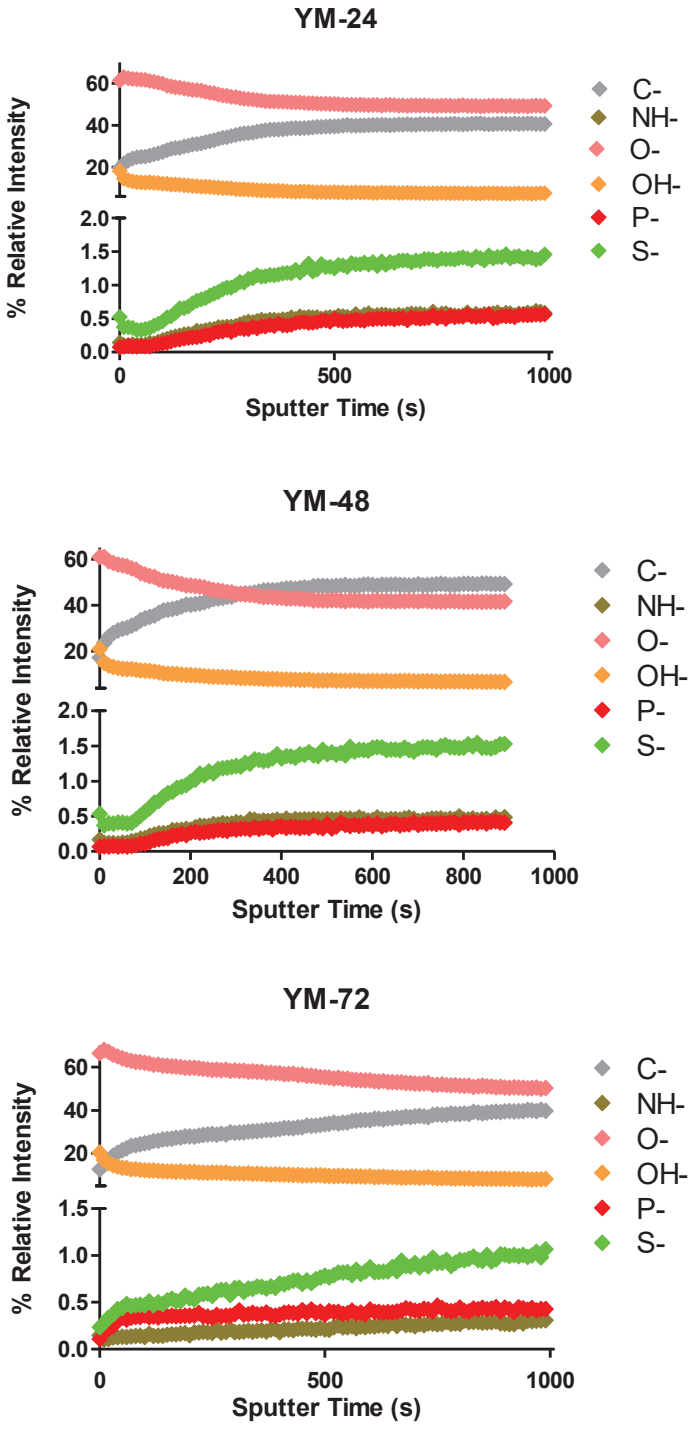

Figure 6.5. TOF-SIMS depth profiling analysis of the SMA strain of *Saccharomyces pastorianus* grown in YM media for 24 h, 48 h and 72 h. The negative atomic ions C^-, NH^-, O^-, OH^-, P^- and S^- were monitored. The intensities were normalized at each sputtering time and are expressed as % relative intensity.

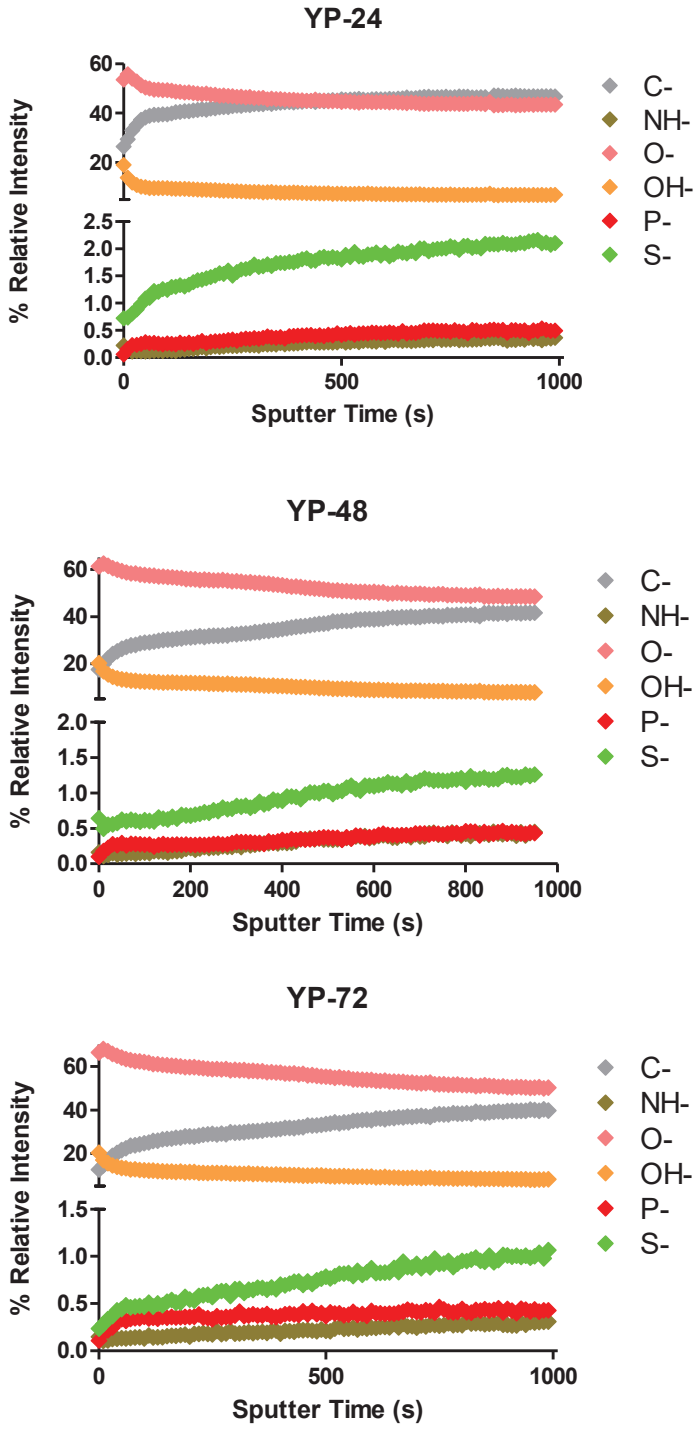

Figure 6.6. TOF-SIMS depth profiling analysis of the SMA strain of *Saccharomyces pastorianus* grown in YPG media for 24 h, 48 h and 72 h. The negative atomic ions C^-, NH^-, O^-, OH^-, P^- and S^- were monitored. The intensities were normalized at each sputtering time and are expressed as % relative amounts.

CSLM using immunofluorescence labelling showed that under fermentation growth conditions, the SMA strain produced progressively more 3-OH oxylipins as fermentation time increased from 24 to 72 h (Fig. 6.7a,c,e). At 72 h, there appeared to be more 3-OH oxylipins present in fermenting (YM) cells than in respiring (YPG) cells (Fig. 6.7e, f); however, after only 24 h growth time, the respiring cells seemed to produce more 3-OH oxylipins (Fig. 6.7b) as compared to fermenting cells of the same age (Fig. 6.7a). The localization and distribution of fluorescence in antibody labelled fermenting cells was compared to respiring cells to determine the influence of bubble formation on 3-OH oxylipin profile. Unfortunately, there was not a clear indication that 3-OH oxylipins localize differently in fermenting or respiring yeasts, but some cells within a FOV did fluoresce more brightly than others. This agrees with the Hi-RES SEM results that suggested certain cells within a population are more active fermenters, and thus more bubble-filled than others.

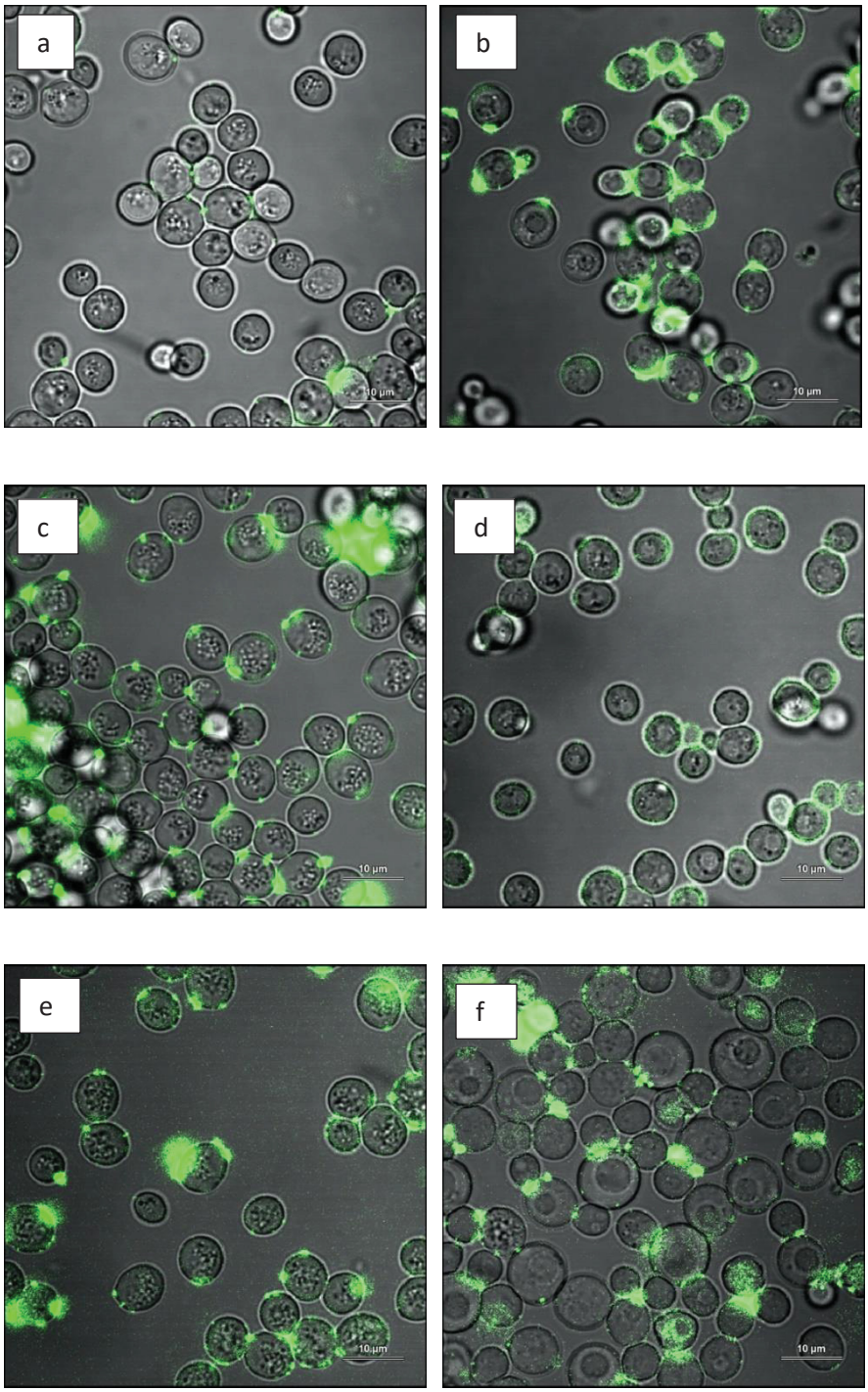

Figure 6.7. CLSM analysis of the SMA strain of *Saccharomyces pastorianus* grown in YM and YPG media for 24 h, 48 h and 72 h. 3-OH oxylipin level increased from 24 h (a), 48 h (c) and 72 h (e) during growth in YM media. In YPG media, there also appeared to be more 3-OH oxylipins after 72 h (f) relative to 24 h (b) and 48 h (d).

6.6 DISCUSSIONS

6.6.1 Light Microscopy

LM results for the SMA strain correspond to those of Swart and co-workers (2012), where gas bubbles were observed as light scattering granules inside YM-grown cells, and YPG-grown cells produced very few light scattering granules. Swart and co-workers (2012) also noted bubble production as intracellular granules in both older and younger cells, which may suggest that bubble production, and thus fermentation, is not strictly linked to cell age. The overall shape of the YM-grown cells proved to be another unexpected result. As the discovery of intracellular gas bubbles inside fermenting yeasts is a recent finding, there have been no coincident examinations of CO_2 bubble formation and other cellular processes. However, given the effect of bubble formation on the overall shape of the cell, it seems plausible that normal organelle and cell function would be influenced/affected. Indeed, intracellular gas accumulation in other biological systems has been shown to have profound physiological effects, including control of cell proliferation, induction of apoptosis, and relaxation and constriction of vascular tissues (Napoli et al., 2013; Yetik-Anacak et al., 2015).

6.6.2 Nano Scanning Auger Microscopy and Hi-Res Scanning Electron Microscopy

In the first applications of NanoSAM AES analysis to biological samples (Swart et al. 2010), elemental abundance of cells of *Nadsonia fulvescens* was reported in terms of intensity. In reality, Auger electron intensities vary between elements and these variations should be taken into account to achieve measurement accuracy. Thus, the elemental abundance values in this study derived using correction factors represent accurate measurements. The earlier NanoSAM AES analysis of *Nadsonia fulvescens* also revealed

pronounced decreases in Os and Au measures at 135 nm etching depths (Swart *et al.,* 2010). A similar reduction in Os and Au % relative concentrations upon etching was not detected in any of the SMA cells grown in this study. While this discrepancy may be explained by variation in sample preparation, it may also point to differences in porosity and rigidity between biological samples. As vegetative brewing yeasts have a propensity to flocculate and are still metabolically active, it would seem that a less rigid cell exterior would be an asset. In contrast, a *Nadsonia fulvescens* ascosopre may be more rigid as this is best suited for durability. The aforementioned Auger-architectomic study of *Nadsonia fulvescens* was also only able to detect trace amounts of N within the cells (Swart *et al.,* 2010). In the current study N was present in very similar amounts as O, and was often more abundant than O (Fig. 6.2b,d).

Hi-Res SEM investigations revealed that the size and complexity of bubble networks within a FOV varied. This suggests that the capacity for fermentation and bubble production may vary across the same across cell population. Heins et al. (2012) reported highly dynamic subpopulation distributions at different growth stages when *Saccharomyces cerevisiae* was grown in a bioreactor and that these subpopulations changed in response to induced glucose and ethanol gradients. Speers et al. (1993) also noted both flocculent and non-flocculent industrial brewing strains had non-normal surface charge distributions. It is possible that heterogeneity in bubble network size may arise from similar gradients or they may occur due to the natural non-synchronous cell populations that arise during fermentations (Speers et al., 1993; Powell et al., 2003; Carlquist et al., 2012). Alternatively, some of the changes in cell ultrastructure may have been obscured during cell preparation or in the ultra-high vacuum environment of the NanoSAM. The Hi-

Res SEM viewed cells were also more wrinkled later in the fermentation, which has been noted in previous SEM studies of older fermenting yeasts (Barker & Smart, 1996; Powell et al., 2000). Another potential contributing factor to the wrinkled cell exteriors may have been the CO_2 anaesthesia effect, where membrane domains in yeast are malformed by dissolution of molecular CO_2 in the lipid bilayer (Isenschmid et al., 1996).

6.6.3 Transmission Electron Microscopy

When the YM-grown SMA strain was subjected to TEM analysis, the 72 h old cells (Fig. 6.4e) were filled with gas bubbles to a greater extent than any other *Saccharomyces cerevisiae* or *pastorianus* fermentation strains tested with this same TEM technique (Swart et al., 2012, 2013). This observation emphasizes the variability between strains with respect to CO_2 production, and identifies the SMA strain as an excellent candidate for future studies on bubble formation. These results also imply growth in YM media and TEM analysis could be a useful screening technique in selecting strains with high intracellular CO_2 production and accumulation. Capture and sequestration of CO_2 is a growing revenue source in beverage and fuel ethanol fermentation operations (Xu et al., 2010). Thus, developing better techniques to select strains with high intracellular CO_2 accumulation could make industrial CO_2 sequestration more efficient.

Given the volume of CO_2 present in the TEM-viewed cells, it is conceivable that the CO_2 anaesthesia effect (Isenschmid et al., 1996) contributed to the formation of the osmiophilic layers which were largest when bubble formation was most prevalent (Fig. 6.4e). No such osmiophilic layers were present in the YPG-grown cells (Fig. 6.4b,d,f), which warrants further investigation of the overall lipid content and fatty acid profile of fermenting vs. respiring cells. In earlier studies Kock et al. (2000) noted the dark-staining,

lipid-rich osmiophilic layers that were present in older fermenting cells, and described how these layers appeared to migrate through cells in a "ghost-like fashion". This observation was made more than a decade before the discovery of intracellular gas bubbles in fermenting yeasts, but was likely indicative of their presence. It is now crucial to address how bubble formation and associated membrane changes during fermentation effect cell stability and flocculation. A wrinkled and rough exterior is thought to promote flocculation in fermenting yeasts (Powell et al., 2003); however, bubble formation and flocculation must now be jointly examined in industrial fermentations to assess if these phenomena are correlated.

The presence of intracellular gas bubbles in both lager and ale fermenting strains also brings into question the current yeast flocculation paradigm. In the case of ale yeast, it is thought that these cells form loose flocs that trap evolved CO_2 bubbles as they rise to the surface of the fermenting medium (Briggs, 2004; Dengis et al., 1995; Vidgren & Londesborough, 2011). However, the work of Swart et al. (2013) discovered that CO_2 also becomes entrapped inside ale yeast cells which could also contribute to the buoyancy of the cells. It is now necessary to examine the effects of intracellular vs. intercellular CO_2 bubbles on ale yeast flocculation. Furthermore, it remains to be elucidated why lager yeast settle to the bottom of a fermentation vessel and ale yeast float to the top when both cells accumulate intracellular CO_2.

6.6.4 Time-of-Flight Secondary Ion Mass Spectrometry and Confocal Laser Scanning Microscopy

As with the NanoSAM results, it was clear that the cell compositions detected by the TOF-SIMS were different at the exterior and within the cell (Fig. 6.5; Fig 6.6). Interestingly,

with NanoSAM AES analysis, N and O were present in near equal amounts during the targeted analysis (Fig. 6.2 b,d), but the TOF-SIMS analysis indicated O^- was much more abundant than NH^- (Fig. 6.5; Fig. 6.6). This discrepancy could arise because the analysis area with the NanoSAM AES technique is much more defined and specific than that achieved with TOF-SIMS. With the NanoSAM AES analysis it was also not possible to detect P or S in reportable amounts, but P^- and S^- could be detected with the TOF-SIMS (Fig. 6.5; Fig. 6.6), with S^- in greater abundance. The prevalence of S^- over P^- may indicate a greater abundance of sulfur-containing amino acids relative to phosphorus-containing cellular lipids (Hershey & Chase, 1952; Choi et al., 2016). In the current yeast flocculation paradigm, hydrophobicity is thought to play an important controlling role in this phenomenon, but there is uncertainty whether cell surface proteins or lipids impart more of a hydrophobic effect (Speers et al. 2006; Potter et al., 2015).

Earlier immunofluorescence experiments examining 3-OH oxylipin distribution in *Dispodascopsis uninucleata* suggested a link between cell cycle, metabolism and 3-OH production as these oxygenated fatty acids were present in the sexual stage, but not during vegetative growth (Kock et al., 1998). Immunofluorescence results of the SMA strain in YM vs. YPG media implied that 3-OH oxylipin production differed in fermenting and respiring cells. For example, after only 24 h growth time, immunofluorescence trials indicated the respiring cells produced more 3-OH oxylipins than the fermenting cells (Fig. 6.7 b). While 3-OH oxylipins are believed to originate during β-oxidation or partial β-oxidation, (Kock et al., 2007, Kock et al., 2013), the exact pathway which gives rise to these hydroxy fatty acids remains unknown. The results of this study indicate that the origin

of 3-OH oxylipins in yeast may be better understood by studying the divergent lipid and fatty acid metabolisms in fermenting vs. respiring cells.

6.7 CONCLUSIONS

In this study the ability of Auger-architectomics to distinguish between elemental compositions of biological tissue was demonstrated based on the previously published atomic concentrations of N in *Nadsonia fulvescens* and the SMA strain of *Saccharomyces pastorianus* examined here. TOF-SIMS depth profiling analysis also revealed that the relative proportions of C^- and O^- varied at the exterior and interior of the cell in the SMA strain and with fermentation or respiration growth metabolisms. Furthermore, the production of 3-OH oxlipins appeared to differ in fermenting and respiring cells, and thus with bubble production, based on investigations with immunofluorescence microscopy and CLSM. Therefore, this study has demonstrated there is a difference in the composition and 3-OH oxylipin profile of bubble-filled and non bubble-filled brewing yeast cells. This study has further indicated that the origin of 3-OH oxylipins in brewing yeast may be revealed by examining the divergent lipid and fatty acid metabolisms in fermenting vs. respiring cells. Finally, the presence of intracellular bubbles in bottom fermenting and top fermenting yeast strains has challenged the current brewing yeast flocculation paradigm. Further investigations examining the influence of intracellular and intercellular CO_2 on brewing yeast aggregation are now required.

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CHAPTER 7 CONCLUSIONS

7.1 GENERAL CONCLUSIONS

Oxylipins are a ubiquitous class of molecule that occurs throughout nature and are often bioactive. 3-OH oxylipins, specifically, are found in fermenting yeasts and are believed to be associated with the flocculation process. In the past these analytes have been primarily identified following a diazomethane-catalyzed methylation during GC-MS analysis; however, the difficulty associated with diazomethane use has required the application of alternative approaches for the analysis of 3-OH oxylipins in fermenting yeasts. Initial attempts to identify 3-OH oxylipins in the SMA strain using a HIP extraction and a H_2SO_4-catalyzed derivatization proved unsuccessful.

Further trials found that 3-OH oxylipins could be analyzed using an ethyl acetate extraction, trimethylsilylation of the total lipid extract and detection using GC-MS. With this approach 3-OH 10:0 was identified in the SMA strain grown in the miniature fermentation assay. Samples were collected at 12, 24, 36, 48 and 60 hours fermentation time and flocculation level and CSH were measured at the same intervals. Detectable levels (as integratable chromatographic peaks) of 3-OH 10:0 appeared at 24 hours fermentation time which was 12 hours before elevated flocculation levels were reached. The oxylipin 3-OH 10:0 was also found to be quite polar based upon poor solvation in hexane and low recovery (0.26 ± 0.18 %) during a HIP extraction. This result seemed in contrast to several earlier reports that described fermenting yeast 3-OH oxylipins as hydrophobic molecules.

In the past, diazomethane-catalyzed methylation has been one of the primary means that FFA were specifically detected with GC and/or GC-MS analysis.

Increasingly, this reagent is becoming more difficult to obtain and may not adhere to the safety codes of many laboratories. It was found that there are numerous means to still specifically detect FFA from other lipid species in a total lipid extract and this can be achieved by selective quantifications/extractions, purifications and alternate derivatizations. Notably, each of methyl iodide and DMP can serve as true alternatives to diazomethane as long as the solvent system is optimized, but there are many different ways to specifically detect FFA without diazomethane highlighted in Chapter 4 of this thesis.

3-OH oxylipins in yeast were also quantitatively assayed in the SMA strain grown in the miniature fermentation assay without diazomethane use by employing a TLC separation, BCl_3-catalyzed methylation and further HFB derivatization followed by analysis using NCI-MS. Using this approach 3-OH 10:0 was undetectable 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 to a maximum of 4.82 ± 0.18 ng/mg dry cell mass at 60 h fermentation time. It was also noted that efficacy of NCI-MS analysis was dependent upon instrument and protocol optimization. Notably, each of i) a buffer wash following HFBA derivatization, ii) background minimization and iii) ion trap and source cleanliness we found to be critical for NCI-MS operation.

Nanotechnological examination of the standard SMA strain using Auger-architechtomics, TOF-SIMS and microscopic examination revealed the presence of intracellular networks of CO_2 in fermenting cells that increased in complexity with fermentation duration. Respiring cells contained very few bubbles and had predominantly

large vacuolar structures in the cell interior. Fermenting cells also produced osmiophilic layers at the cell exterior that became more apparent as the fermentation proceeded. Similar osmiophilic layers were absent in respiring cells in the SMA strain. TOF-SIMS analysis further revealed a compositional difference at the exterior and interior of SMA cells and in fermenting vs. respiring cells. Bubble-filled fermenting cells also appeared to have a different 3-OH oxylipin profile than respiring cells when the cells were examined with immunofluorescence microscopy.

7.2 CONTRIBUTIONS TO THE ADVANCEMENT OF KNOWLEDGE

When this research project began 4 years ago, the initial goal was to investigate 3-OH oxylipin accumulation and thus, CSH as a force implicated in brewing yeast flocculation. The methods and techniques to assay these hydroxy fatty acids in yeast and other biological systems were, however, not well defined. In initial correspondence with Dr. Ivo Feussner, a plant oxylipin expert at the University of Goettingen, he cautioned that 3-OH oxylipin analysis in yeast was challenging and that researchers at the same institution had struggled with this detection. Similarly, Kock and co-workers noted the presence of 3-OH oxylipins at flocculation onset, but had not monitored their formation over the course of fermentation. Thus, this project's initial contribution to the advancement of knowledge was to track the presence of 3-OH oxylipins in brewing yeast over the course of lab-scale fermentations.

In a similar early correspondence at the outset of this project, Prof. Kock had noted that the Lipid Biotechnology group had abandoned analysis of yeast 3-OH oxylipins due to the lack of available diazomethane, which he suggested was a crucial

part of detecting these analytes. Much of the initial work of this project was devoted to devising analytical methods for 3-OH oxylipins in yeast with approaches that did not require diazomethane. Therefore, the second contribution to the knowledge achieved in this project was the detection of 3-OH oxylipins in yeast using two approaches that did not require diazomethane: i) silylation of the total lipid extract and attention to the diagnostic m/z 233 ion and ii) purification of 3-OH oxylipins with TLC, conversion to HFB methyl esters and subsequent analysis with NCI-MS. During the method development work, it also became apparent that a compendium of methods for detection of FFA without diazomethane would be useful in the lipid science literature. The third contribution of this thesis to the knowledge base was to compile an analysis guide for NEFA with approaches that did not require diazomethane.

In the early stages of this project a dialogue was also initiated in the literature between our lab and researchers at UFS (Chapter 2). In their letter Prof. Kock and colleagues suggested they had only been able to qualitatively establish the presence of 3-OH oxylipins in most yeasts. The fourth contribution of this thesis to the advancement of knowledge was to accomplish the first quantitative analysis of 3-OH oxylipins in yeast using NCI-MS analysis of the HFB methyl ester derivatives. Since discontinuiung chemical analysis of yeast 3-OH oxylipins, the Lipid Biotechnology group has recently embarked on an extensive nanotechnology research program which, for the first time, uncovered the presence of CO_2 bubbles inside fermenting yeasts. Auger-architectomics has only been applied to a limited number of industrial fermentation yeasts and has not yet been applied to any standard test strains. Therefore, the final contribution of this

project was to characterize the standard SMA strain with Auger Architectomics and TOF-SIMS.

7.3 FUTURE WORK

This fermentation yeast 3-OH oxylipin research project has focused on addressing, examining and solving some of the analytical challenges that occur when working with these medium-chain hydroxy fatty acids. Furthermore, the SMA strain, used in all the early chapters of this thesis, was later examined with the nascent nanotechnological techniques used at UFS in an attempt to better understand how fermentation (bubble production) and respiration are related to 3-OH oxylipin production and the industrially important flocculation phenomenon. Nonetheless, many details around 3-OH oxylipin synthesis, secretion and function remain unknown and more study is needed to completely understand the exact role of these compounds in the flocculation process.

To analyze oxylipins and other fatty acids in the SMA strain, the total lipid extract was trimethylsilyated, but this detection method does have the drawbacks that silyl esters and ethers are hydrolytically unstable and can only be stored for short periods of time. In the future, the 3-OH oxylipins could be analyzed with a number of the other approaches described in the literature review/analysis guide (Chapter 4) so the samples may be stored and re-analyzed as part of a more comprehensive study. In particular, both methyl iodide and DMP could have been used as a substitute for diazomethane to selectively methylate the FFA, provided the reaction conditions were optimized.

Detection of 3-OH 10:0 as the HFB methyl ester using NCI-MS provided the first quantitative analysis of 3-OH oxylipins in yeast, but further studies may still be done in

conjunction with this quantitative analysis. Since the total lipid extract was purified using TLC and 80:20:2 (v/v/v) hexane: diethyl ether: acetic acid, the neutral lipids extracted with the ethyl acetate extraction were separated into their respective lipid classes. Later research projects may quantify the levels of 3-OH oxylipins in fermenting yeast and coincidently monitor the fatty acid profiles of the different lipid classes. By changing the solvent systems in the TLC protocol to include a phospholipid separation, the different phospholipids, which are the most abundant lipid class in the yeast cell membrane, and their fatty acid constituents may be analyzed. Furthermore, a better separation of 3-OH oxylipins will likely be achieved with this approach as the 3-OH 10:0 and 11:0 standards had very similar Rf values to the phospholipids. The new analytical technique should also be coupled with assays for β-oxidation activity and substrate consumption. It is also now important to study the effect of 3-OH oxylipins on fermenting cell ultrastructure, considering their highly polar nature and their abundance relative to oxylipins in other biological systems.

A number of follow up studies are also now required based upon the results of the nanotechnological examination of the SMA strain. Given the absence of osmiophilic layers in the YPG-grown cells, the overall lipid content and fatty acid profile of fermenting bubble-filled cells compared to respiring cells which contain far fewer bubbles should be studied. It is also crucial to address how bubble formation and associated membrane changes during fermentation effect cell stability and flocculation. In particular, bubble formation and flocculation must now be tracked in industrial fermentations to assess if these processes are correlated. The effects of intracellular vs. intercellular CO_2 bubbles on ale yeast flocculation should be studied as well as determining why lager yeast settle to the

bottom of a fermentation vessel and ale yeast float to the top even though both cells accumulate intracellular CO_2. Finally, the 3-OH oxylipin signatures of fermenting and respiring yeasts must be analyzed because immunofluorescence results have implied the respective profiles are different. TOF-SIMS would be a convenient platform to study the 3-OH signatures and future work may use this technique. Such an analysis would provide a chemical signal to help understand the phycial (or intercellular) differences identified in repsiring and fermention cells.

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APPENDIX B CHROMATOGRAM AND MASS SPECTRA FROM SILYLATED LIPID EXTRACT

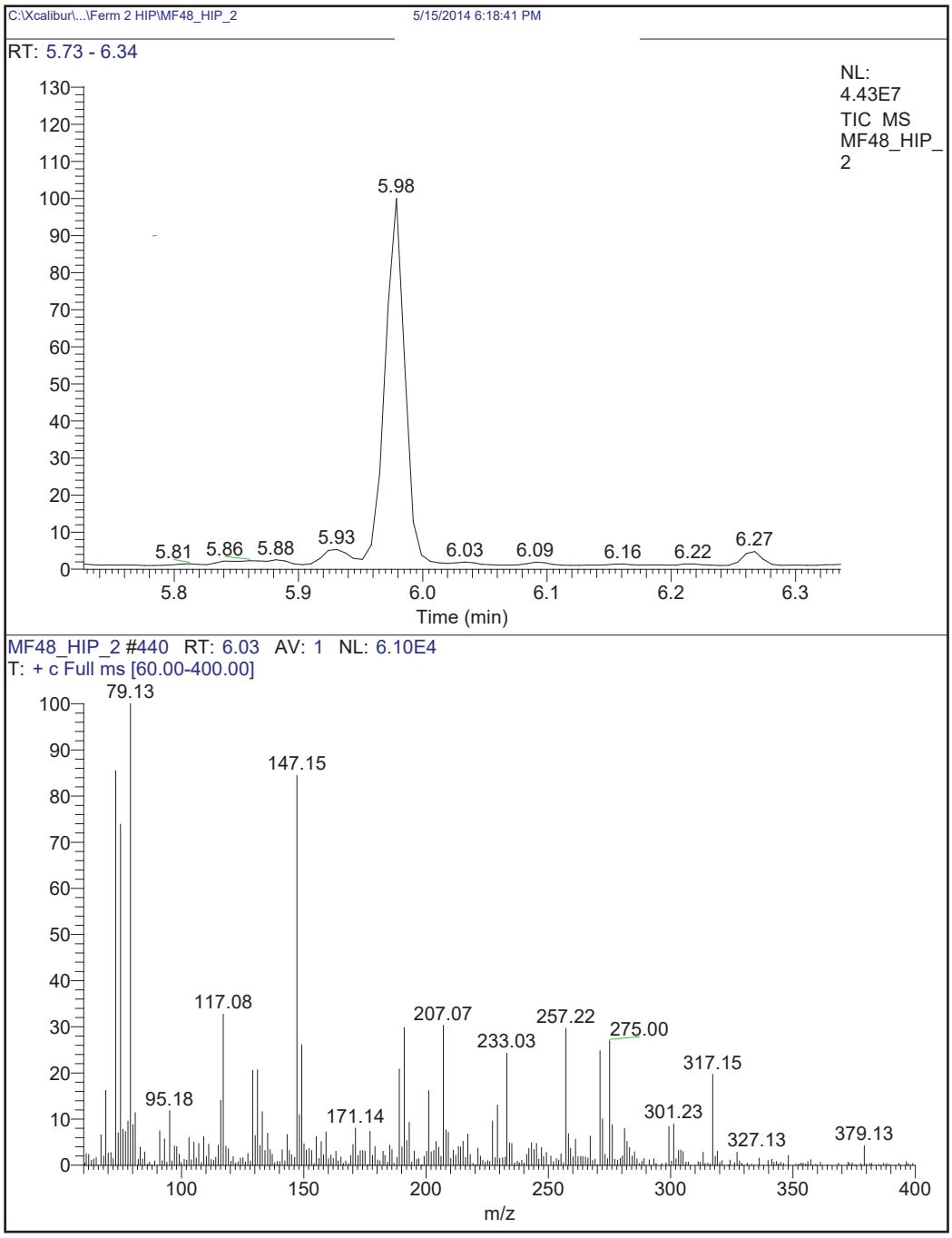

Figure A1. A section of a total ion chromatogram trace produced from cells grown in the miniature fermentation assay for 48 hours where the lipids were HIP extracted The mass spectra at 6.03 min is shown which is the retention time for double silylated 3-OH 10:0. The diagnostic m/z 233 fragment ion is visible and a positive match for double silylated 3-OH 10:0 was obtained when this spectra was compared to the NIST database.

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