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Calibration curve-free GC-MS method for quantitation of amino and non-amino organic acids in biological samples	
Nature of contribution by PhD candidate	Experimental work, data analysis and writing
Extent of contribution by PhD candidate (%)	75%

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David Greenwood	Assistance with experimental design and manuscript edition
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Please indicate the chapter/section/pages of this thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work.	Rapid Quantitative Fatty Acid Profiling of Biological Samples by Gas Chromatography-Mass Spectrometry
Nature of contribution by PhD candidate	Experimental work, data analysis and writing
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Nature of contribution by PhD candidate	Experimental work, data analysis and writing
Extent of contribution by PhD candidate (%)	80%

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Please indicate the chapter/section/pages of this thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work. Effect of Fatty Acids on Sauvignon Blanc Fermentation

Nature of contribution by PhD candidate	Experimental work, data analysis and writing
Extent of contribution by PhD candidate (%)	75%

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Name	Nature of Contribution
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CHAPTER I

General Introduction

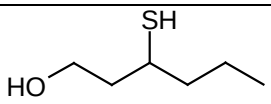
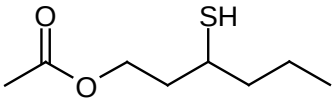
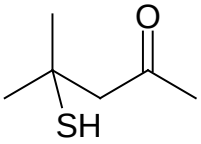
1.1. From the origins of wine to modern winemaking trends in New Zealand

The mastery of wine making is one of the oldest activities that involves microorganisms in fermentation processes alongside cheese making and beer brewing (Robinson, 2006). According to historians and archaeological findings, the grape juice was purposely fermented in different parts of the world as early as 7000 BC (McGovern, 2003; McGovern *et al.*, 2004). Gradually, the idea of producing wine – a beverage with an unusual, pleasant, psychotropic effect (Pretorius *et al.*, 2012), spread throughout the Roman Empire, Greece, Northern Europe, and by the 16^{th}-17^{th} century reached North, Middle and South America (von Bassermann-Jordan, 1923). The first arrival of grape vines to New Zealand was documented in the early 1800's with the first vine planted in 1819 by Samuel Marsden (Scott, 1964).

Vitis vinifera L. cv. Sauvignon Blanc originated from Bordeaux and Loire Valley regions in France, before becoming globally cultivated (USA, South Africa, Chile, Australia and New Zealand) (Jackson, 2008) and used in production of one of the most distinctive aromatic dry white wines. The first New Zealand Sauvignon Blanc (SB) wine was produced in 1974, resulting in the first SB vintage in 1979 (Cooper, 2008). Produced from grapes grown in cool sunshine climate and moderately fertile soil, Sauvignon Blanc is today New Zealand's flagship wine style, recognised locally and internationally for its distinguishable fruity, tropical flavours (Lund *et al.*, 2009). The recognised value of SB wine on an international level and its increasing consumer demand, make SB New Zealand's most planted grape varietal. SB wine made up 85.5% of New Zealand's wine exports, shipping to over 80 countries, with a total export value upwards of $1.33 billion in 2014 (New Zealand Winegrowers, 2014). New Zealand Sauvignon Blanc wine has earned international recognition mainly for its high levels of volatile thiols, such as 3-mercaptohexyl acetate (3MHA), 3-mercaptohexan-1-ol (3MH) and 4-mercapto-4-methylpentan-2-one (4MMP) (**Table 1.1**), which are extremely odorous molecules responsible for

passionfruit, grapefruit and box tree (cat's pee) aromas, respectively (Benkwitz *et al.*, 2012).

Table 1.1. Structure and sensory characteristics of volatile thiols in SB wine[1]

Volatile thiol	Structure	Description	Perception threshold, ng/L
3-mercaptohexan-1-ol (3MH)		Passionfruit	60
3-mercaptohexyl acetate (3MHA)		Grapefruit	4
4-mercapto-4-methylpentan-2-one (4MMP)		Box tree, cat's pee	0.8

[1] Benkwitz *et al*, 2012

In 2004, the multidisciplinary SAUVIGNON BLANC PROGRAMME was initiated as collaboration between NZ Government, wine industry and leading research organisations, in order to produce wine with consistent quality to satisfy a consumer-driven market, which is essential for maintaining a strong international reputation and sustainable growth of the NZ wine industry (http://www.sbprogramme.co.nz/). Ongoing collaboration between research teams from The University of Auckland (http://www.winescience.auckland.ac.nz/), Lincoln University (http://www.lincoln.ac.nz/), Plant and Food Research (http://www.plantandfood.co.nz/), and Marlborough Wine Research Centre (http://www.mrc.org.nz/) aim to provide tools for the NZ wine industry to maintain

an exclusive NZ Sauvignon Blanc style, and to design original and distinctive bouquets and aromas from Sauvignon Blanc grapes (http://www.sbprogramme.co.nz/).

1.2. Sauvignon Blanc wine aroma

The aroma of Sauvignon Blanc wine comprises hundreds of different odorous compounds with concentrations varying from ng/L to mg/L (Pinu *et al.*, 2014) creating an endless combination of flavours (Lambrechts *et al.*, 2000). SB has a distinctive aroma usually described as citrus, capsicum, gooseberry, mandarin peel, grapefruit and passionfruit (Parr *et al.*, 2005; Nicolau *et al.*, 2006). The ensemble of these compounds is responsible for specific aroma of SB wine, however, the key aromas of SB wines are predominantly from a considerably small group of odorous compounds such as methohypyrazines, acetate and ethyl esters, C_6 alcohols and aldehydes, higher alcohols, fatty acids, terpenes, and volatile thiols (Jouanneau *et al.*, 2012).

Secondary metabolites of amino acid metabolism in plants (Ribéreau-Gayon *et al.*, 2006) – methoxypyrozines - are nitrogenated heterocycles found in grape juice and highly odorous compounds with remarkably low perception thresholds as low as 2 ng/L (Buttery *et al.*, 1969; Murray *et al.*, 1970; Seifert *et al.*, 1970). Methoxypyrozine compounds add “green” and “vegetable” (i.e. asparagus and capsicum) aromas to SB wines (Allen *et al.*, 1991). Ethyl and acetate esters are another important group of aroma compounds in SB wine, mainly responsible for fruity and floral aromas (Pinu *et al.*, 2014). C_6 compounds develop during grape ripening and through the enzymatic oxidation of unsaturated fatty acids during fermentation (Oliveira *et al.*, 2006), adding “grassy” flavours to SB wines. C_6 compounds have high perception threshold (up to 2 mg/L) (Peinado *et al.*, 2004), therefore playing an important role in NZ SB wine aroma (Benkwitz *et al.*, 2012).

Unlike other aroma compounds, volatile thiols – sulphur-containing compounds with an -SH group attached to a carbon atom – have the most prominent impact on the bouquet of NZ Sauvignon Blanc wine, adding the pronounced "tropical" fruit aroma (Pinu *et al.*, 2014). Volatile thiols are known to be the products of yeast metabolism during alcoholic fermentation (Pinu *et al.*, 2014), nevertheless, trace amounts of 3MH were also detected in grape juice prior to fermentation (Capone *et al.*, 2011). Potential biosynthetic pathways and putative precursors for volatile thiol production during fermentation have been studied extensively (Allen *et al.*, 2011; Capone *et al.*, 2011; Fedrizzi *et al.*, 2009; Harsch *et al.*, 2013; Schneider *et al.*, 2006). It was found however that concentrations of 3MH, 3MHA and 4MMP have low correlations with their cysteinylated (Cys-3MH, Cys-4MMP) and glutathionylated (GSH-3MH, GSH-4MMP) putative precursors in grape juice (Pinu *et al.*, 2012; Roland *et al.*, 2010) - demonstrating a weak relationship between thiols and their corresponding direct precursors (**Figure 1.1**). It is evident that despite the extensive research, the biogenesis of these thiols still remains poorly understood.

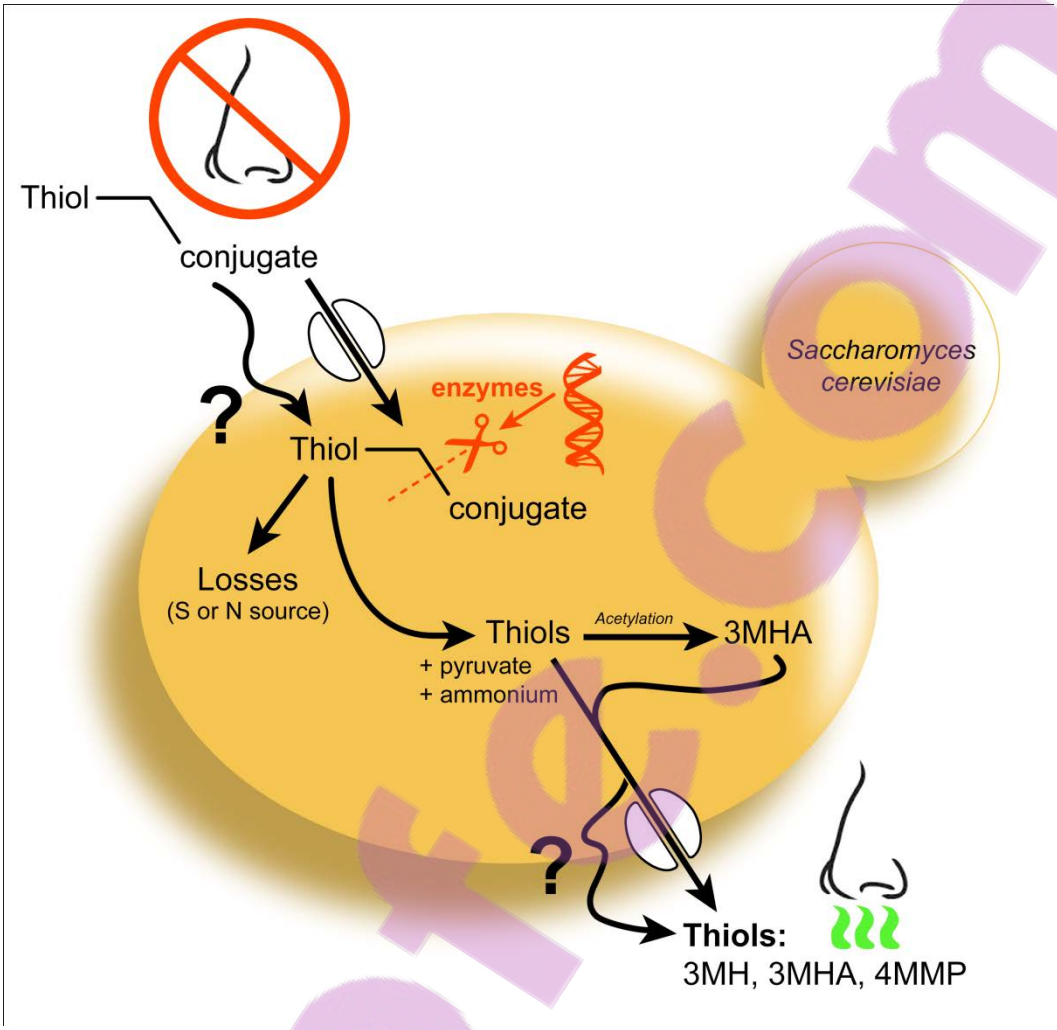


Figure 1.1. Proposed volatile thiol biosynthesis by yeast. Question marks represent processes which are not fully understood

1.3. The influence of different factors on volatile thiol concentration in Sauvignon Blanc wine

Saccharomyces cerevisiae produce a wide range of odorous compounds in small quantities which contribute to a specific aroma of fermented wine (Cordente *et al.*, 2007). However, major alterations in wine aroma can be induced by a range of parameters such as: the biochemical composition of grape juice itself (result of viticultural practices), fermentation conditions (different yeast species (Masneuf *et al.*, 2002) and strains (Howell *et al.*, 2004; Murat *et al.*, 2001; Swiegers *et al.*, 2009), and temperature (Howell *et al.*, 2004; Masneuf-Pomarede *et al.*, 2006; Murat *et al.*, 2005). Masneuf *et al.* (2002) showed that fermentations with different indigenous wine yeast strains and hybrids (*S.bayanus*) resulted in higher amounts of 3MH and 4MMP. Peyrot des Gachons *et al.* (2002) found that Cys-3MH (3MH precursor) was more abundant in grape skin, whilst Cys-4MMP (4MMP precursor) was found to be equally presented in the berry and skin. Thus, it was proposed that longer contact of grape skin with juice will increase the concentration of 3MH precursor in the must (Maggu *et al.*, 2007; Murat *et al.*, 2001; Peyrot des Gachons *et al.*, 2002) and therefore, will increase the concentration of 3MH during the wine fermentation. Temperature also has an impact on the production of 4MMP (Howell *et al.*, 2004), causing the increase of this thiol during fermentation at higher temperatures. Despite these findings, Lee *et al.* (2008) observed that the biochemical composition of grape juice was the predominant factor affecting the development of 3MH and 3MHA in fermented wine.

In order to understand the complex biogenesis of volatile thiols and other aroma compounds developed during wine fermentation to produce wine with desired properties, different manipulation (viticultural, oenological, genetic) strategies could be applied (Swiegers *et al.*, 2006; Cordente *et al.*, 2007; Gimeno-Alcaniz *et al.*, 2001). Howell *et al.* (2005) were the first to report about the genetic regulation of 4MMP production followed by a single-gene deletion experiment to validate their

hypothesis. Swiegers *et al.* (2006) described that 3MHA – an acetate ester of 3MH, is biosynthesised from 3MH under *ATF1* gene regulation. Based on juice manipulation experiments, Pinu *et al.* (2012 and 2014) proposed that low conversion yield of direct precursors into volatile thiols could be affected by the level of metabolites in grape juice involved in the respective biochemical reactions and regulated by genes expression, thus, introducing metabolomics, with its non-targeted, quantitative approach, as a powerful tool for investigating the influence of individual juice metabolites on the development of volatile thiols during wine fermentation. Pinu *et al.* (2014) performed a pioneering study where the comprehensive metabolomic analysis coupled to statistical analysis was employed in order to identify metabolites that had a high impact on volatile thiol development.

1.4. Metabolomics

Metabolomics is a relatively new "omics" approach with its first mention in a paper published by Oliver Fiehn in 2001 (Fiehn, 2001) aiming to provide untargeted and unbiased analysis of metabolites in biological samples (Villas-Bôas, 2013; Villas-Bôas *et al.*, 2005). In contrast to the traditional targeted metabolite analyses, which cover a defined number of analytes of interest (Halket *et al.*, 2004), metabolomics allows for investigations into the complete metabolic functioning of a system at the small molecule level (<1,500 Daltons), covering a wider range of metabolites and thus, broadening our understanding of biological systems, creating a niche for discovery work and hypothesis generation (Villas-Bôas, 2013; Vielhauer *et al.*, 2011). Metabolomics describes a biological system closest to the phenotype through the combination of information from both the underlying metabolism of a living system, as well as the influence of the environment (Villas-Bôas, 2013; Reaves *et al.*, 2011). In comparison to targeted metabolite analysis which is employed in analytical chemistry and biochemistry, metabolomics analyses a number of metabolite classes, using a range of analytical platforms, and most importantly,

demands powerful bioinformatics tools for high-throughput processing of spectral and chromatographic data considering all detected analytes (Gummer *et al.*, 2009). Comprehensive metabolite profiling became feasible as a result of recent technological progress in separation and identification of metabolites (Wishart, 2008), and can be achieved by using different analytical methods based on coupling of chromatographic separation (gas chromatography (GC), liquid chromatography (LC), and capillary electrophoresis (CE)), with various detection methods (mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR)) (Koek *et al.*, 2011). Metabolite profiling methods (fingerprinting and footprinting) have served their purpose in providing descriptive information about biological systems through qualitative and relative semi-quantitative data (Kvitvang *et al.*, 2011). However, quantitative characterisation of a system cannot be fully achieved without employing absolute metabolite concentrations, an area which is lacking in most current metabolomics platforms (Kvitvang *et al.*, 2011). Furthermore, an increased interest in dynamic modelling studies employing metabolomics makes absolute quantification of metabolites essential (Vielhauer *et al.*, 2011), providing information for the kinetic description of a system and helping make inter-laboratory data more readily comparable (Koek *et al.*, 2011).

1.5. GC-MS-based Metabolomics

GC-MS has been one of the most commonly employed analytical platforms for conducting metabolomics studies (Villas-Boas, 2013). GC-MS instruments with linear quadrupole analysers have been used for many years, performing highly robust analysis (Sugitate *et al.*, 2012). Developments in manufacturing of longer capillary GC columns (30, 60 and 100 m) together with controlled thickness of column stationary phase have led to improved and more efficient capillary GC separation of complex biological mixtures (Hernandez *et al.*, 2012). Demand for high-throughput metabolite analysis has led to the coupling of GC to a mass analyser with faster scan

rates – time-of-flight MS (TOF-MS). Recently, comprehensive two-dimensional gas chromatography, GCxGC, coupled to fast scan TOF MS analyser has emerged as a valuable tool for metabolomics, providing sensationally high chromatographic separation efficiency together with high MS resolution. Considering the amount of data generated using this platform, this may exceed the capacity of current bioinformatics tools for data processing, posing a significant limitation for data analysis (Halket *et al.*, 2004).

As it was mentioned above, GC-*single quadrupole*-MS is widely applied in non-targeted analyses of complex biological samples. However, due to complexity of biological samples, metabolite co-elution is a common phenomenon in routine GC analysis. In order to distinguish signals from co-eluting metabolites, a mathematical model called deconvolution must be applied, thus providing retention time and MS fragmentation information for metabolite identification and quantification (Tsugawa *et al.*, 2014). In case of metabolites with identical retention time and similar fragmentation pattern (e.g. *cis*- and *trans*-isomers of fatty acids) deconvolution cannot distinguish compounds leading to metabolite misidentification and loss in accurate quantification. Selected reaction monitoring (SRM) analysis applied using GC triple-quadrupole mass spectrometer (GC-QqQ-MS) has become a valuable technique in order to address this problem (Tsugawa *et al.*, 2014). In SRM analysis the signal for individual metabolite can be efficiently distinguished from co-eluting peak and background noise using precursor (MS1) and product (MS2) ion-pairs, therefore providing selectivity in metabolite identification (Tsugawa *et al.*, 2014).

Nowadays, there are a number of GC-MS instruments readily available on the market. However, the main drawback for GC-based platforms is that it can be employed only for separation of volatile and semi-volatile analytes. As most metabolites are non-volatile (e.g. amino and organic acids, amines, sugars), this adds an extra step in the sample preparation procedure – chemical derivatisation, which facilitates the volatilisation of metabolite derivatives (Kvitvang *et al.*, 2011). Not all

metabolites, however, can be made volatile, especially larger metabolites which form strong intermolecular interactions (e.g. glycerophospholipids, ceramides).

GC-MS-based techniques are an attractive candidate for absolute quantitation due to their remarkable chromatographic resolution combined with reproducible MS detection using electron impact ionisation (EI) (Koek *et al.*, 2011). The high sensitivity of the single quadrupole mass detector, reproducible ionisation and fragmentation pattern of EI for a broad range of metabolite derivatives, informative mass spectra (Vielhauer *et al.*, 2011), availability of mass spectra libraries (Koek *et al.*, 2011), equipment affordability and low maintenance cost, make GC-MS an ideal analytical platform (Villas-Bôas *et al.*, 2011) for quantitative metabolomics applications.

1.6. Alternative methods for Metabolomics

1.6.1. LC-MS-based Metabolomics

There are several other analytical platforms based on chromatography methods coupled to MS that are readily available and used for metabolomics studies. Among these, liquid chromatography-mass spectrometry (LC-MS) is the most common. Compared to GC, the chemical derivatisation of metabolites is not required in LC-MS and the sample is able to be analysed directly (Gummer *et al.*, 2009; Halket *et al.*, 2004). The LC-MS platform has advantages over GC-MS in its ability to analyse native non-derivatised metabolites, and its ability to analyse high molecular weight metabolites of different polarity (e.g.; lipid analysis) as well as thermolabile metabolites (e.g. phosphorylated sugars, nucleotides and nucleosides) (Vielhauer *et al.*, 2011). Recent developments in ultra performance liquid chromatography (UPLC) have initiated a breakthrough in metabolite analysis utilising LC-MS - as smaller UPLC columns have higher separation efficiency compared to traditional HPLC columns, resulting in shorter analysis time and lower solvent consumption (Viant *et al.*, 2013). However, issues with reproducibility and precision of quantification for

LC-MS analysis originate from complexity of the analysed sample, where stronger matrix effect can lead to reduced ionisation efficiency of co-eluting metabolites (Bruheim *et al.*, 2013).

Previously mentioned, chemical derivatisation of metabolites for further analysis via LC-MS is not required, however, it can serve as an alternative strategy for absolute quantification of selected subclass of metabolites. Thus, Boughton *et al* (2011) described an approach in which specific subset of metabolites (primary and secondary amines) are chemically modified and analysed using LC-MS, demonstrating robustness of such strategy.

1.6.2. CE-MS-based Metabolomics

Capillary electrophoresis-mass spectrometry (CE-MS) emerged as a powerful approach for profiling of charged analytes in biological samples (Ramautar *et al.*, 2013) and plays its main role as a complementary analytical platform to LC-MS in metabolite analysis (Gummer *et al.*, 2009). In capillary electrophoresis charged metabolites are characterised by electrophoretic mobility μ_0 – function of applied electric field, and separated based on their charge-to-mass ratio (z/m) (Ramautar *et al.*, 2009). CE has a remarkable separation efficiency (unlimited number of theoretical plates), high sensitivity, and has the ability to analyse metabolites without chemical derivatisation (Robledo *et al.*, 2014). CE-MS has been found as a powerful technique in many biomedical and clinical applications which required non-targeted approach (Ramautar *et al.*, 2015). Thus, CE-MS methods were developed for metabolite profiling of rat urine (Kok *et al.*, 2014), rat serum (Naz *et al.*, 2013), and also for biomarker screening in prostate cancer (Soliman *et al.*, 2012). However, majority of developed methods suffer from low retention time reproducibility caused by electrophoretic mobility shift. Moreover, high maintenance cost of CE-MS systems make this technique a much less desirable platform for metabolite analysis (Gummer *et al.*, 2009). A review of the CE-MS platform in metabolomics published

in 2014 (Robledo *et al.*, 2014) concludes that CE-MS is only a powerful, application driven alternative to other chromatographic techniques.

1.6.3. Direct infusion

Direct injection or infusion mass spectrometry (DI-MS) is based on direct introduction of metabolite extracts of complex biological samples into mass spectrometer without traditional chromatographic separation (Gonzalez-Dominguez *et al.*, 2014). This introduction of the sample into the ion source with no chromatographic separation has several advantages over classical hyphenated methods. DI-MS provides with non-targeted metabolite coverage, therefore, increasing high-throughput screening capacity of DI-MS-based methods (Gonzalez-Dominguez *et al.*, 2015). Together with metabolic fingerprinting (Kirwan *et al.*, 2013) and shotgun lipidomics (Ejsing *et al.*, 2009), direct infusion mass spectrometry emerged as the most suitable platform in clinical applications (Lokhov *et al.*, 2014; Southam *et al.*, 2014). This approach entirely depends on the resolving power (i.e. ability of separating of two narrow mass spectral peaks (Scigelova & Makarov, 2006)) of the mass analyser. In the case of high resolution MS, DI-MS can be almost as efficient as hyphenated methods. Because many LC systems have an option of direct sample introduction without prior separation, with high resolution mass analysers (e.g. FT-ICR-MS, Q-TOF, Orbitrap™), this approach is always an optional method for non-targeted analysis. Advantages of using DI-MS include the high throughput as samples may be analysed in only a few minutes, and a somewhat more unbiased approach due to the lack of sample preparation. Disadvantages of using DI-MS are in lack of isobars (i.e. atomic or molecular species with the same nominal mass but different exact masses (Murray *et al.*, 2013)) resolution, difficulty in quantification in absence of stable-isotope internal standards (Gonzalez-Dominguez *et al.*, 2015) and MS sensitivity suffering due to ion suppression (Kirwan *et al.*, 2013).

1.6.4. NMR-based Metabolomics

Nuclear magnetic resonance spectroscopy emerged in the middle of the last century and since has served as a powerful physico-chemical method mainly for structure elucidation of unknown compounds (Mahrous & Farag, 2015). NMR spectroscopy allows for detailed structural information as it measures the properties of nuclei under certain experimental conditions (Mahrous & Farag, 2015). Developments in NMR spectroscopy in the past decade have been driven mainly by its wide applications in the process of drug discovery (Leenders *et al.*, 2015) and plant metabolomics (Bingol & Bruschweiler, 2015). NMR can be described as non-destructive technique (i.e. the sample can be recovered after the analysis) providing absolute quantification through integration of metabolite ^{1}H NMR signals (Simmler *et al.*, 2014). However, high equipment and maintenance cost, low sensitivity, relatively low number of identified metabolites and lack of existing spectral data bases are the major drawbacks for NMR-based metabolomics and require further significant improvements for metabolomics purposes (Leenders *et al.*, 2015).

1.7. Quantitative Metabolomics

In the past decade metabolomics community has been discussing intensively the importance of accurate quantitative description of biological systems. Thus, quantitative metabolomics, the ultimate goal of which is to describe the system via absolute metabolite levels (i.e. concentrations) and keeping the approach non-targeted (Koek *et al.*, 2010; Noack *et al.*, 2014), has become a major direction in further developments in Metabolomics (Dias *et al.*, 2015). Modern analytical platforms utilised for metabolomics studies are able to provide robust absolute quantification of metabolites within the concentration dynamic ranges which are finite. However, the robustness of absolute quantification of each individual

metabolite in complex mixtures can be dramatically affected by fluctuating concentration of other metabolites in the sample. This interfering on signals from many metabolites is driven by various physico-chemical mechanisms (e.g. yield of chemical derivatization, ion suppression), commonly known as matrix effect (Redestig *et al.*, 2011). Matrix effect in complex biological samples poses a great concern regarding the reliability of generated quantitative data using all MS-based analytical platforms. In the absence of a reference biological sample, using a mixture of internal standards can help to evaluate the matrix effect and, therefore, provide accurate quantification. Nevertheless, preparation of internal standard mixtures can be very laborious and moreover, the availability of required internal standards could be an issue, particularly when analysing a metabolome that could comprise of hundreds of metabolites (Redestig *et al.*, 2011; Buscher *et al.*, 2009).

To address this problem, a few approaches were described to provide the internal standardisation for each component of the metabolome. Two main approaches in the literature are isotope dilution mass spectrometry (IDMS) (Bueschl *et al.*, 2014; Neumann *et al.*, 2014) (**Figure 1.2**) and isotope-coded derivatisation (ICD) (Kvitvang *et al.*, 2011; Bruheim *et al.*, 2013) (**Figure 1.3**). IDMS uses uniformly (U) ^{13}C-labeled metabolites (e.g. ^{13}C-U-glucose) that are fed to microorganisms (yeast, bacteria, algae) and enter into their metabolism to distribute and incorporate ^{13}C into metabolites. Cell extracts are then used as a mixture of internal standards spiked to analysed biological samples for accurate metabolite quantification (Vielhauer *et al.*, 2011). This method is widely used for accurate metabolite quantification employing LC-MS, however, it is significantly costly and laborious (Vielhauer *et al.*, 2011).

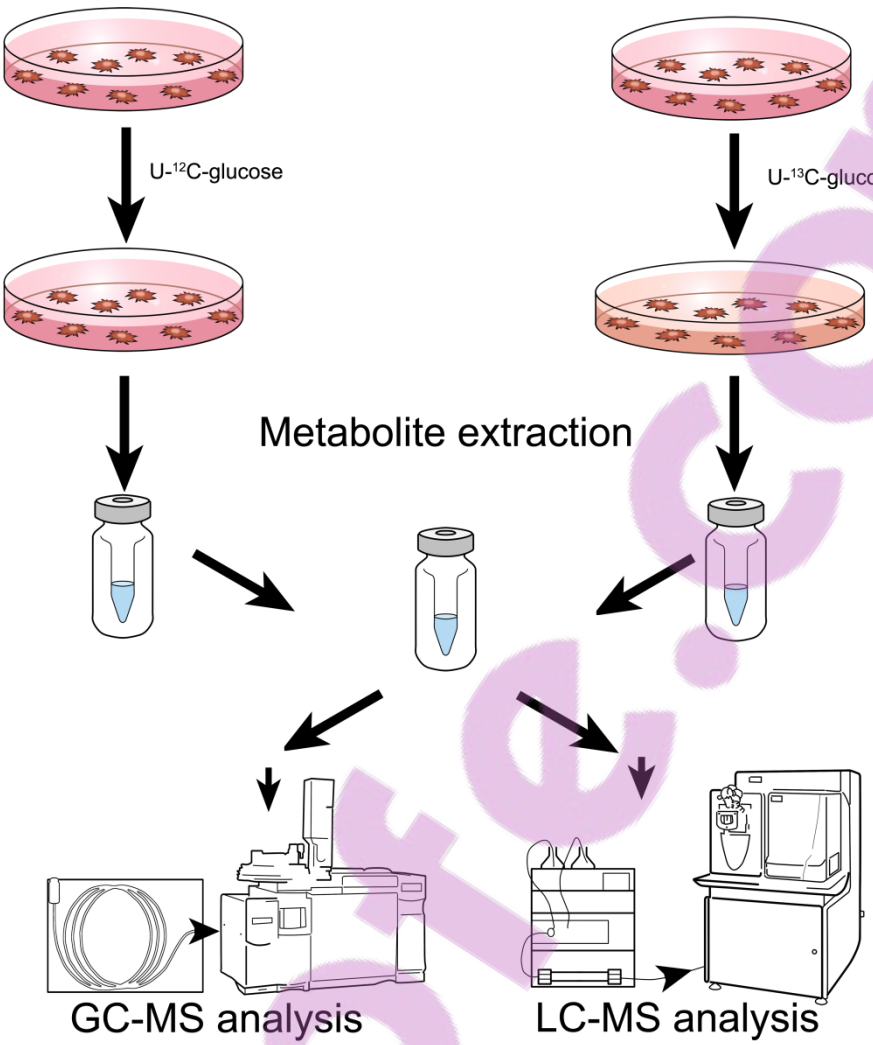


Figure 1.2. Schematic representation of isotope dilution mass spectrometry (IDMS) approach

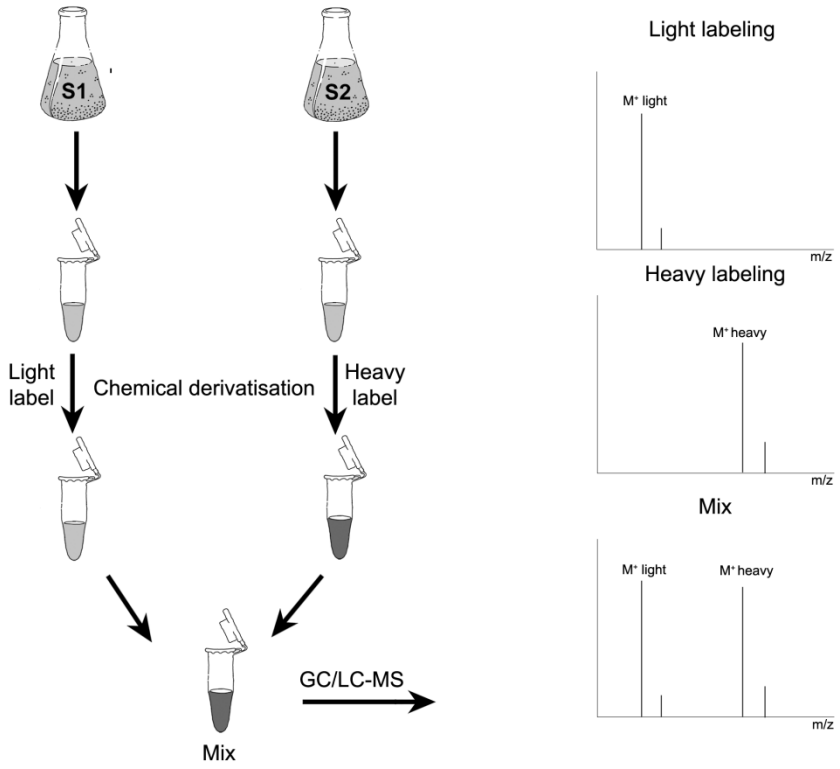


Figure 1.3. Schematic representation of isotope coded derivatisation strategy (ICD)

The ICD strategy was adopted for metabolomics studies utilising GC-MS, however, the availability of some derivatisation reagents is still an issue and often require custom organic synthesis. Chemical derivatisation of metabolites prior to metabolomic analysis is a common step in metabolomics studies utilising a GC-MS analytical platform, in order to increase volatility of analysed metabolites (Bruheim *et al.*, 2013). ICD strategy involves the chemical derivatisation of metabolites with a stable isotope-labelled derivatisation reagent generating labelled metabolite derivatives which are used then as internal standards for absolute quantification (Kvitvang *et al.*, 2011; Bruheim *et al.*, 2013).

1.8. Lipidomics

Lipidomics is a rapidly developing scientific discipline that involves the comprehensive qualitative and quantitative characterisation of the cellular lipidome (Herzog *et al.*, 2011). Despite the fact that the lipidome is the part of metabolome (Griffiths *et al.*, 2011), the important physiological role of the lipidome (e.g. component of cellular membranes, storage of energy and signalling function (Ejsing *et al.*, 2009)), its complexity (eukaryotic cells could comprise of hundreds of different lipid classes with total number of different lipid species reaching 100,000 (Herzog *et al.*, 2011), and its substantial differences in physical-chemical properties from polar metabolites, mean that distinctive methods are required for separation and analysis of lipids (Griffiths *et al.*, 2011), with a heavy reliance on high resolution mass spectrometry.

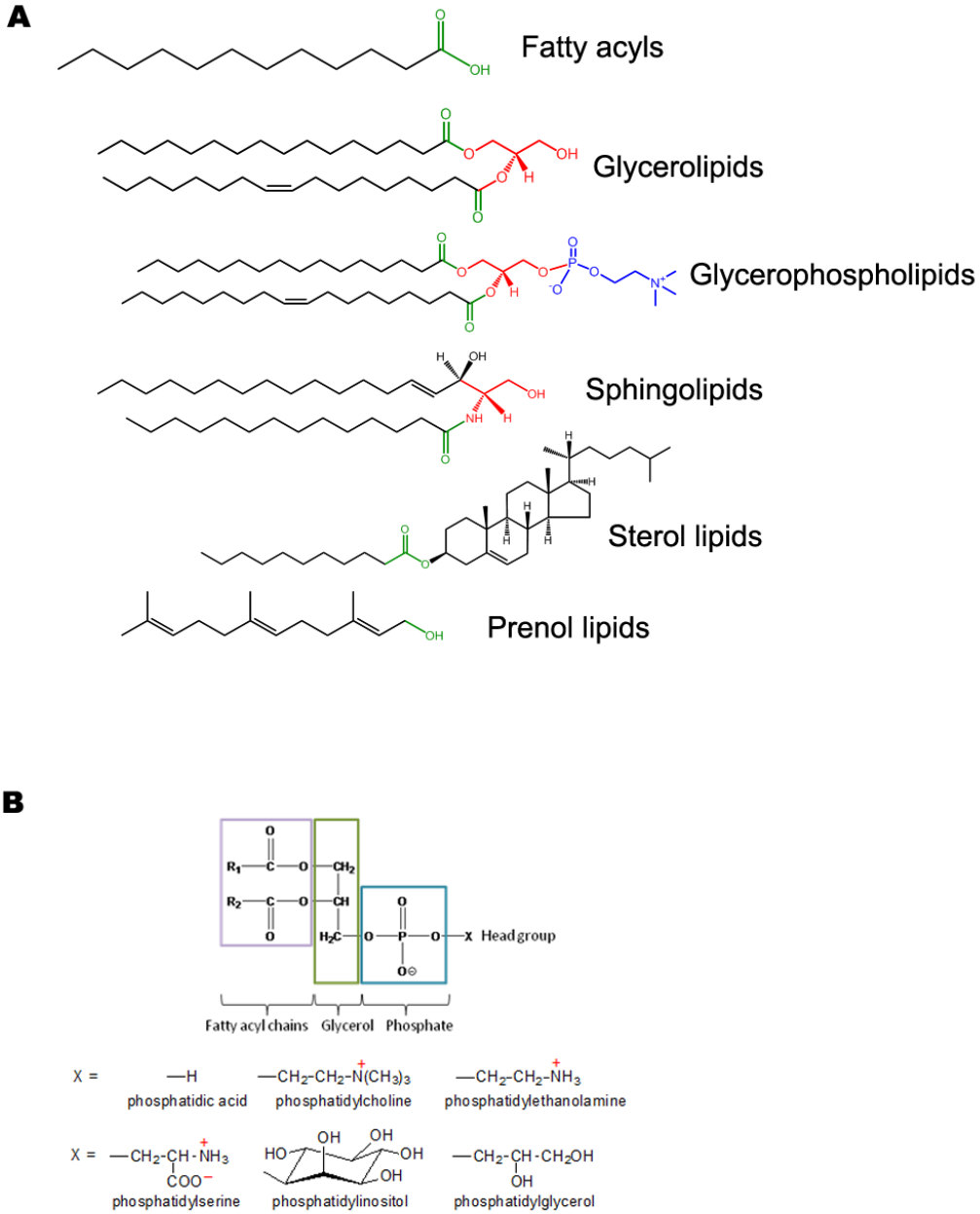


Figure 1.4. (A) Lipid classification according to LipidMaps, (B) classification of glycerofospholipids (adopted from Kelly, K. and Jacobs, R. (2011))

Traditionally, the GC-MS platform was widely employed for lipid analysis (Sjovall, 2004) and covered only a few classes of lipids (fatty acyls and sterols). However, because of significant structural diversity of lipid species the development of the state-of-the-art high resolution MS (e.g. Orbitrap™) allowed for the detailed investigation into all major classes of the cellular lipidome (Shevchenko *et al.*, 2010; Schuhmann *et al.*, 2011). Lipidomics analyses can be performed in either a targeted or untargeted (global) way (Griffiths *et al.*, 2011). Targeted lipidomics focuses on the analysis of a certain class of lipids and is based on a specific type of lipid extraction and chromatographic separation (Griffiths *et al.*, 2011). Global or shotgun lipidomics aims to characterise the system through the identification of the majority of lipids in the sample, and is usually performed with a lipid extraction step followed by direct infusion of crude lipid extract into high resolution MS analysis (Griffiths *et al.*, 2011).

1.9. The role of Metabolomics and Lipidomics in wine research

Analysis of metabolites in grape juice and aroma compounds in wine is not a new phenomenon, and has been performed in a targeted manner in order to characterise the object (Webb, 1964). However, comprehensive metabolite profiling with its non-targeted approach has been applied in order to describe the vintage effect (Lee *et al.*, 2009), chemical composition and quality of grape juice (Atanassov *et al.*, 2009), and the molecular interactions of different *S.cerevisiae* species during wine fermentation (Howell *et al.*, 2006). Moreover, global metabolite profiling of grape juice and its fermentation product – wine, together with correlation analysis, have served as a powerful hypothesis generating tool employed in juice manipulation experiments. One recent metabolite profiling study revealed an important role of individual metabolites in juice on the development of aroma compounds in wine (Pinu *et al.*, 2014).

Despite the plethora of studies demonstrating the strong effect of amino- and organic acids in grape juice on yeast metabolism during the wine fermentation process, it was shown that the grape juice lipidome with its trace quantities has also a significant effect on aroma compound development (Pinu *et al.*, 2014). An absence of studies describing the grape juice lipidome and its potential effect on yeast metabolism during fermentation created a niche for extensive research activity through juice manipulation experiments, which were described in this PhD work.

1.10. Aims and objectives

The main aim of this thesis was to improve our capabilities in absolute quantification of metabolites in different biological samples. The developed approach was then implemented for absolute quantification of metabolites and lipids in grape juice and wine. Based on generated quantitative data, hypotheses were created which have been validated through juice manipulation experiments in order to better understand the biogenesis of wine aroma compounds. This thesis represents a collection of multidisciplinary research work including the development of analytical methods, organic synthesis, analytical biochemistry, hypothesis generation, juice manipulation experiments, and wine fermentation using different microbiological and biochemical approaches, programming, and the creation of bioinformatics tools for metabolomics data analysis. To achieve these goals, this PhD project had the following specific objectives:

- Method development for the accurate quantification of metabolites in different biological samples including grape juice and wine, using a GC-MS analytical platform combined with methyl chloroformate derivatisation;

- Method development for accurate quantification of free and total fatty acid composition of different biological samples including grape juice and wine, using a GC-MS;

- The validation of lipidomics findings by performing juice manipulation experiments through the addition of different lipids, enzymes and co-cultured wine fermentations in order to assess the effect of different lipids components on *S.cerevisiae* metabolism and, therefore, development of volatile thiols and other aroma compounds during wine fermentation.

CHAPTER II

Calibration curve-free GC-MS method for quantification of amino and non-amino organic acids in biological samples

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The content of this chapter has been submitted for publication

2.1. Abstract

Technological advances in the area of analytical chemistry and the development of state-of-the-art analytical instrumentation have allowed for a shift in the focus from a previously targeted approach to chemical analysis more towards the approach adopted in metabolomics, the essence of which is non-targeted providing an unbiased analysis of metabolites in biological samples. Metabolite profiling methods have served their purpose in providing descriptive information about biological systems through qualitative and relative semi-quantitative data. However, quantitative characterisation of a system cannot be fully accomplished without using absolute metabolite concentrations, an area which is lacking in most current metabolomics platforms. Herein, we present a novel calibration curve-free GC-MS method based on isotope-coded derivatisation for absolute non-targeted quantification of polar metabolites. A new R-based package *MetabQ* was created for automated data processing of GC-MS data files performing data extraction and calculation of absolute metabolite values according to the described approach. The introduced R package significantly increased the throughput in the data analysis process, extensively reducing the time required to perform the task manually. The new method requires metabolite response factors which should be calculated prior to the analysis. The method showed high reproducibility and accuracy, and was validated for metabolite quantification of different biological matrices. Our novel approach gives the potential to identify and quantify hundreds of metabolites, far exceeding the capabilities of any absolute quantitative targeted metabolite analysis, limited only by the size of the in-house mass spectral library available.

2.2. Introduction

Metabolomics is commonly defined as the untargeted and unbiased analysis of metabolites in biological samples (Villas-Bôas, 2013; Villas-Bôas *et al.*, 2005). Metabolomics is a relatively new approach to analytical biochemistry with its first mention in a paper published by Oliver Fiehn in 2001 (Fiehn, 2001). In contrast to the traditional targeted metabolite analyses, metabolomics allows for investigations into the complete metabolic functioning of a system at the small molecule level (<1,500 Daltons), broadening our understanding of biological systems and creating a niche for discovery work and hypothesis generation (Villas-Bôas, 2013; Vielhauer *et al.*, 2011). Metabolomics complements other omics studies, providing a description of a biological system closest to the phenotype which combines information from both the underlying metabolism of a living system, as well as the influence of the environment (Villas-Bôas, 2013; Reaves *et al.*, 2011). Comprehensive metabolite profiling can be achieved by using different analytical methods developed for metabolomics which include the coupling of chromatographic separation (GC, LC, CE) with various detection methods (MS, NMR) (Gummer *et al.*, 2009; Koek *et al.*, 2011). Technological advances in the area of analytical chemistry and the development of state-of-the-art analytical instrumentation has allowed for a shift in the focus from a highly targeted analysis approach towards a comprehensive identification and quantification strategy for hundreds of compounds in biological samples (Vielhauer *et al.*, 2011; Koek *et al.*, 2011). Metabolite profiling methods (fingerprinting and footprinting) have served their purpose in providing descriptive information about biological systems through qualitative and relative semi-quantitative data (Kvitvang *et al.*, 2011). However, quantitative characterisation of a system cannot be fully accomplished without using absolute metabolite concentrations, an area which is lacking in most current metabolomics platforms (Kvitvang *et al.*, 2011). Furthermore, an increase in dynamic

modelling studies employing metabolomics makes absolute quantification of metabolites essential (Vielhauer *et al.*, 2011), providing information for the kinetic description of a system and helping make inter-laboratory data more readily comparable (Koek *et al.*, 2011).

GC-MS and LC-MS have become two major analytical techniques employed by metabolomics (Halket *et al.*, 2004; Bruheim *et al.*, 2013). Classically, both techniques have been extensively applied in targeted analysis of metabolites providing high accuracy when the available isotope-labelled internal standards have been used, however, in the past decade with accelerated development in the field of metabolomics these analytical platforms started to serve mainly for untargeted profiling with little reports of targeted analysis in the literature. Nevertheless, these trends hold true despite the presence of isotope labelled internal standards, revealing a significant limitation to the development of quantitative untargeted MS-based methods. GC-MS-based techniques are an attractive candidate for absolute quantification due to their remarkable chromatographic resolution combined with reproducible MS detection using electron impact ionisation (EI) (Koek *et al.*, 2011). The high sensitivity of the single quadrupole mass detector, reproducible ionisation and fragmentation pattern of EI for a broad range of metabolite derivatives, informative mass spectra, availability of mass spectra libraries, equipment affordability and low maintenance cost make GC-MS an ideal analytical technique for quantitative metabolomic applications (Vielhauer *et al.*, 2011; Koek *et al.*, 2011; Villas-Bôas *et al.*, 2011).

In this chapter, we describe a method using single quadrupole GC-MS for a calibration curve-free quantitative analysis of amino- and non-amino organic acids in biological samples, coupled to easy-to-use software capable of performing automatic data extraction and processing. The analytical approach described here employs an isotope coded derivatisation (ICD) strategy (Kvitvang *et al.*, 2011; Bruheim *et al.*, 2013)

that was significantly upgraded and extended for quantification of untargeted metabolites. The R package *MetabQ* was designed and created in-house to facilitate the extraction of quantitative data directly from data files generated by the GC-MS machine and has been made available on-line on the Web page of the Metabolomics Group from the University of Auckland (http://metabolomics.auckland.ac.nz/).

2.3. Experimental Section

2.3.1. Chemicals

Methanol, chloroform, sodium hydroxide, potassium hydroxide, acetone, sodium bicarbonate and dibutyl phthalate (DBP) were obtained from MERCK (Darmstadt, Germany). Pyridine, toluene, the derivatisation reagent methyl chloroformate (MCF), bis(trichloromethyl) carbonate (BTC, triphosgene), tetrabutylammonium chloride, calcium chloride anhydrous, sodium sulphate anhydrous were purchased from Sigma-Aldrich (St. Louis, USA). The internal standard 2,3,3,3-d_4-alanine as well as other metabolite standards were obtained from Sigma-Aldrich (St. Louis, USA). Isotopically labelled methanol (methanol-d_4) for chemical synthesis of deuterium labelled methyl chloroformate (d-MCF) was obtained from Cambridge Isotope Laboratories, Inc (Andover, USA). All chemicals were of analytical grade.

2.3.2. Standards

For the current study we used representative metabolite standards of different chemical classes based on Villas-Boas *et al.* (2011) (**Table 2.1**). All standards were prepared at a final concentration of 10 mM using appropriate solvents. The standard mixtures were freeze-dried using BenchTop K manifold freeze dryer (VirTis, SP Scientific, Warminster, PA, USA) before chemical derivatisation was performed.

Table 2.1. List of metabolite standards used for validation of quantitative approach

Metabolite class	Solvent	Metabolite representative and abbreviation
Neutral amino acids	Water	Alanine, valine, glutamine
Basic amino acids	Water	Lysine
Acidic amino acids	1 M NaOH	Glutamic acid, aspartic acid
Aromatic amino acids	Water	Phenylalanine, tryptophan
Sulfur-containing amino acids	Water	Methionine
Monocarboxylic acids	Water	2-Hydroxybutyric acid, lactic acid, phosphoenolpyruvic acid
Dicarboxylic acids	Water	Succinic acid, fumaric acid
Tricarboxylic acids	Water	Citric acid
Aromatic carboxylic acids	1 M NaOH	*trans*-Cinnamic acid, ferulic acid
2-oxo acids	Water	2-oxoglutaric acid, 3-methyl-2-oxovaleric acid
Fatty acids	Methanol	Myristic acid, palmitic acid
Internal standard	Water	L-Alanine-2,3,3,3-d_4 (alanine-d_4)

2.3.3. Biological Materials

Grape juice, *S. cerevisiae* biomass, rat liver, plasma and urine were used to validate the proposed analytical approach using different biological matrices. Sauvignon Blanc grape juices from three different harvest seasons were used to study seasonal and geographical variations in grape juice composition employing the quantitative approach. Grape juice samples were collected from Marlborough, a New Zealand wine growing region. Grape juice samples were provided by Plant & Food Research, supplemented with internal standard alanine-d_4, frozen, and then transported on dry ice to the laboratory where they were kept frozen at -20°C until use. For analysis, grape juice was kept at 4°C until defrosted and 1 mL of each juice sample was used for method validation. Slow defrosting of grape juice at low temperature preserves grape juice from

substantial degradation of amino acids and amines and allows dissolving precipitated malic and tartaric acids which are present in grape juice at high concentration.

Saccharomyces cerevisiae CEN.PK was grown aerobically under carbon-limited continuous culture on glucose as described by Han *et al*. (2013). The continuous fermentation was carried out at 30°C, pH 5, 20% dissolved oxygen, 300 rpm agitation, compressed air flow rate of 1.25 L/min and dilution rate was set to 0.3 (10 mL/min). Samples were quenched after 3 residential times under steady-state growth using cold-glycerol saline (Smart *et al.*, 2010). *S. cerevisiae* quenched biomass (n=6) was used to extract intracellular metabolites as described in Smart *et al*. (2010). An internal standard, alanine-d_4, was added to each sample before extraction and the extracted samples were freeze-dried prior to derivatisation.

Male Sprague-Dawley rats were fed a standard 18% plant protein derived rodent diet (Harlan Teklad 2018, Madison, WI, USA) for a period of 8 weeks from weaning. The animals were kept under a 12-hour light/dark cycle (50-70% humidity, 22 ± 2°C) with access to food and water *ad libitum*. After 8 weeks, the rats were culled under general anaesthesia, induced with Isoflurane (2-5%; 2 L/min O_2 via nasal cone) in an induction chamber. The abdominal cavity was opened in a midline fashion and blood was harvested from the inferior vena cava using a 14 g angiocath. Plasma samples were obtained after blood was collected into BD vacutainer Lithium-Heparin collection tubes and spun for 1600 *g* for 15 minutes at 4°C. Urine sample was collected by inserting a sterile needle into the bladder and collecting in a sterile Eppendorf (Axygen Eppendorf) and centrifuging at 1600 *g* for 10 min. A section of the left lateral lobe of the liver was harvested and frozen in liquid nitrogen and wrapped in a sterile aluminium foil for further metabolomics analysis. All experiments were approved by the University of Auckland Animal Ethics Committee (#R965). Frozen rat liver was pounded in liquid nitrogen using a mortar and pestle. Powdered liver then was transferred into pre-weighed

eppendorf tubes and spiked with 20 µL of 10 mM solution of alanine-d_4 prior to metabolite extraction (adopted from Masson *et al.* (2010). Liver metabolite extracts were then freeze-dried prior to derivatisation. 200 µL aliquot of both plasma and urine were used for analysis. Metabolites from plasma were extracted as described by de Seymour *et al*. (2014). Plasma metabolite extract was freeze-dried prior the derivatisation. Urine samples were spiked with the internal standard alanine-d_4 and also freeze-dried prior to derivatisation.

2.3.4. Synthesis of Deuterium Labelled Methyl Chloroformate

Due to the high toxicity of phosgene, the chemical synthesis of d-MCF was carried out in a well-ventilated hood. The procedure included two steps and had a total yield of 70%. 5.1 g of triphosgene was mixed with 10 ml of toluene and 40 mg of tetrabutylammonium chloride in a round-bottom flask (Pasquato *et al.*, 2000). The reaction flask was equipped with a condenser and connected to a condensation flask with a PVC hose. This recovery flask was dipped in dry ice/acetone bath (-70°C) to liquefy the phosgene gas and connected to atmosphere through a drying tube filled with anhydrous calcium chloride. The triphosgene/toluene mixture was periodically heated to 40°C under constant stirring for 10-15 min. After 15 min the decomposition of triphosgene started and was followed by the release of phosgene gas, which was collected in the condensation flask. If the phosgene formation stopped, the reactor flask was heated gently. The reaction ended when all solid triphosgene had completely decomposed. The flask with liquid phosgene was weighed. Methanol-d_4 was added drop-wise over 10 min to the phosgene containing flask in a dry ice/acetone bath. The reaction mixture was stirred for 1 hour then warmed to room temperature. The product was refluxed for 15 min to allow evaporation of deuterochloric gas and excess phosgene. d-MCF was distilled and the fraction with boiling point 71-73°C was collected. The

mass of d-MCF was 2.3 g**.** The structure and purity were confirmed by ^{1}H and ^{13}C NMR (in deuterochloroform $CDCl_3$). As d-MCF does not have any protons in the molecule, ^{1}H spectrum should have a signal from solvent only (**Appendix 2.1**). ^{13}C NMR spectrum confirms the structure of synthesized d-MCF (decoupled ^{13}C NMR ($CDCl_3$): δ 150.35 ppm (singlet (*s*), C=O), 56.72 ppm (septet (*sep*), J(C-D) 22.9 Hz, CD_3)). Singlet peak 77.0 ppm corresponds to chloroform ($CHCl_3$) (**Appendix 2.2**). **Figure 2.1** shows the scheme for d-MCF synthesis.

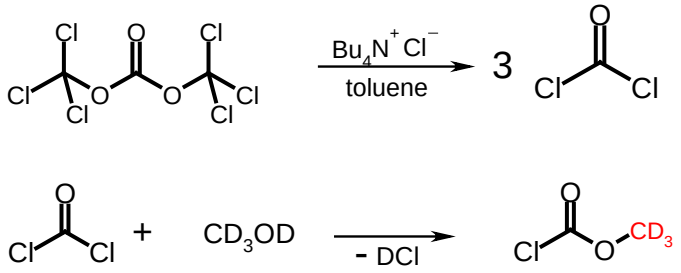

Figure 2.1. The two-step protocol used to synthesise d-MCF. The phosgene gas was generated by decomposition of triphosgene with a catalytic amount of tetrabutylammonium chloride in toluene followed by liquefaction of the gas. The second step included mixing methanol-d4 with liquid phosgene, producing d-MCF

2.3.5. Sample Analysis

2.3.5.1. Quantification approach

Our quantification approach is based on calculating metabolite absolute concentrations using their respective response factors (RF) to an internal standard and the corresponding detected abundances of each metabolite corrected for losses incurring during sample preparation and by the sample matrix effect. Alanine-d_4 was used as the internal standard during sample preparation to correct for sample preparation losses

(quenching, extraction and concentration), while isotope-labelled metabolite derivatives were prepared and spiked into each sample for correction of the matrix effect. To achieve absolute quantification of metabolites, the response factor ratios of all metabolites to dibutyl phthalate were used. The whole approach is illustrated in **Figure 2.2.**

In summary, an aliquot of each sample in a given batch to be analysed was mixed together to make a pooled sample (**Figure 2.2A**). This pooled sample contained an equal representation of each sample in the batch, i.e. any metabolite from any sample can be potentially found in the pooled sample. Pooled samples were freeze-dried as normal samples to avoid dilution of lower abundant metabolites. Each individual sample was then derivatised by a standard MCF protocol as described below (Smart *et al.,* 2010), while the pooled sample was derivatised using d-MCF and methanol-d_4. This pooled sample contained deuterium-labelled metabolite derivatives and represented in essence a mixture of internal standards, one for each metabolite in the analysed samples. A scheme of metabolite chemical derivatisation with MCF (a) and d-MCF (b) is shown in **Figure 2.3**. Only amino- , non-amino acids and some phenolic compounds will be derivatised by MCF. Thus, sugars (i.e. mono-, di- and trisaccharides) will not be modified by MCF and for their analysis silylation chemical modification should be used. Then, each MCF derivatised sample was spiked with an equal aliquot of d-MCF derivatives obtained from the derivatised pooled sample. After this step, each sample received an equal amount of internal standard, an aliquot of dibutyl phthalate solution in chloroform as a mass reference (**Figure 2.2B**). Therefore, the final sample consisted of 100 µL of MCF derivatised sample, 50 µL of d-MCF derivatised pooled sample and 20 µL of 1 mM solution of DBP in chloroform (see **Figure 2.2**).

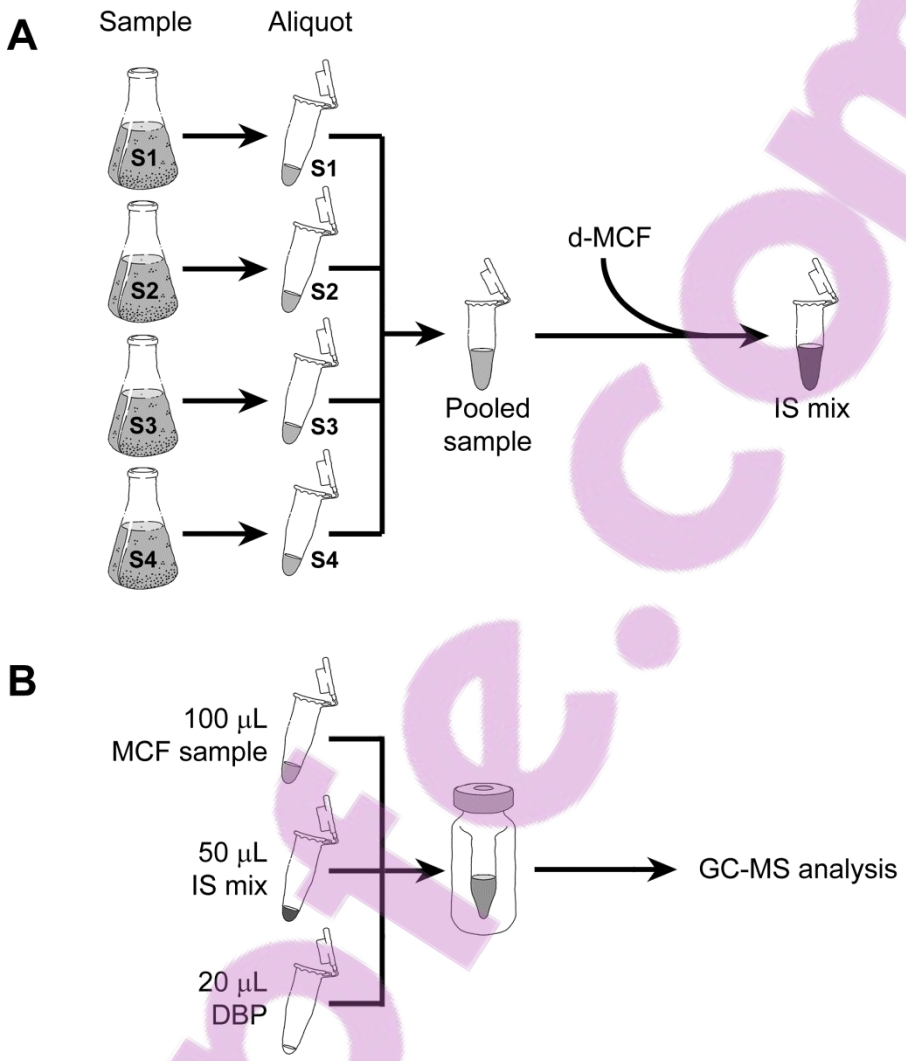

Figure 2.2. Sample preparation workflow for the absolute quantitative approach

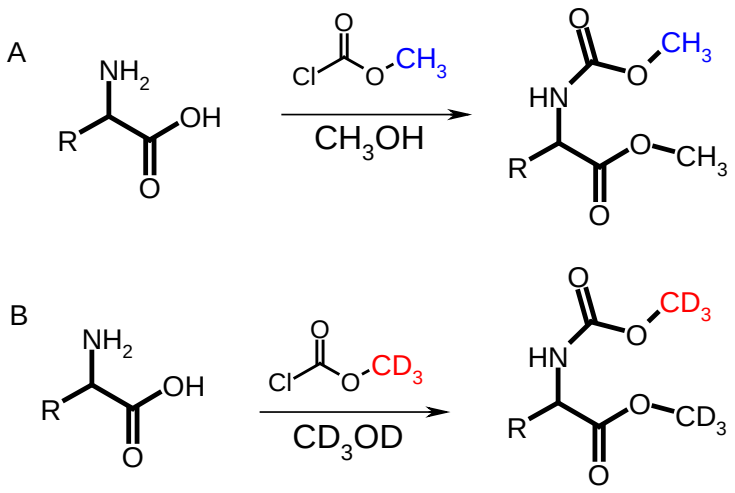

Figure 2.3. Using MCF derivatisation (A), the carboxylic group of a metabolite was converted to a methyl ester and the amino group to a carbamate. D-MCF derivatisation (B) introduced an isotopic label into the metabolite molecule

2.3.5.2. Sample derivatisation

The chemical derivatisation procedure of samples using MCF was adapted from the protocol described by Smart *et al*. (2010). In brief, a freeze-dried sample (grape juice and rat urine) or metabolite extract (yeast biomass, rat liver and rat plasma) was resuspended in 200 µL of 1 M sodium hydroxide and transferred to 6 mL silanised borosilicate tubes (CTS-1275, Thermo Fisher Scientific, USA). The sample in the silanised tubes was mixed with 68 µL of pyridine and 334 µL of methanol, followed by the addition of 40 µL of methyl chloroformate MCF under vigorous stirring (vortexing). After 30 seconds, a second 40 µL aliquot of MCF was added to the mixture, followed by 30 seconds of mixing. Thereafter, 400 µL of chloroform and 800 µL of 50 mM solution of sodium bicarbonate were added to each sample, thence constant vortexing for 20 seconds. The mixture was then centrifuged and the upper aqueous layer discarded. The chloroform fraction was dried with anhydrous sodium sulphate and used for further

analysis. The same procedure was applied for chemical derivatisation using d-MCF by using deuterated methanol and d-MCF.

2.3.5.3. GC-MS analysis and metabolite identification

GC-MS analysis was performed using a Thermo Trace Ultra GC system coupled with a Thermo ISQ mass selective detector (EI) operating at 70 eV. The column used for all analyses was a ZB-1701 (30 m x 250 µm id x 0.15 µm with 5 m guard column, Phenomenex). The GC-MS parameters were set according to Smart *et al*. (2010). 1 µL of sample was injected using pulsed splitless mode with the injector temperature at 290°C. The GC oven program was as described in **Table 2.2**. The gas carrier was helium and the flow rate was set at 1.0 mL/min. The interface temperature was 250°C, while the quadrupole temperature was 200°C. The mass spectrometer was operated in scanning mode with a mass range of 38 to 550 m/z. Automated Mass Spectral Deconvolution and Identification System (AMDIS, v2.71, NIST, Boulder, CO, USA) software was used for metabolite identification by comparing compound retention times and mass spectra against our in-house MS spectral library. Unique ion for each metabolite was used for quantification. Kovats Indices were calculated for identified metabolites, however, locking of retention time wasn't used in this GC-MS method as metabolite retention times were highly reproducible.

Table 2.2. GC temperature program for metabolite analysis

Start temperature (°C)	Ramp (°C/min)	End temperature (°C)	Hold time (min)
45	-	45	2
45	9	180	5
180	40	240	11.5
240	40	280	2

2.3.6. *MetabQ* Development

2.3.6.1. Automated Data Processing

The described approach for absolute metabolite quantification includes complex mathematical calculations in order to extract absolute metabolite concentration values. To facilitate the process of data extraction and calculation of absolute concentrations, we designed and developed an in-house software package *MetabQ* written in R (R Core Team, 2014), based on the calculations described below. The package accepts raw GC-MS data in CDF format and produces a table in CSV (*comma-separated values*) file with the absolute concentrations of metabolites. This package is available for download from the Web page of the Metabolomics Group, The University of Auckland. *MetabQ* package requires *xcms* library (Smith *et al.*, 2006) to be installed from *Bioconductor* and both *tcltk* and *scatterplot3d* libraries from *CRAN*. AMDIS software is used for deconvolution of GC-MS chromatograms, preliminary metabolite identification, and generation of a report that is used by *MetabQ* package. AMDIS software settings must be change to 75% for minimum match factor and by deselecting the option of multiple identifications per compound in order to avoid multiple ID for analyte and thus to provide correct identification and quantification for metabolite. Only metabolites identified by AMDIS will be further quantified, therefore, any unknown peaks (e.g. contaminants or yet to be identified metabolites) will be removed for *MetabQ* analysis. Thermo GC-MS generated data files (*.raw) are converted into CDF format using *Thermo Xcalibur* software.

The package *MetabQ* includes four functions: settings(), relative(), correct() and quant(). The default settings file *MetabQ.settings csv* needs to be customised by user. Function *settings()* operates as an input function to provide the script with required information and generates the spreadsheet as *_lib.csv file which contains the list of identified metabolites and their analytical parameters. Function *relative()* operates as a

data extraction function, resulting in the *_auto.csv file containing abundances of identified metabolites. Additionally, this function generates graphical files of metabolite peaks.

Correct() function should be used for manual correction of the time window, in case the top of the metabolite peak is out of the time frame window. This function overwrites the file *_auto.csv with metabolite abundances (function *relative*) replacing data with corrected values and generates a new file *_correct.csv. The *quant()* function performs the calculations according to the quantitative approach described below. As a result, the script generates the final spreadsheet with absolute metabolite concentration in mg/L. Instructions on how to install and run *MetabQ* are included in **Appendix 2.3**.

2.3.6.2. Calculation of Response Factors

The slope of a linear function built by plotting the ratio of metabolite concentration to concentration of DBP (internal standard and mass reference) against the ratio of corresponding abundances represents the response factor for each respective metabolite. The response factor remains constant within this linear concentration range function.

2.3.6.3. Calculation of Metabolite Concentrations

The first calculation step includes correction of the observed metabolite abundance caused by the sample matrix effect. The ratio between deuterated metabolite derivative signal and signal from DBP represents the coefficient proportional to the matrix effect (ME) of a sample (**Equation 2.1**):

$$k_{ME} = {Ab_D}/{Ab_{DBP}} \quad (2.1),$$

where k_{ME} is a coefficient proportional to the sample matrix, Ab_D (the abundance of deuterated metabolite derivative) and Ab_{DBP} (the abundance of DBP).

Multiplication of the observed metabolite abundance to k_{ME} provides information of the signal aliquot lost due to the matrix effect. Consequently the matrix effect corrected metabolite abundance Ab_H^{ME} has a value, as calculated in **Equation 2.2**:

$$Ab_H^{ME} = Ab_H \times (1 + k_{ME}) \quad (2.2),$$

where Ab_H is the observed metabolite abundance.

Alanine-d_4 was used as an internal standard to calculate losses during sample preparation (SP). We assumed that the loss of each metabolite equals the equivalent loss of alanine-d_4 and can be determined as follows:

$$k_{SP} = 1 - (\frac{Ab_{D4-ALA}^{ME}}{Ab_{D4-ALA}}) \quad (2.3),$$

where k_{SP} is the coefficient for sample preparation losses, Ab_{D4-ALA}^{ME} – the matrix effect corrected abundance of alanine-d_4 in **Equation 2.2** and Ab_{D4-ALA}, the expected abundance of alanine-d_4.

The final correction step can be represented as:

$$Ab_H^{ME,SP} = Ab_H^{ME} \times (1 + k_{SP}) \quad (2.4),$$

where $Ab_H^{ME,SP}$ is the final corrected metabolite abundance. Using this value the absolute concentration of metabolite $c_H$can be calculated:

$$c_H = (\frac{Ab_H^{ME,SP}}{Ab_{DBP}} - y_{intercept}) \times \frac{c_{DBP}}{RF} \quad (2.5),$$

where $y_{intercept}$ and RF are calculated as the y-axis intercept and metabolite response factor respectively and c_{DBP} is the concentration of DBP standard added.

2.3.7. Matrix effect, metabolite recovery and method reproducibility

Each biological sample described above was spiked with a mixture of metabolites (**Table 2.1**) in order to assess the influence of sample matrix on the metabolite recovery. Metabolite recovery was determined by comparison of respective samples spiked and non-spiked with the metabolite mixture. The method reproducibility was assessed by determining the relative standard deviation (RSD) for quantified metabolite concentrations in the samples, each analysed at least in triplicate, using **Equation 2.6**:

$$RSD = {}^{SD}/_{mean} \times 100 \qquad (2.6).$$

2.3.8. Metabolite quantification in different sample matrices

All identified metabolites in rat urine, extracts from yeasts, rat plasma and liver were quantified using the approach described. Metabolites in grape juice were quantified using both the described approach and a traditional calibration curve method. Calibration curves were built up using pure metabolite standards derivatised with MCF (Smart *et al.*, 2010).

2.4. Results and Discussion

2.4.1. Rationale of the method

In classical quantification of targeted metabolites, the response factor (RF) of each analyte is calculated via calibration curves obtained with standard solutions analysed in parallel to every sample batch. This is necessary because machine performance changes over time and the RFs change depending on the sample matrix. However, in our approach the RF of each analyte in the mass spectra library needs to be determined only once for each individual GC-MS machine. Our RFs are determined in relation to a very stable internal standard, dibutyl phthalate (DBP), that does not require chemical derivatisation. Moreover and the matrix effect and technical variability of the sample are corrected at every sample batch by spiking samples with deuterium-labeled internal standard derivatives. The deuterium-labeled derivatives are *de novo* synthesised for every sample batch by mixing an aliquot of each sample into a sample mix followed by chemical derivatisation of a mix using deuterium-labeled derivatising reagents. Therefore, we calculated the RF for the 155 metabolites present in our GC-MS library (**Table 2.3**). The RFs ranged from 0.0003 for glutamine to 0.7591 for undecanoic acid. The RFs remained constant within the range of quantification. Most of the amino acids and organic acids showed a wide quantification range (200 fold) – between 10 µM and 10 mM for different metabolites , whilst sulfur and phosphate-containing metabolites showed a narrower quantification range (15-35 fold) (**Table 2.3**). Noticeably, higher values of RFs were observed for fatty acids. In general, odd-numbered chain fatty acids showed higher RFs compare to even-numbered chain fatty acids. We also observed that unsaturated fatty acids presented lower RF values compared to saturated ones with the same number of carbon atoms, and an increase in the number of double bonds in fatty acids resulted in lower RF values. These observations clearly indicate a retention-structure correlation of metabolite described previously through mosaic and/or bond

increments (Santiuste *et al.*, 2003) as well as Kovats retention indices (RI) (Takacs, 1991). However, RFs are also related to derivatisation yield and, consequently depends on the matrix effect of a sample, which is corrected in our method with a deuterium-labeled internal standard for each analyte.

Table 2.3. Analytical characteristics of metabolites used to perform quantitative method. All metabolites listed were analysed as MCF derivatives

Metabolite	RI	RF	Y-intercept	Quantification range, mM	m/z
10-Heptadecenoic acid	1055	0.2125	-0.0016	0.02-4	55
10-Pentadecenoic acid	1043	0.2015	-0.0027	0.02-4	55
11,14,17-Eicosatrienoic acid	1068	0.1287	0.0009	0.01-2	79
11,14-Eicosadienoic acid	1067	0.1431	0.0006	0.01-2	67
13,16-Docosadienoic acid	1077	0.1109	-0.0027	0.01-2	67
1-Aminocyclopropane-1-carboxylic acid	1025	0.0345	-0.0020	0.01-1	141
1-Phenylethanol	1014	0.0214	-0.0010	0.01-1	107
2,3-Butanediol	1001	0.0423	-0.0012	0.01-1.5	45
2,4-Diaminobutyric acid	1055	0.0443	0.0001	0.02-4	114
2,6-Diaminopimelic acid	1076	0.0596	-0.0029	0.05-10	200
2-Aminoadipic acid	1046	0.0243	-0.0124	0.05-10	114
2-Aminophenylacetic acid	1040	0.0647	-0.0064	0.02-4	164
2-Hydroxybutyric acid	1016	0.0449	-0.0028	0.05-10	117
2-Hydroxycinnamic acid	1050	0.0678	-0.0034	0.05-10	161
2-Hydroxyisobutyric acid	1012	0.0754	-0.0023	0.02-4	73
2-Isopropylmalic acid	1023	0.0678	-0.0016	0.05-10	145
2-Methyloctadecanoic acid	1060	0.1374	0.0067	0.02-4	88
2-Oxoadipic acid	1028	0.0536	0.0003	0.01-2	129
2-Oxobutyric acid	1001	0.0497	0.0001	0.05-10	57
Glutaric acid	1015	0.0147	-0.0012	0.05-10	100
Glutathione	1045	0.0126	-0.0163	0.05-1.5	142
Glyceric acid	1020	0.0024	0.0014	0.01-1	119
Glycerol	1016	0.0069	0.0027	0.01-1	61
Glycine	1019	0.0875	-0.0034	0.05-10	88
Glyoxylic acid	1018	0.0027	0.0047	0.01-1	75
Gondoic acid	1067	0.0946	-0.0034	0.02-4	55
Heneicosanoic acid	1073	0.315	0.0067	0.01-2	74
Hexanoic acid	1002	0.3236	-0.0174	0.02-4	74
Hippuric acid	1050	0.0678	-0.0037	0.05-10	105
Histidine	1068	0.0027	-0.0074	0.05-10	139
Homocysteine	1056	0.0039	-0.0145	0.02-4	114
Indole-3-butyric acid	1065	0.0212	-0.0087	0.01-1	130
Isocitric acid	1048	0.0215	-0.0067	0.05-10	129
Isocitric acid secondary peak	1047	0.0234	-0.0057	0.05-10	129
Isoleucine	1027	0.0842	-0.0057	0.05-10	115
Itaconic acid	1015	0.0378	-0.0079	0.05-10	127
Lactic acid	1012	0.0195	0.0006	0.02-4	103
Leucine	1028	0.1136	-0.0067	0.05-10	144

Table 2.3 (continued)

2-Oxoglutaric acid	1027	0.0158	-0.0034	0.05-10	115	Levulinic acid	1011	0.0987	-0.0012	0.05-10	99
2-Oxovaleric acid	1006	0.0202	-0.0049	0.01-1.5	71	Lignoceric acid	1088	0.0979	0.0067	0.01-2	74
2-Phosphoenolpyruvic acid	1029	0.0435	-0.0121	0.05-1	109	Linoleic acid	1058	0.2379	-0.0001	0.02-4	67
2-Phosphoglyceric acid	1036	0.0497	-0.0169	0.01-1	169	Lysine	1065	0.0856	-0.0092	0.05-10	142
3,5-Diiodo-L-tyrosine	1019	0.0348	-0.0160	0.01-1	429	Malic acid	1019	0.034	-0.0057	0.05-10	103
3-Hydroxybenzoic acid	1037	0.0312	-0.0012	0.05-10	135	Malonic acid	1007	0.0167	-0.0029	0.05-10	101
3-Hydroxydecanoic acid	1031	0.0856	0.0027	0.05-10	103	Margaric acid	1055	0.6007	0.0037	0.02-4	74
3-Hydroxyoctanoic acid	1022	0.1815	0.0039	0.02-4	103	Methionine	1040	0.0394	0.0096	0.08-2.8	147
3-Hydroxypropionic acid	1025	0.0894	-0.0125	0.05-10	87	Myristic acid	1038	0.3293	-0.0037	0.02-4	74
3-Methyl-2-oxopentanoic acid	1008	0.1097	-0.0024	0.05-10	57	Myristoleic acid	1038	0.2418	0.0042	0.01-2	55
3-Oxoadipic acid	1028	0.1036	-0.0021	0.05-10	69	N-Acetylcysteine	1050	0.0674	0.0047	0.02-4	176
4-Aminobenzoic acid	1056	0.0107	-0.0014	0.05-10	178	N-Acetylglutamic acid	1043	0.0117	-0.0027	0.02-4	116
4-Aminobutyric acid	1029	0.2089	-0.0214	0.05-10	102	N-alpha-Acetyllysine	1074	0.0128	-0.0016	0.01-1	129
4-Hydroxycinnamic acid	1053	0.2578	-0.0274	0.02-4	161	Nicotinamide	1002	0.0059	0.0074	0.1-5	57
4-Hydroxyphenylacetic acid	1043	0.2348	-0.0147	0.05-10	121	Nicotinic acid	1017	0.0297	-0.0168	0.01-1	137
4-Hydroxyphenylethanol	1043	0.3498	-0.0367	0.02-4	121	Nonadecanoic acid	1063	0.2579	0.0064	0.02-4	74
4-Methyl-2-oxopentanoic acid	1008	0.1167	-0.0067	0.02-4	85	Norvaline	1025	0.107	-0.0037	0.05-10	130
5-Hydroxy-L-lysine	1078	0.166	-0.0197	0.05-10	101	O-Acetylserine	1033	0.1237	-0.0167	0.01-2	100
5-OH-methyl-2-furaldehyde	1028	0.2974	-0.0168	0.01-2	168	Octanoic acid	1012	0.4003	-0.0054	0.02-4	74

Table 2.3 (continued)

5-Methoxytryptophan	1109	0.0119	-0.0678	0.05-10	160	Oleic acid	1058	0.1112	0.0023	0.02-4	55
5-Oxotetrahydrofuran-2-carboxylic acid	1028	0.1978	-0.0497	0.01-2	85	Ornithine	1061	0.0291	-0.0016	0.05-10	128
9-Heptadecenoic acid	1055	0.1267	-0.0074	0.02-4	55	Oxalic acid	1002	0.0578	-0.0069	0.05-10	59
Adipic acid	1021	0.2978	-0.0064	0.05-10	114	Oxaloacetic acid	1014	0.0023	0.0069	0.01-1	101
Adrenic acid	1076	0.1079	0.0037	0.01-2	79	Palmitic acid	1050	0.2337	0.0048	0.02-4	74
Alanine	1018	0.1046	-0.0312	0.05-10	102	Palmitoleic acid	1049	0.0971	-0.0019	0.02-4	55
Anthranilic acid	1029	0.2014	-0.0247	0.05-10	146	para-Toluic acid	1019	0.0678	-0.0069	0.05-10	119
Arachidic acid	1068	0.2099	-0.0067	0.01-2	74	Pentadecanoic acid	1043	0.5671	0.0031	0.02-4	74
Arachidonic acid	1066	0.1316	0.0021	0.02-4	79	Phenylalanine	1046	0.0671	0.0260	0.05-10	162
Asparagine	1036	0.2478	-0.0678	0.01-1	127	Pimelic acid	1027	0.0269	0.0079	0.05-10	115
Aspartic acid	1034	0.1491	-0.0200	0.05-10	160	Proline	1030	0.1815	-0.0036	0.05-10	128
Azelaic acid	1035	0.0328	-0.0047	0.05-10	185	Putrescine	1051	0.0113	-0.0057	0.05-10	88
Behenic acid	1077	0.1782	-0.0041	0.01-2	74	Pyroglutamic acid	1035	0.0874	-0.0125	0.01-1	84
Benzoic acid	1013	0.0159	-0.0024	0.05-10	105	Pyruvic acid	1003	0.0062	0.0087	0.02-2	89
bishomo-γ-Linolenic acid	1066	0.1393	0.0027	0.02-4	79	Quinic acid	1035	0.0011	-0.0057	0.05-10	191
Caffeine	1057	0.2487	-0.0157	0.02-4	194	Salicylic acid	1036	0.0269	-0.0006	0.05-10	135
cis-4-Hydroxyproline	1045	0.043	-0.0712	0.02-4	144	Sebacic acid	1039	0.0978	0.0057	0.05-10	199
cis-Aconitic acid	1032	0.0126	-0.0167	0.05-10	153	Serine	1038	0.0095	-0.0067	0.05-10	100
cis-Vaccenic acid	1058	0.1794	-0.0067	0.02-4	55	Sinapic acid	1076	0.0031	-0.0049	0.05-10	296
Citraconic acid	1015	0.0677	-0.0234	0.05-10	127	Stearic acid	1059	0.3396	0.0024	0.02-4	74
Citramalic acid	1017	0.2147	-0.0067	0.05-10	117	Suberic acid	1031	0.0678	0.0058	0.05-10	129
Citric acid	1034	0.0741	-0.0213	0.05-10	143	Succinic acid	1012	0.0781	0.0095	0.05-10	115
Citric acid secondary peak	1033	0.0645	-0.0216	0.05-10	101	Syringic acid	1057	0.0672	0.0042	0.05-10	211
Creatinine	1036	0.1678	0.0004	0.05-10	202	Tartaric acid	1047	0.0458	-0.0023	0.05-10	59
Cystathionine	1092	0.0024	0.0021	0.1-3.5	160	Threonine	1032	0.0106	-0.0021	0.05-10	115
Cysteine	1046	0.0046	0.0014	0.07-2.5	192	trans-4-Hydroxyproline	1039	0.043	-0.0012	0.05-10	216
Decanoic acid	1022	0.4678	0.0032	0.02-4	74	trans-Cinnamic acid	1028	0.0959	-0.0377	0.05-10	162

Table 2.3 (continued)

DHA	1076	0.0893	-0.0023	0.01-2	79	Tricosanoic acid	1082	0.1503	-0.0032	0.01-2	74
Dipicolinic acid	1040	0.2687	-0.0032	0.05-10	137	Tridecanoic acid	1034	0.4957	0.0023	0.01-2	74
Dodecanoic acid	1030	0.4525	0.0049	0.01-2	74	Tryptophan	1087	0.0992	-0.0198	0.05-10	130
DPA	1076	0.0799	0.0002	0.01-2	79	Tyrosine	1073	0.0326	-0.0127	0.05-10	236
EDTA	1069	0.0197	-0.0024	0.05-10	174	Undecanoic acid	1026	0.7591	-0.0014	0.01-2	74
EPA	1067	0.1387	-0.0017	0.01-2	79	Valine	1023	0.1502	-0.0010	0.05-10	130
Erucic acid	1076	0.0978	0.0041	0.01-2	55	Vanillic acid	1048	0.0071	-0.0016	0.05-10	165
Ferulic acid	1063	0.0415	-0.0049	0.05-10	222	α-Linolenic acid	1058	0.2087	0.0031	0.02-4	79
Fumaric acid	1012	0.0835	-0.0049	0.05-10	113	β-Alanine	1023	0.0411	-0.0065	0.05-10	88
Glutamic acid	1040	0.0295	-0.0112	0.05-10	174	γ-Linolenic acid	1059	0.1806	0.0021	0.02-4	79
Glutamine	1063	0.0003	-0.0004	0.5-7.5	84						

RI, Kovats retention index; RF, response factor; m/z, quantifier ion

It is important to mention that the RFs calculated based on data generated by different GC-MS instruments (Agilent and Thermo GC-MS) showed different values (**Appendix 2.4**). As a result, the listed RF values in **Table 2.3** cannot be used as a universal standard set. RF values should be re-calculated separately for each machine. In addition, our mass reference standard (DBP) is a phthalate compound, and phthalates can often leak from plasticware and are common contaminants in GC-MS analysis (Reid *et al.*, 2007). We did not observe DBP in any of our samples (data not shown), but we recommend using deuterated DBP as mass reference standard when different plasticware is used during sample preparation. DBP-d_4 is commercially available and produces an abundant ion fragment of m/z 153 (unlabeled DBP gives m/z 149), which can be easily deconvoluted by AMDIS software.

In principle, our method does not have a limitation on the number of metabolites that could be quantified as long as the metabolite RF is determined prior to the analysis. In addition, the deuterium-labeled derivative for each analyte in the sample can be easily distinguished from the non-labeled counterpart through GC-quadrupole-MS. Garcia-Dominguez *et al* (1997) showed that Kovats coefficients and molecular structural coefficients are two components of the retention index, which can be calculated with high precision using mosaic and bond increment algorithms but only for certain classes of compounds (Santiuste *et al.*, 2003). Mosaic and bond increments can be defined as compound molecular parameters (structure and atom composition - for mosaic, and number and nature of bonds (i.e. σ- and π-bonds) in molecule – for bond, respectively) correlating to retention parameters of stationary phase during chromatographic analysis. To the best of our knowledge, there are no studies showing connection or correlation between Kovats retention indices and analyte response factors. We hypothesise however that if the RF could be calculated using RI and other related analytical parameters for GC (Castello *et al.*, 1996; Gerbino *et al.*, 1995; Donovan, 1996), our described approach

could be used to quantify all chromatographically detectable and identified metabolites without having to calculate experimentally the RFs for each analyte in the MS library. In that line, Katritzky *et al* (1994) published a study showing that retention time and response factor can be calculated using a six-parameter equation with high precision. A described quantitative structure-property relationship (QSPR) treatment was tested on different chemical classes of compounds including amines, fatty acids, alcohol and esters.

2.4.2. Method accuracy and reproducibility

Based on the recovery of 22 metabolites which were spiked in the different biological samples (**Table 2.1**), we observed accurate metabolite recovery for most metabolites independent of sample matrix (**Table 2.4**). We obtained full recovery of 18 metabolites out of the 22 spiked in different sample matrices, whilst the recovery of methionine and phosphoenolpyruvic acid were between 75-80 % across all tested matrices. We therefore corrected the level of methionine using a correction factor of 1.33 and phosphoenolpyruvic acid using a correction factor of 1.25. The concentration of the amino acid glutamine was significantly underestimated, whilst the glutamic acid concentration was found overestimated. We assume that during MCF derivatisation glutamine could be converted to glutamate resulting in about 30% of glutamic acid overestimation. Thus, using the correction factors of 1.43 and 0.7 for glutamine and glutamic acid respectively, it was possible to obtain accurate quantitative values for both amino acids (**Table 2.4**). Out of 155 identified metabolites conversion only of glutamine into glutamate was validated by analysing metabolites as individual compounds. In addition, the method showed excellent reproducibility with RSD < 5% for all 22 metabolites in all biological samples (**Table 2.4**). Citric and isocitric acids form two derivatives upon MCF derivatisation. In this case each derivative (e.g. citric acid and

citric acid secondary peak) is treated as individual metabolite with calculation of its concentration following the integration of calculated concentrations. We observed overestimation of palmitic acid that can be easily explained by common contamination with this fatty acid from plasticware used for sample preparation (Tumanov *et al.*, 2015). The concentration of palmitic acid can be corrected by subtracting the abundance of palmitic acid peak from procedure blank. The 22 metabolites used for an assessment of the method represented different metabolite classes presenting different physico-chemical properties. Although the accurate quantification of metabolites could be compromised due to oxidation, volatility or matrix interference, the presence of the respective d-MCF metabolite derivative allowed us to correct the data, providing a better estimation of metabolite concentration without the need to build calibration curves for each metabolite every time a batch of samples was analysed.

Table 2.4. Recoveries (%) of the metabolite standards spiked to biological samples. Recoveries were presented as mean value (RSD% shown in brackets, n=3)

Metabolite	Standard metabolite mix	Rat plasma	Rat urine	Rat liver	Grape juice	Yeast extract
2-Hydroxybutyric acid	100.39 (4.21)	102.50 (1.25)	96.09 (2.82)	96.51 (3.85)	100.07 (3.41)	99.82 (3.38)
2-Oxoglutaric acid	94.66 (1.33)	94.63 (0.70)	95.49 (2.21)	94.07 (0.86)	95.60 (1.30)	95.74 (0.17)
3-Methyl-2-oxopentanoic acid	98.17 (2.83)	99.02 (1.58)	103.73 (0.93)	101.17 (1.90)	99.66 (3.76)	101.87 (2.03)
Alanine	102.36 (1.99)	97.73 (0.93)	101.43 (1.13)	96.43 (0.88)	99.23 (2.12)	102.17 (1.91)
Aspartic acid	99.36 (0.93)	95.43 (0.57)	97.87 (0.60)	95.23 (0.40)	97.85 (1.46)	103.50 (1.08)
Citric acid	103.20 (1.84)	101.79 (1.44)	106.10 (0.72)	103.23 (2.46)	99.32 (1.54)	102.80 (2.87)
Ferulic acid	93.05 (1.09)	94.08 (0.94)	94.78 (1.22)	93.43 (0.49)	94.14 (1.10)	93.74 (1.55)
Fumaric acid	98.27 (4.13)	98.95 (1.00)	98.98 (3.45)	98.10 (4.38)	98.94 (3.10)	100.95 (2.78)
Glutamic acid	101.36 (1.00)	102.87 (2.37)	97.29 (1.99)	99.25 (2.23)	104.46 (3.44)	106.63 (1.79)
Glutamine	95.62 (1.54)	91.07 (1.40)	98.09 (1.21)	98.64 (1.44)	97.25 (4.07)	97.44 (2.46)
Lactic acid	101.83 (4.73)	99.34 (4.10)	102.36 (0.57)	101.96 (1.20)	103.65 (1.97)	102.94 (1.68)
Lysine	97.63 (1.50)	95.53 (0.43)	99.35 (2.02)	95.11 (1.08)	95.48 (1.68)	96.26 (0.96)
Methionine	100.48 (4.29)	99.80 (3.71)	103.18 (1.74)	102.69 (1.10)	93.24 (1.57)	93.14 (0.65)
Myristic acid	105.34 (4.20)	104.48 (1.32)	102.01 (2.61)	101.82 (2.76)	100.52 (2.07)	103.23 (0.80)
Palmitic acid	108.45 (3.40)	103.77 (3.35)	103.59 (2.12)	106.54 (4.39)	101.70 (1.92)	104.83 (2.58)
Phenylalanine	99.00 (1.60)	97.96 (3.39)	102.15 (3.22)	97.26 (0.93)	99.73 (1.38)	99.20 (2.23)
Phosphoenolpyruvic acid	85.02 (1.57)	90.36 (3.43)	93.75 (1.33)	88.90 (3.98)	87.25 (2.87)	89.62 (4.49)
Succinic acid	98.70 (2.63)	100.52 (4.13)	101.82 (4.24)	99.71 (2.26)	96.98 (3.13)	97.82 (4.42)
trans-Cinnamic acid	98.93 (2.38)	97.14 (2.13)	97.17 (3.04)	99.91 (3.06)	95.07 (1.19)	99.14 (3.20)
Tryptophan	104.28 (1.17)	104.21 (0.77)	103.35 (0.44)	104.56 (0.47)	100.45 (2.99)	97.11 (0.88)
Valine	101.16 (2.55)	97.49 (0.86)	100.74 (1.55)	96.58 (0.75)	100.93 (1.73)	100.10 (2.08)
Alanine-d_4 (IS)	*99.69 (1.20)*	*99.65 (0.91)*	*99.22 (1.56)*	*100.03 (0.42)*	*99.02 (0.35)*	*98.87 (0.52)*

2.4.3. Metabolite quantification in different sample matrices

To demonstrate the applicability of the method we quantified metabolites in a wide range of biological samples (**Table 2.5** and **Appendix 2.5**). Total ion current (TIC) chromatogram is displayed on **Appendix 2.**6. The concentrations found were with the expected order of magnitude for majority of metabolites in all biological samples. Moreover, the concentrations of common grape juice metabolites by this new method were comparable to the concentrations obtained by using a classical calibration curve method for the same grape juice sample with the majority of quantified values being in agreement with the concentration range previously reported for white grape juices (Huang *et al.*, 1991; Spayd *et al.*, 1996; Hernandes-Orte, 2003; Munos-Robredo *et al.*, 2011; Escobal *et al.*, 1996; Gregan *et al.*, 2012; Soyer *et al.*, 2003; Bouloumpasi *et al.*, 2002). Deuterated metabolites were detected for all identified juice metabolites except for the amino acids cysteine and glutamine. The concentration of glutamine due to deamination was underestimated and lay outside of reported concentration range. Among all other metabolites, the %RSD was below 9%. Similarly, the intracellular metabolite concentrations in yeast cells were also comparable to reported concentrations determined under very similar growth conditions described by Canelas *et al*. (2009). Relative standard deviations were also below 10% with the highest value (9.96 %) observed for glutamic acid. Among all biological samples analysed, the average variability was 4.03%.

Table 2.5. List of the most common metabolites quantified in biological samples used for method validation. Results were presented as mean value for (A) grape juice (%RSD shown in brackets, n=3), (B) yeast extract (%RSD shown in brackets, n=6). No spike recovery method was applied for described biological samples

A

Metabolites	Concentration, mg/L	Calibration curve results, mg/L	Range reported, mg/L	Reference
Amino acids				
Alanine	68.8 (1.82)	62.4 (2.12)	26.8-180.0	1-3, 6, 8
Asparagine	7.3 (0.29)	6.7 (1.29)	3.7-60.0	2-3, 6
Aspartic acid	64.7 (7.62)	66.1 (5.39)	8.2-30.0	1-3, 6, 8
Cysteine	1.47 (1.27)	1.06 (2.74)	0.7-7.1	2-3, 6
GABA	3.8 (5.85)	3.5 (6.34)	0.0-109.7	3, 8
Glutamic acid	153.16 (10.37)	213.4 (8.58)	22.5-150.0	2-3, 6
Glutamine	19.6 (7.29)	13.8 (3.78)	47.0-200.0	1, 3, 6
Glycine	9.4 (5.95)	8.7 (1.78)	1.6-16.0	3, 6, 8
Histidine	78.2 (2.97)	79.7 (3.78)	6.9-65.0	1-3, 6, 8
Isoleucine	59.4 (3.44)	53.7 (5.78)	5.0-53.3	1-3, 6
Leucine	93.0 (2.20)	87.5 (3.70)	7.0-80.4	1-3, 6
Lysine	3.2 (1.03)	2.8 (1.78)	1.2-12.0	1-3, 6
Methionine	4.9 (4.87)	3.7 (6.37)	1.5-21.6	1-3, 6
Ornithine	2.4 (7.17)	2.1 (2.78)	2.0-18.5	1, 8
Phenylalanine	39.1 (4.23)	34.8 (1.97)	5.2-66.6	1-3, 6, 8
Proline	441.0 (4.87)	412.9 (5.04)	122.0-610.0	1-3, 6
Serine	39.1 (3.21)	34.7 (3.47)	5.2-95.0	1-3, 6, 8
Threonine	12.6 (6.92)	10.9 (2.96)	6.7-97.3	1-3, 6, 8
Tryptophan	14.7 (2.82)	12.0 (7.17)	0.1-31.2	4, 6
Tyrosine	37.6 (3.32)	32.8 (5.78)	10.0-18.0	1-3, 6
Valine	94.0 (5.54)	86.2 (8.02)	4.5-123.0	1-3, 6, 8
Organic acids				
Citric acid	405.4 (5.74)	384.1 (4.97)	5.0-250.0	1, 4, 7
Lactic acid	1.4 (3.27)	1.7 (5.74)	118.0	5
Malic acid	2215.3 (6.78)	2019.4 (8.79)	390.0-3550.0	4-5, 7
Tartaric acid	405.0 (4.67)	367.7 (6.02)	1003.0-7480.0	4-5, 7

B

Metabolites	Concentration, [9] mg/g_{DW}	Concentration reported[10], [a] mg/g_{DW}
Amino acids		

Table 2.5 (continued)

Alanine	2.25 (2.72)	1.76
Asparagine	0.54 (2.02)	0.60
Aspartic acid	2.17 (4.92)	2.10
Glutamic acid	15.23 (9.96)	17.95
Glutamine	nd	6.59
Glycine	0.31 (4.68)	0.17
Histidine	1.03 (3.46)	0.82
Isoleucine	0.31 (3.66)	0.23
Leucine	0.16 (5.34)	0.13
Lysine	1.24 (7.87)	0.64
Methionine	nd	0.03
Ornithine	0.42 (8.54)	0.52
Phenylalanine	nd	0.08
Proline	0.47 (4.50)	0.45
Serine	0.85 (5.60)	0.42
Threonine	0.44 (4.21)	0.49
Tryptophan	0.06 (1.69)	0.08
Tyrosine	0.49 (3.25)	0.26
Valine	1.04 (5.56)	0.95
Organic acids		
Citric acid	1.33 (5.39)	1.34
Fumaric acid	0.14 (2.07)	0.08
Malic acid	0.44 (3.26)	0.40

nd, not detected, [1]Huang *et al.*, 1991; [2]Spayd *et al.*, 1996; [3]Hernandes-Orte, 2003; [4]Munos-Robredo *et al.*, 2011; [5]Escobal *et al.*, 1996; [6]Gregan *et al.*, 2012; [7]Soyer *et al.*, 2003; [8]Bouloumpasi *et al.*, 2002; [9]Concentration in mg per g of yeast cell dry weight; [10]Canelas *et al.*, 2009

2.5. Conclusion

We presented here a reproducible, accurate and sensitive GC-MS method for the quantification of polar metabolites in biological samples coupled to software developed for high-throughput data analysis. The method does not require the use of calibration curves using standards to be analysed in parallel with every sample batch. However, there is a small group of metabolites where their quantification required additional steps of correction due to their chemical instability (e.g. cysteine, glutamine/glutamic acid, methionine, phosphoenolpyruvate). We also demonstrated that this new method can be applied to a wide range of biological matrices such as animal tissues, body fluids, microbial cell extracts and fruit juices with a high sugar content.

2.6. Acknowledgment

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CHAPTER III

Rapid quantitative fatty acid profiling of biological samples by gas chromatography-mass spectrometry

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This chapter presents preliminary results not submitted for publication

3.1. Abstract

The esterification of free fatty acids into fatty acid methyl esters is a traditional modification that is widely employed for lipid profiling using gas chromatography-mass spectrometry (GC-MS). Although several methods have been developed for fatty acid profiling using GC-MS, they involve multistep sample preparation procedures which are often very laborious and time consuming. We report here a novel protocol for rapid and accurate fatty acid profiling which includes a lipid saponification step followed by methylation of fatty acids through methyl chloroformate derivatisation and GC-MS analysis, which does not require anhydrous conditions. By using this method, a batch of samples can be prepared within 90 minutes using widely available chemical reagents.

3.2. Introduction

Fatty acids are hydrophobic metabolites that exist as discrete entities (free fatty acids, FFAs) or are incorporated into complex lipids. FFAs play key functional roles as signaling molecules, and they can be diverted to triglycerides to be stored in cells via cascade of lipogenic pathways (Tumanov *et al.*, 2015). Fatty acid molecules are also important structural components of cells when forming part of glycerophospholipids, which regulate the physical properties of cell membranes (Quehenberger *et al.*, 2011).

The important physiological role of these lipophilic molecules encourage the development of high-throughput and robust fatty acid profiling methods. The principle of these methods is based on GC-MS analysis of fatty acid methyl esters (FAMEs) or their analogues generated by the derivatisation of FFAs after the saponification of complex lipids. Although a number of methods have been described for fatty acid profiling, they often involve laborious procedures with several extraction steps and a combination of different solvents, anhydrous conditions and freezing cycles, which make sample preparation very time consuming (Quehenberger *et al.*, 2011; Wiesman *et al.*, 2009; Connerth *et al.*, 2009; Abdulkadir *et al.*, 2008; Akoto *et al.*, 2008; Armstrong *et al.*, 2008; Cantellops *et al.*, 1999; Glaser *et al.*, 2010; Mazalli *et al.*, 2007; Paterson *et al.*, 1997; Rodriguez-Palmero *et al.*, 1997; Ulberth *et al.*, 1995; Olmstead *et al.*, 2013).

Here we report a new protocol for rapid quantitative fatty acid profiling of lipid samples, which combines a fast and efficient method for lipid saponification, with a well-established chemical derivatisation method for esterification of organic acids followed by GC-MS analysis (Smart *et al.*, 2010). This method is simple, does not require anhydrous conditions, and has been validated using both standard mixtures of triglycerides and phospholipids as well as complex biological samples such as yeast cells and human serum.

3.3. Experimental Section

3.3.1. Reagents and standards

Methanol, chloroform, pyridine, potassium hydroxide, 2,6-*bis*(1,1-dimethylethyl)-4-methylphenol (butylated hydroxytoluene, BHT), anhydrous sodium sulphate, and methyl chloroformate (MCF) were purchased from Sigma-Aldrich. Standard mixtures of fatty acid methyl esters (FAME37) and both GLC-458 and GLC-455 standard mixtures were purchase from Sigma-Aldrich and Nu-Check Prep, Inc., respectively. The triglyceride standards (tritridecanoin and trinonadecanoin) were obtained as pure standards (99+%) from Nu-Check Prep, Inc., USA; whilst the phospholipid standards were obtained as a mixture of natural glycerophosphocholines (Avanti Polar Lipids, Inc., USA #840051P) isolated from chicken eggs.

3.3.2. Conditions for base-catalised hydrolysis

The conditions for lipid saponification were adopted from Juárez *et al.* (2008). Lipid samples were hydrolysed at 60°C with 1.3 M potassium hydroxide in methanol-water 9:1, v/v (methanolic base). To check the completeness of lipid hydrolysis, triglyceride standards (tritridecanoin and trinonadecanoin), wet and dry yeast biomass and plasma samples (n=6) were heated with methanolic base during 1 hour. Each 10 minutes an aliquot was taken and the amount of total free fatty acid was quantified in order to determine the endpoint time of hydrolysis.

3.3.3. Biological samples for method validation

Human serum sample was obtained from the National Institute of Standards and Technology as a part of Interlaboratory Analytical Comparison Study. Yeast biomass (*Saccharomyces cerevisiae* EC1118) was obtained by harvesting yeast cells

from exponentially growing liquid culture under aerobic growth conditions. Biomass was collected by centrifugation and washed twice with phosphate buffered saline (PBS) solution. To validate the method both wet and freeze-dried yeast biomass were used and results were compared.

3.3.4. Sample preparation

Standard solutions of tritridecanoin (TAG13:0/13:0/13:0), trinonadecanoin (TAG19:0/19:0/19:0) (20 mM) and phospholipids (5 mg/mL) were prepared in chloroform. Then, 20 μL of each solution was transferred to 2 mL amber GC-MS vials (n=6) and mixed with 400 μL of methanolic base containing 50 μg of BHT, which was added to prevent lipid oxidation. The vials were closed and then incubated at 60°C for 30 min. The contents of each vial were transferred to 6 mL silanised borosilicate test tubes (CTS-1275,Thermo Fisher Scientific, USA). The vials were washed with 100 μL of water-methanol (1:1, v/v) and the washing solution was combined with the lipid hydrolysate in the tubes for further chemical derivatisation.

To determine the fatty acid profile of yeast cells, 0.5 mL (n=6) of resuspended yeast biomass in PBS was centrifuged and analysed as “wet biomass” and 0.5 ml (n=6) was freeze-dried. Both wet and dry biomass was mixed with 400 μL of methanolic base, 20 μL of 5 mM trinonadecanoin chloroform solution (internal standard) and 50 μg of BHT, followed by the saponification step described above. Each sample contained approximately 6.2 mg dry weight of biomass. After incubation at 60°C for 30 min, the samples were transferred into 6 mL borosilicate tubes, as described above, for chemical derivatisation (**Figure 3.1**).

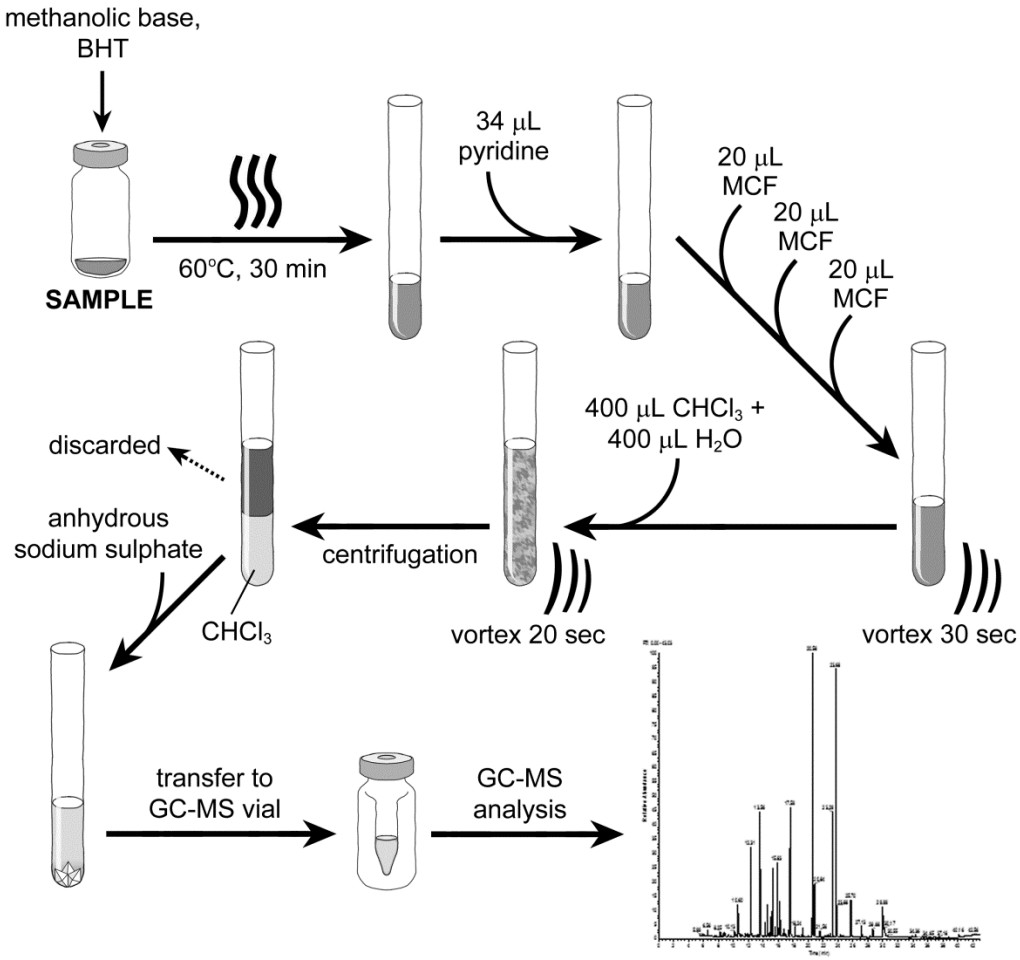

Figure 3.1. Schematic representation of the workflow for rapid fatty acid profiling protocol. Lipid extract or dry cell biomass/tissue in GC-MS vial is mixed with methanolic base (1.3M potassium hydroxide solution in methanol-water 9:1, v/v) and a solution of butylated hydroxytoluene (BHT) followed by incubation at 60°C for 30 min. The hydrolysate is then transferred to a silanised glass tube and mixed with 34 µL of pyridine followed by MCF derivatisation. After aliquots of chloroform and water are added to the reaction, and the mixture is centrifuged, the top (aqueous) layer is discarded. The chloroform layer is dried with anhydrous sodium sulphate and transferred into GC-MS vials for analysis

A modified Bligh and Dyer method (Bligh *et al.*, 1959) was applied for lipid extraction from human serum sample. 200 µL of human serum (n = 6) was mixed with 750 µL of chloroform-methanol mixture (1:2, v/v), 50 µg of BHT and 20 µL of internal standard (5 mM trinonadecanoin solution in chloroform). The mixture was homogenised by vortex mixing for one minute. Thereafter, 250 µL of chloroform was added to the mixture and vortex mixed for another minute. Finally, 250 µL of distilled water was added to the mixture followed by vortex mixing (1 min). The mixture was then centrifuged at 1258 g (5810/5810R Eppendorf centrifuge at 2500 rpm) and the lower chloroform fraction was collected and transferred to 2 mL amber GC-MS vials for chloroform evaporation under nitrogen gas. The solid lipid fraction was mixed with 400 µL of methanolic base, followed by the saponification step. After incubation at 60°C for 30 min, the samples were derivatised.

3.3.5. Chemical derivatisation and GC-MS analysis

CAUTION! Chemical derivatisation is performed using methyl chloroformate. Methyl chloroformate is a highly reactive compound and has unpleasant pungent odor. MCF decomposes at high temperatures releasing acid and extremely toxic phosgene gas. Handling and other manipulations with MCF should be made only in fume hood!

Chemical derivatisation of free fatty acids was performed based on a modified protocol described by Smart *et al.* (2010). In brief, the lipid hydrolysate in the silanised tubes was mixed with 34 µL of pyridine, followed by the addition of 20 µL of methyl chloroformate under vigorous stirring (vortexing). After 30 seconds, a second 20 µL aliquot of MCF was added to the mixture, followed by 30 seconds of mixing, and a third aliquot of MCF was further added to the mixture followed by another 30 seconds of mixing. Thereafter, 400 µL of chloroform and 400 µL of water were added to each sample, followed by constant vortexing for 20 seconds. The mixture was centrifuged and the upper aqueous layer was discarded. The chloroform

fraction was dried with anhydrous sodium sulfate and transferred into the GC-MS vials for further analysis. Chemical derivatisation undergoes rapidly and in presence of antioxidant (BHT) amber glass for borosilicate tubes is not required. The main steps of this protocol are summarised in **Figure 3.1**. The MCF added to derivatise FFAs also neutralises the excess of potassium hydroxide present in the samples. Potassium chloride, which is formed during the derivatisation step, dissolves in the water layer and helps to ensure extraction of FAMEs into the chloroform layer.

GC-MS analyses were performed using a Thermo Trace Ultra GC system coupled with a Thermo ISQ MS. The GC-MS parameters were set up as described by Smart *et al.* (2010).

Identification of FAMEs was achieved by running FAME37 mix from Supelco using AMDIS software (National Institute of Standards and Technology, USA, http://chemdata.nist.gov/mass-spc/amdis/). Peak heights were used for absolute quantification. *MetabQ* was employed to extract abundances of analytes from chromatograms. *MetabQ* is an in-house software written in R-environment (http://www.r-project.org/) that generates a .csv file with individual FAME abundances using AMDIS report. This script requires library XCMS (Smith *et al.*, 2006) and can process data files in NetCDF and mzXML formats. The quantification of fatty acids was performed using calibration curves of pure standards.

3.3.6. Recovery of fatty acids and method reproducibility

The recovery of fatty acids from triglyceride and phospholipid standards was determined based on the ratio between the quantified amount of fatty acids obtained experimentally with the real (expected) amounts. Expected amount of fatty acids for phospholipid mixture was based on the specification provided by the manufacturer (Avanti Polar Lipids, Inc., USA #840051P). In addition, fatty acid recovery from biological matrices was also assessed by determining the recovery of nonadecanoic

acid obtained from the hydrolysis of the internal standard (trinonadecanoin) spiked into the samples.

The method reproducibility was assessed by determining the relative standard deviation of each fatty acid in standard mixtures and biological samples using 6 replicates according to the equation below:

$$\%RSD = \frac{SD}{mean} \times 100\%, \quad (3.1)$$

where SD is the standard deviation.

3.3.7. Fatty acid quantification

The absolute quantification of all identified fatty acids has been achieved by building calibration curves using fatty acid standards (**Appendix 3.1**).

3.4. Results

3.4.1. Lipid hydrolysis.

Completeness of saponifcation was confirmed by determination of total free fatty acids (**Figure 3.2**). Hydrolysis of lipid standards (n=6) was completed within 10 minutes, while yeast biomass (n=6) and plasma lipids (n=6) had their lipid hydrolysis completed within 30 minutes.

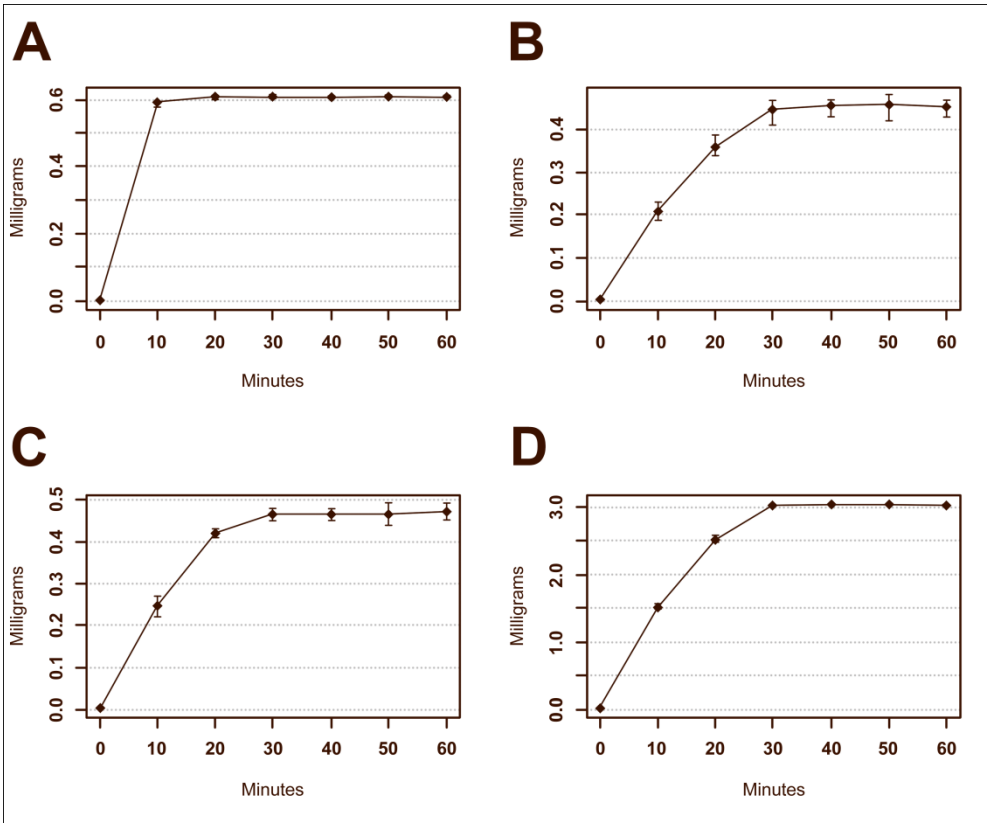

Figure 3.2. Validation of hydrolysis conditions on (A) mixture of lipid standard (tritridecanoin and trinonadecanoin); (B) wet yeast biomass; (C) freeze-dried yeast biomass and (D) plasma sample (n=6)

3.4.2. Analysis of lipid standards.

Recovery of tridecanoic and nonadecanoic acid from the TAG standard mixture was 98% and 97.5% respectively, considering that 20 µL of 20 mM of TAG solution generates 0.257 mg of tridecanoic acid and 0.358 mg of nonadecanoic acid (**Table 3.1A**). The technical variability of quantified fatty acids was 1.23% and 1.82% for tridecanoic and nonadecanoic acids respectively. Using the phospholipid standard mixture, stearic acid showed the highest recovery rate of 98.4%, while the lowest recovery rate was obtained for oleic acid with 95.7% (**Table 3.1B**). Nonetheless, all of them were above 95%. The technical variability of quantified fatty acids was between 0.9% (palmitoleic acid) and 3.7% (oleic acid), which is excellent (< 5%). Quantification of fatty acids was achieved using calibration curves of standards and the range of quantification for detected analytes was found to be considerably broad, showing 500 fold for tridecanoic and nonadecanoic acid and 100-200 fold for the rest of FAs. The limit of detection (based on signal-to-noise ratio 3:1) of the tested compounds were in the range 1 – 8.72 µM under the conditions used, which highlights the high sensitivity of our method.

Table 3.1. Quantification and recovery of fatty acids from lipid containing samples

A Mixture of tritridecanoin (TAG13:0/13:0/13:0) and trinonadecanoin (TAG19:0/19:0/19:0)

Fatty acid	Chain length	Concentration (mg)	%RSD[1] (n=6)	Reference cocentration (mg)	Recovery (%)
Tridecanoic acid	13:0	0.252	1.23	0.257	98.0
Nonadecanoic acid	19:0	0.349	1.82	0.358	97.5

B Standard mixture of natural glycerophosphocholines isolated from chicken egg

Fatty acid	Chain length	Ratio (w/w)	%RSD (n=6)	Reference ratio (w/w)	Recovery, %
Myristic acid	14:0	0.19	3.2	0.2	96.4
Palmitic acid	16:0	31.85	1.2	32.7	97.4
Palmitoleic acid	16:1n7	1.06	0.9	1.1	96.3
Stearic acid	18:0	12.10	1.4	12.3	98.4
Oleic acid	18:1n9	30.62	3.7	32.0	95.7
Linoleic acid	18:2n6	16.48	1.7	17.1	96.4
Eicosadienoic acid	20:2n6	0.19	1.3	0.2	96.7
Bishomo-γ-Linolenic acid	20:3n6	0.29	2.4	0.3	97.1
Arachidonic acid	20:4n6	2.65	1.1	2.7	98.2
Docosahexenoic acid	22:6n3	0.38	2.6	0.4	95.9

[1]Relative standard deviation (standard deviation/mean x 100).

3.4.3. Analysis of yeast biomass and human plasma lipids.

Both freshly harvested wet yeast biomass as well as freeze-dried yeast cells were used to determine the ability of our method to efficiently profile fatty acids composition of biological samples. Identical results were obtained for both wet and dry yeast biomass. We identified seven of the most abundant fatty acids in *S. cerevisiae* biomass (**Table 3.2A**). Based on the quantified amount of nonadecanoic acid, the recovery level of fatty acids from wet and freeze-dried yeast cells was 97.6% and 98.1% respectively. Palmitoleic and oleic acids were the two most abundant unsaturated fatty acids with relative distributions of 37.95% and 29.31% w/w respectively for wet yeast biomass, while distribution of these fatty acids in dry yeast biomass were 38.36% and 28.45% w/w respectively. Palmitic acid was the most abundant saturated fatty acid present. The variability of the quantified fatty acids was in the range of 0.95-4.01% for wet yeast biomass and 1.03-3.05 % for dry yeast biomass. This fatty acid profile is in agreement with other studies where the fatty acid composition of *S. cerevisiae* cells was characterised (Torija *et al.*, 2003; Lamackaa *et al.*, 1998; Cocito *et al.*, 1999).

Total fatty acid composition of human serum is shown in **Table 3.2B**. More than 40 individual peaks can be observed on chromatogram, however, only 20 different fatty acids have been identified and quantified using our method: six saturated, five monounsaturated and nine polyunsaturated fatty acids. Typical TIC chromatogram is displayed on **Appendix 3.2**. Palmitic acid showed the highest abundance and the lowest technical variability in serum among saturated fatty acids. Linoleic acid was the unsaturated fatty acid detected with the highest abundance in serum. All variation in fatty acid quantification was below 7% (**Table 3.2B**). The quantified amount of nonadecanoic acid (internal standard) was used as a reference for the recovery of fatty acids from serum and was 95.7%. The quantified amount of serum fatty acids corresponded to reference data provided by NIST (**Table 3.2B**).

Reference data were distributed by NIST and represented a concentration consensus median between laboratories participating in study for each fatty acid.

Table 3.2. Fatty acid composition of different biological samples

A *Saccharomyces cerevisiae* biomass

Fatty acid	Chain length	Ratio in wet biomass (w/w)	%RSD wet biomass (n=6)	Ratio in dry biomass (w/w)	%RSD dry biomass (n=6)	Reference range data , w/w^{1-3}
Decanoic acid	10:0	2.61	1.03	2.57	1.42	1.58-14.6
Lauric acid	12:0	3.78	1.07	3.67	1.09	2.15-8.08
Palmitic acid	16:0	16.38	1.53	16.44	2.03	10.5-43.6
Palmitoleic acid	16:1n7	37.95	3.05	38.36	4.01	26.1-38.7
Stearic acid	18:0	8.45	2.34	9.15	2.13	6.80-20.2
Oleic acid	18:1n9	29.31	1.36	28.45	0.95	26.1-39.9
Linoleic acid	18:2n6	1.52	2.11	1.36	3.15	0.70-4.90

B Human serum

Fatty caid	Chain length	Concentration (mg/L)	%RSD (n=6)	Reference concentration (mg/L)
		Saturated fatty acids		
Myristic acid	14:0	34.71	1.97	44.72
Palmitic acid	16:0	720.82	1.35	762.1
Stearic acid	18:0	231.01	5.26	228.9
Arachidic acid	20:0	3.92	2.45	4.27
Docosanoic acid	22:0	11.21	3.87	14.41
Lignoceric acid	24:0	11.50	2.90	14.60
Total		*1013.17*		*1069.0*
		Unsaturated fatty acids		
Myristoleic acid	14:1n5	3.03	6.45	3.84
Palmitoleic acid	16:1n7	51.90	3.49	52.66
Oleic acid	18:1n9	560.93	3.45	562.2
Linoleic acid	18:2n6	862.20	1.97	954.5
α-Linolenic acid	18:3n3	32.03	5.21	28.96

Table 3.2
(continued)

γ-Linolenic acid	18:3n6	12.53	2.97	11.70
Eicosenoic acid	20:1n9	5.87	2.97	5.24
Eicosadienoic acid	20:2n6	4.84	2.14	6.05
Bishomo-γ-Linolenic acid	20:3n6	34.33	3.08	31.57
Arachidonic acid	20:4n6	208.29	3.40	193.97
Eicosapentaenoic acid	20:5n3	90.15	2.87	81.37
Docosapentaenoic acid	22:5n3	21.75	1.08	21.72
Docosahexaenoic acid	22:6n3	97.59	3.02	98.25
Nervonic acid	24:1n9	22.88	4.20	21.37
Total		*2008.32*		*2073.40*
Total Fatty Acids		***3021.49***		***3142.40***

[1]Torija *et al.*, (2003); [2]Lamackaa *et al.*, (1998); [3]Cocito *et al.*, (1999)

Therefore, we successfully combined a reliable extraction and saponification method with a robust derivatisation protocol, which allowed us to carry out a rapid and robust quantitative analysis of the total fatty acid composition in different biological samples. The method is highly reproducible (**Table 3.1, Table 3.2**), sensitive and with a wide linearity range (**Appendix 3.1**).

3.5. Discussion

Quantitative fatty acid profiling requires complete lipid hydrolysis and formation of FAMEs. Transesterification of fatty acids can be performed as "one-pot" reaction where hydrolysis and methylation undergo in conditions of basic or acidic catalysis, or as a series of procedures to achieve saponification and esterification. There are many protocols for FA profiling and quantification based on transesterification using sodium or potassium methoxide catalysis and catalysis with methanolic BF_3 (Christie, 1993).

Alkaline methoxide reagents are known to be used for fast FA transesterification and can be prepared by dissolving alkaline metal in methanol. Marinetti (1966) showed that cleavage of FAMEs and glycerol occurs within 5 minutes at room temperature in presence of sodium methoxide in methanol-chloroform. However, these reagents have short shelf life and their preparation involves high chemical hazards (Christie, 1993).

The Lewis acid, BF_3 in methanol, presents powerful catalytic properties for FA esterification (Christie, 1993). Connerth *et al.* (2009) reported a protocol where dried lipid extract was treated with BF_3-methanol for 40 min at 95°C followed by several extraction steps (30 min each) using benzene and petroleum ether. However, Morrison & Smith (1964) indicated that using BF_3-methanol can lead to losses of unsaturated esters due to isomerisation side reactions. Wiesman & Chapagain (2009) reported a method in which one of the steps of sample preparation was the stratification of lipid extract at -20°C for a few hours. The main disadvantage of these methods is the amount of time spent for sample preparation which makes these protocols very labor intensive for studies involving large numbers of samples.

The main requirement for transesterification reactions catalysed by alkaline methoxide or BF_3-methanol is a water-free solvent environment. However, our method proposed here allows processing both dry and wet biological samples that makes it applicable for a wider range of biological samples. MCF has been widely

used as derivatisation reagent for the analysis of amino acids and non-amino organic acids (Leggio *et al.*, 2012). Regardless of high reactivity of MCF with water, Villas-Bôas *et al.* (2003) has shown quantitative conversion of metabolites into corresponding derivatives in water-based solution.

Another promising reagent for fast transesterification of lipids was described by Olmstead *et al.*, 2013. Ready-to-use reagent Meth-Prep II is mixture of tertiary ammonia base in methanol that does one-pot re-esterification in mild conditions. Nevertheless, all described methods cannot distinguish between free fatty acid and total fatty acid pools, making MCF derivatisation platform a perfect technique for studies described in **Chapters 4** and **5**.

Our described methodology allows complete saponification of a sample within 30 minutes in addition to 2 minutes sample derivatisation. Our experience shows that the amount of time spent for sample preparation can be reduced to 1-1.5 hours of lab work to process a batch of 20-25 samples and including the time involved in lipid extraction, which makes this a rapid protocol. Moreover, our protocol makes use of commonly available low-cost reagents (potassium hydroxide, methyl chloroformate, BHT, methanol, pyridine, chloroform), which make it easy to implement in most laboratories with access to a GC-MS platform.

3.6. Acknowledgments

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CHAPTER IV

Comprehensive Lipidome Profiling of Sauvignon Blanc Grape Juice

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4.1. Abstract

This study presents a comprehensive lipidome analysis of Sauvignon Blanc grape juice by combining GC-MS based fatty acid profiling with shotgun lipidomics strategy. We observed that despite grape juice being a water based matrix it contains a diverse range of lipid species, including common saturated and unsaturated free and intact fatty acids as well as odd-numbered and hydroxy fatty acids. Based on GC-MS quantitative data of 217 grape juices from three different vintages, we found that the total lipid content could be as high as 2.8 g/L. The majority of lipids were present in the form of complex lipids with relatively small amount of free fatty acids (<15%). Therefore we concluded that the lipidome should be considered an important component of grape juice with the potential to impact on fermentation processes as well as on the sensorial properties of fermented products. This work serves as a hypothesis generating tool, the results of which justify follow-up studies to explore the influence of the grape juice lipidome and lipid metabolism in yeast on the aroma profile of wine.

4.2. Introduction

Grape juice is a water-based sugar-rich medium containing different classes of metabolites with concentrations ranging from mg/L (methionine, lysine) (Huang *et al.*, 1991) to g/L (malic acid, tartaric acid) (Mato *et al.*, 2007), which provides a nutritive environment under s tressful anaerobic conditions for yeasts during the alcoholic fermentation process. *Saccharomyces cerevisiae* can grow in the presence of a small spectrum of indispensable organic and inorganic compounds but cell growth under the hypoxic conditions of wine fermentation is limited without an exogenous source of unsaturated fatty acids (UFAs) and ergosterol (Varela *et al.*, 2012). Anaerobic conditions combined with poor lipid supplementation govern a chain of morphological changes in *S. cerevisiae* cells (Landolfo *et al.*, 2010) that lead to the activation of biochemical pathways requiring NADPH and subsequently, the production of high levels of reactive oxygen species (ROS) (Rosenfeld *et al.*, 2003; Landolfo *et al.*, 2010) producing oxidative stress on the yeast cells (Landolfo *et al.*, 2010; Landolfo *et al.*, 2008). Biosynthesis of fatty acid molecules is important for the maintenance of structural cell components such as glycerophospholipids, which regulate the physical properties of cell membranes under metabolic stress (Quehenberger *et al.*, 2011; Landolfo *et al.*, 2010). It has previously been observed that an increase in unsaturation index in plasma membrane lipids provides yeast cells with higher ethanol tolerance during fermentation (Alexandre *et al.*, 1994; Thomas *et al.*, 1978; You *et al.*, 2003). However, under hypoxic conditions the biosynthesis of unsaturated fatty acids is repressed, reducing the viability of yeasts (Mannazzu *et al.*, 2008; Landolfo *et al.*, 2010). The inability of *S. cerevisiae* cells to acquire complex lipids (i.e. triacylglycerols) from the extracellular medium makes them highly dependent on exogenous sources of unsaturated fatty acids (Dyer *et al.*, 2002). Since *S. cerevisiae* cannot utilise complex lipids (Dyer *et al.*, 2002), exogenous phospholipids and glycerolipids can be a potential source of free fatty acids (FFAs) for yeast utilisation, which are liberated through lipolytic activity of enzymes

supplemented to grape juice or through co-culturing with lipase-secreting microorganisms.

Despite a plethora of studies reporting the amino- and organic acid profile of grape juices and their effect on yeast metabolism in wine fermentation, there is no study that we are aware of characterising comprehensively the lipid profiling of grape juice. This is somewhat surprising since previous studies have shown that the availability of free fatty acids in grape juice affects yeast metabolism, significantly altering the production of varietal aroma compounds, which is clearly pertinent to wine production (Varela *et al.*, 2012; Pinu *et al.*, 2014). Varela *et al.* (2012) demonstrated that unsaturated fatty acids and ergosterol supplementation stimulates the production of esters, higher alcohols and volatile fatty acids in Chardonnay wines and Pinu *et al.* (2014) reported a reduction in the level of acetate esters and 3-mercaptohexyl acetate (passionfruit aroma) in Sauvignon Blanc wines with the direct supplementation of linoleic acid to the juice prior to fermentation. Pinu *et al.* (2014) also indicated that the aroma profiles were affected differently depending on the amount of linoleic acid supplemented in the juice (Pinu *et al.*, 2014).

The important physiological role of lipid molecules in yeast cells and recent discoveries of their influence in the wine aroma profile provide convincing reasons to explore the lipidome of grape juice. Although a number of methods have been described for fatty acid profiling (Quehenberger *et al.*, 2011; Wiesman *et al.*, 2009; Connerth *et al.*, 2009; Abdulkadir *et al.*, 2008; Akoto *et al.*, 2008; Armstrong *et al.*, 2008; Cantellops *et al.*, 1999; Glaser *et al.*, 2010; Mazalli *et al.*, 2007; *et al.*, 1997; Rodriguez-Palmero *et al.*, 1997; Ulberth *et al.*, 1995), they often involve laborious procedures with several extraction steps and a combination of different solvents, anhydrous conditions and freezing cycles, which make sample preparation very time consuming. Moreover, the high sugar content in grape juice poses a significant problem to profile and quantify lipids at their trace levels due to the matrix effect. These factors have encouraged the optimisation of high-throughput and robust fatty acid profiling methods. The principle of these methods is based on GC-MS analysis

of fatty acid methyl esters (FAMEs) or their analogues generated by the derivatisation of FFAs after the saponification of complex lipids.

In this work, we present a comprehensive Sauvignon Blanc grape juice lipidome study that quantifies and differentiates free fatty acids from total fatty acids available in a wide range of Sauvignon Blanc grape juices harvested in different geographical locations in New Zealand over three consecutive vintages as well as shotgun lipidomics data of selected juices which together provide a resource for wine science research.

4.3. Experimental Section

4.3.2. Chemicals

Methanol, chloroform, pyridine, potassium hydroxide, 2,6-bis(1,1-dimethylethyl)-4-methylphenol (butylated hydroxytoluene, BHT), anhydrous sodium sulphate, nonadecanoic acid, d_4-alanine and methyl chloroformate (MCF) were purchased from Sigma-Aldrich. Standard mixtures of fatty acid methyl esters (FAME37) and both GLC-458 and GLC-455 standard mixtures were purchased from Sigma-Aldrich and Nu-Check Prep, Inc., respectively. Internal standard trinonadecanoin (TAG 19:0/19:0/19:0) was purchased from Nu-Check Prep, Inc. Internal standard triethanolamino trimyristate (TEM) was purchased from Omics Biochemicals Limited (Auckland, New Zealand).

4.3.3. Grape juice samples

In total 217 Sauvignon Blanc grape juices were used in this study including 10 juices from 2010, 105 juices from 2011 and 102 juices from 2012 vintages. Selected wineries from across New Zealand, with an emphasis on Marlborough, were invited to contribute juices from their vineyard blocks to participate in the Juice Index project. This project was part of the Sauvignon Blanc II Programme (C11X1005) which was started in 2010-2011. That year approximately 180 different juices were analysed and made into wine. For the 2011-2012 seasons, mostly juices from the same vineyard blocks as the previous year were used.

Commercial fruit was harvested and processed by each company in its own particular way with its own sulphur additions. At this point no yeasts are added to grape juice. For each vineyard block juice, three new 1 L bottles were sent to the wineries. All wineries were asked to fill these bottles with clear juice (after cold stabilisation, just before fermentation) with each juice clearly coded. The juices were

kept in at 4^{o}C at the wineries until collection the same day by Plant and Food Research (PFR).

After overnight cold storage in the PFR laboratory, the three bottles of juice were consolidated into one sample before sub-sampling for the individual analyses and winemaking. For the chemical analyses of the juice, exactly 60 mL of juice was decanted in new 70 mL specimen bottles to which 0.48 mL of 50 mM solution d_4-alanine was added as internal standard. For winemaking, one litre plastic bottles were filled with 700 mL of juice and all the air was removed from the bottles by squeezing them in order to prevent oxidative processes before the juice would actually be frozen. All juice samples were frozen at -20 ^{o}C, until processing. Juice samples were thawed at 4°C for 8 hours prior to sample preparation.

4.3.4. Quantitation of free fatty acids

1 mL sample of each grape juice in triplicates were mixed with an aliquot of nonadecanoic acid which was used as an internal standard. The juice sample was then freeze-dried using a BenchTop K manifold freeze dryer (VirTis, SP Scientific, Warminster, PA, USA) and derivatised using methyl chloroformate (MCF) as described previously (Smart *et al.*, 2010), followed by GC-MS analysis.

4.3.5. Quantitation of total fatty acids

4.3.5.1. Lipid extraction

A modified Bligh and Dyer method (Bligh *et al.*, 1959) was applied for lipid extraction from grape juice samples. In summary, 3 mL of each grape juice sample in triplicate were mixed with 3 mL of chloroform-methanol mixture (1:2, v/v), 50 µg of BHT to prevent lipid oxidation and 20 µL of internal standard (5 mM

trinonadecanoin solution in chloroform). The mixture was homogenised using a vortex mixer for 3 minutes. Thereafter, 1 mL of chloroform was added to the mixture and vortex mixed for another minute. Finally, 1 mL of distilled water was added to the mixture, followed by vortex mixing (1 min). The mixture was then centrifuged at 1258 *g* (5810/5810R Eppendorf centrifuge at 2500 rpm) and the lower chloroform phase was collected and transferred to 2 mL amber GC-MS vials for chloroform evaporation under nitrogen gas. The solid lipid residue was then saponified using conditions adapted from (Juarez *et al*, 2008). Lipid samples were hydrolysed at 60°C with 1.3 M potassium hydroxide in methanol-water 9:1, v/v (methanolic base). The vials were closed and then incubated at 60°C for 30 min. The contents of each vial were transferred to 6 mL silanised borosilicate test tubes (CTS-1275, Thermo Fisher Scientific, USA). The vials were washed with 100 µL of water-methanol (1:1, v/v) and the washing solution combined with the lipid hydrolysate in the tubes for further chemical derivatisation.

4.3.5.2. Chemical derivatisation

Chemical derivatisation of total fatty acids in the samples was performed based on a modified protocol described by Smart *et al*. (2010). In brief, the lipid hydrolysate in the silanised tubes was mixed with 34 µL of pyridine, followed by the addition of 20 µL of methyl chloroformate (MCF) under vigorous stirring (vortexing). After 30 seconds, a second 20 µL aliquot of MCF was added to the mixture, followed by 30 seconds of mixing, and a third aliquot of MCF was further added to the mixture followed by another 30 seconds of mixing. Thereafter, 400 µL of chloroform and 400 µL of water were added to each sample, followed by constant vortexing for 20 seconds. The mixture was centrifuged and the upper aqueous layer was discarded. The chloroform fraction was dried with anhydrous sodium sulfate and transferred into GC-MS vials for further analysis.

4.3.5.3. GC-MS and resulting data analysis

GC-MS analyses were performed using a Thermo Trace Ultra GC system coupled with a Thermo ISQ MS. The GC-MS parameters were set up as described by Smart *et al*. (2010). Identification of FAMEs was achieved using AMDIS software (National Institute of Standards and Technology, USA, http://chemdata.nist.gov/mass-spc/amdis/) and an in-house MS library of MCF standards. Peak heights were used for quantification. *MetabQ* was used to extract abundances of analytes from chromatograms. *MetabQ* is an in-house software written in R-environment (http://www.r-project.org/) that generates a .csv file with individual FAME abundances using AMDIS report. This script requires the XCMS library (Smith *et al.*, 2006) and can process data files in NetCDF and mzXML formats. The quantification of the different fatty acids was performed using calibration curves of pure standards.

4.3.5.4. Shotgun lipidome analysis of selected grape juice samples

Based on the quantitative fatty acid profile of grape juices, the 5 most distinctive grape juice samples (with high and low lipid content) from each year were used for shotgun lipidomics. Lipids were extracted using the procedure described in Section 4.3.5.1. 300 µL of each lipid extract was mixed with 20 µL of 5 mM chloroform solution of TEM (the internal standard for the positive ionisation mode) and 20 µL of 0.1 M chloroform solution of nonadecanoic acid (the internal standard for the negative ionisation mode) followed by sample dilution with 400 µL of 10 mM ammonium formate solution in methanol/chloroform (2:1 v/v). Samples were then subjected to direct infusion MS analysis.

A Q-Exactive™ orbitrap mass spectrometer (Thermo Scientific™, San Jose,CA) equipped with an electrospray ion source and Xcalibur™ 2.2 system software was employed to perform shotgun lipidome analysis of juice lipid extracts.

Prepared lipid extracts were directly infused into the ESI source with a syringe pump at a flow rate of 5 μL/min. Lipid classes were analysed in two different modes: positive and negative ion ESI. Tanden MS was performed for the Top 100 most abundant ion peaks in both charge modes. The electrospray settings were as follows: sheath gas flow rate 6 (arbitrary units), auxiliary gas flow rate 5 (arbitrary units), sweep gas flow 4 (arbitrary units), spray voltage 3.1 kV for positive ion ESI and 4.0 kV for negative ion ESI, capillary temperature 150°C. The mass spec resolution was set to 140,000 and automatic gain control was set to $3*10^6$ with a maximum injecton time of 200 ms. The scan range was 100-1,500 *m/z* for positive mode and 100-1,000 *m/z* for negative mode. Data extraction and lipid identification were conducted using Lipid Mass Spectrum Data Analysis (LIMSA) v1.0 software in conjunction with an in-house XCMS-based R script.

4.4. Results and discussion

4.4.1. Free fatty acid profile in grape juice and their concentrations

Based on our GC-MS analysis, we detected almost a hundred chromatographic peaks in each analysed sample. Using our in-house MS library of fatty acid standards we were able to identify and quantified only four free fatty acids in juice samples from 2010, five in juices from 2011 and six in 2012 juices (**Tabel 4.1A** and **Appendix 4.1**). Our MCF library of derivatised fatty acid standards contains MS spectra of 43 different fatty acids, ranging from C6 to C24. Therefore, those non-identified peaks are probably MCF derivatives of other juice metabolites such as amino and non-amino organic acids, but certainly it could include uncommon fatty acids not found in our library such as branched and hydroxy fatty acids. Palmitic, stearic, linoleic, and γ-linolenic acids were the four common free fatty acids detected in all 217 juice samples. Palmitic acid was the most abundant free fatty acid found in Sauvignon Blanc grape juices. The unsaturated fatty acids linoleic and γ-linolenic acids showed a reduced range of variation in concentration when comparing different vintages. The lowest and the highest concentration of linoleic and γ-linolenic acid across all analysed samples were 0.06/8.57 and 0.04/4.31 mg/L, respectively (**Table 4.1A**). Myristic acid was identitfied only in juices from 2011, whilst both hexanoic and oleic acids were found only in juices from 2012. Therefore, based on our data, the total content of free fatty acids in the Sauvignon Blanc grape juice samples lies in the range of 0.56 to 28.45 mg/L over the three consecutive vintages studied. The lowest level of free fatty acid was observed in juice samples from 2010 (1.07-4.85 mg/L), whilst free fatty acid content in juice samples from 2011 and 2012 ranged between 0.87-26.01 mg/L and 0.56-28.45 mg/L, respectively (**Table 4.1A**).

Table 4.1. Concentration range of identified (A) free fatty acids and (B) total fatty acids (free fatty acids + fatty acids from complex lipid molecules) in Sauvignon Blanc grape juices

A	**Juice vintage**		
Fatty acid	**2010 (n=10)**	**2011 (n=105)**	**2012 (n=102)**
(C6:0) Caproic acid	nd	nd	0.01-0.15
(C14:0) Myristic acid	nd	0.17-0.96	nd
(C16:0) Palmitic acid	0.58-2.42	0.39-11.19	0.24-13.61
(C18:0) Stearic acid	0.11-0.21	0.21-9.73	0.02-0.18
(C18:1n-9) Oleic acid	nd	nd	0.15-1.63
(C18:2n-6,9) Linoleic acid	0.07-1.61	0.06-3.27	0.08-8.57
(C18:3n-6,9,12) γ-Linolenic acid	0.31-0.61	0.04-0.86	0.06-4.31
Range of total free fatty acid content	1.07-4.85	0.87-26.01	0.56-28.45

B	**Juice vintage**		
Fatty acid	**2010 (n=10)**	**2011 (n=105)**	**2012 (n=102)**
(C6:0) Caproic acid	0.84-34.07	7.97-100.68	3.95-37.69
(C8:0) Caprylic acid	0.74-1.01	nd	nd
(C14:0) Myristic acid	7.49-9.38	7.05-8.39	3.69-69.16
(C15:0) Pentadecanoic acid	2.7-3.32	2.58-2.94	3.65-17.02
(C15:1n-5) Pentadecenoic acid	0.63-0.85	nd	nd
(C16:0) Palmitic acid	53.12-258.70	1.49-213.23	3.56-935.16
(C16:1n-7) Palmitoleic acid	5.58-9.56	5.44-13.53	3.91-16.58
(C17:0) Margaric acid	8.35-9.95	8.20-9.24	4.70-11.90
(C17:1n-7) Heptadecenoic acid	5.38-6.86	nd	nd

Table 4.1 (continued)

(C18:0) Stearic acid	10.40-41.29	0.99-51.99	4.23-219.23
(C18:1n-9) Oleic acid	2.66-150.42	26.18-162.26	3.86-225.41
(C18:2n-6,9) Linoleic acid	1.46-125.16	1.26-89.07	3.60-423.82
(C18:3n-6,9,12) γ-Linolenic acid	3.79-857.28	3.02-26.74	3.62-245.96
(C20:0) Arachidic acid	nd	nd	4.34-22.44
(C20:2n-6,9) Eicosadienoic acid	2.52-432.58	3.60-293.81	3.59-409.58
(C20:3n-3,6,9) Eicosatrienoic acid	0.92-186.07	2.68-57.29	3.63-245.96
Range of total fatty acid content	106.58-2126.50	70.46-1029.17	50.33-2879.91

nd, not detected

4.4.2. Total fatty acid profile of juice lipidome

The profile of total fatty acids obtained from grape juice lipid extracts (free fatty acids + fatty acids from complex lipids) showed a higher diversity of fatty acids when compared to the profile of free fatty acids alone (**Table 4.1B** and **Appendix 4.2**), which suggests that most fatty acids in the Sauvignon grape juice are not readily available for yeast fermentation because they are part of complex lipid molecules. Overall, we identified and quantified 15 fatty acids in juice samples from 2010, 12 from 2011 and 13 in 2013 juices. The fatty acid profiles for all samples were found to be simular, however, caprylic, pentadecenoic and heptadecenoic acids were identified only in 2010 juice whilst arachidic acid was detected only in juices from the 2012 vintage. Among the unsaturated fatty acids, we identified and quantified 8 in the 2010 samples, and 6 in the 2011 and 2012 juice samples. Palmitic acid was the most abundant fatty acid found in analysed grape juices both in the free fatty acid pool as well as the pool derived from lipid hydrolysis. In 2010 juice samples γ-linolenic acid showed the largest concentration variation (more than 220-fold), whilst in both 2011 and 2012 juice samples, palmitic acid showed the largest variation (143-fold and 262-fold, respectively) (**Table 4.1B**). Therefore, based on the profile of total fatty acids in the grape juice samples, the variation in lipid content in these water based samples was found to be in the range of 50.33-2879.91 mg/L (**Table 4.1B**).

In order to analyse the lipid content of grape juice by means of total fatty acid profiling we used 3 ml of grape juice, whilst for free fatty acid analysis only 1 ml was used. We observed that GC-MS analysis of 1 ml of lyophilised grape juice and lipid extract from 3 ml of juice sample gave an equal number of identified free fatty acids (data not shown). This information helped us to generate quantitative free fatty acid and polar metabolite (i.e. amino acids, organic acids and amines) profiles and minimise the matrix effect caused by high sugar concentration.

4.4.3. Percentage of free fatty acids over total fatty acid content in juice

Our quantitative GC-MS approach showed that the free fatty acids in grape juice are present in trace quantities (**Table 4.1A**) with a maximum total free fatty acid concentration of 28.45 mg/L (≈30 ppm). The majority of these FFAs are saturated fatty acids, predominantly palmitic and stearic acids. Higher levels of fatty acids in grape juice were present esterified as complex lipids (i.e. glycerolipids, glycerophospholipids) with the highest concentration being almost 2.9 g/L (**Table 4.1B**). Based on quantitative data for the free and total fatty acid content of grape juice (**Appendix 4.3**), we observed that only a small amount of total lipid specifically in the form of free fatty acids is readily available for yeast consumption during grape juice fermentation (**Figure 4.1**). These data were calculated as the ratio between total free fatty acid content and total lipid content for each juice sample. Thus, juices of the 2010 vintage showed the lowest percentage of FFAs (<1%), whilst 2011 juices showed the highest at 15%.

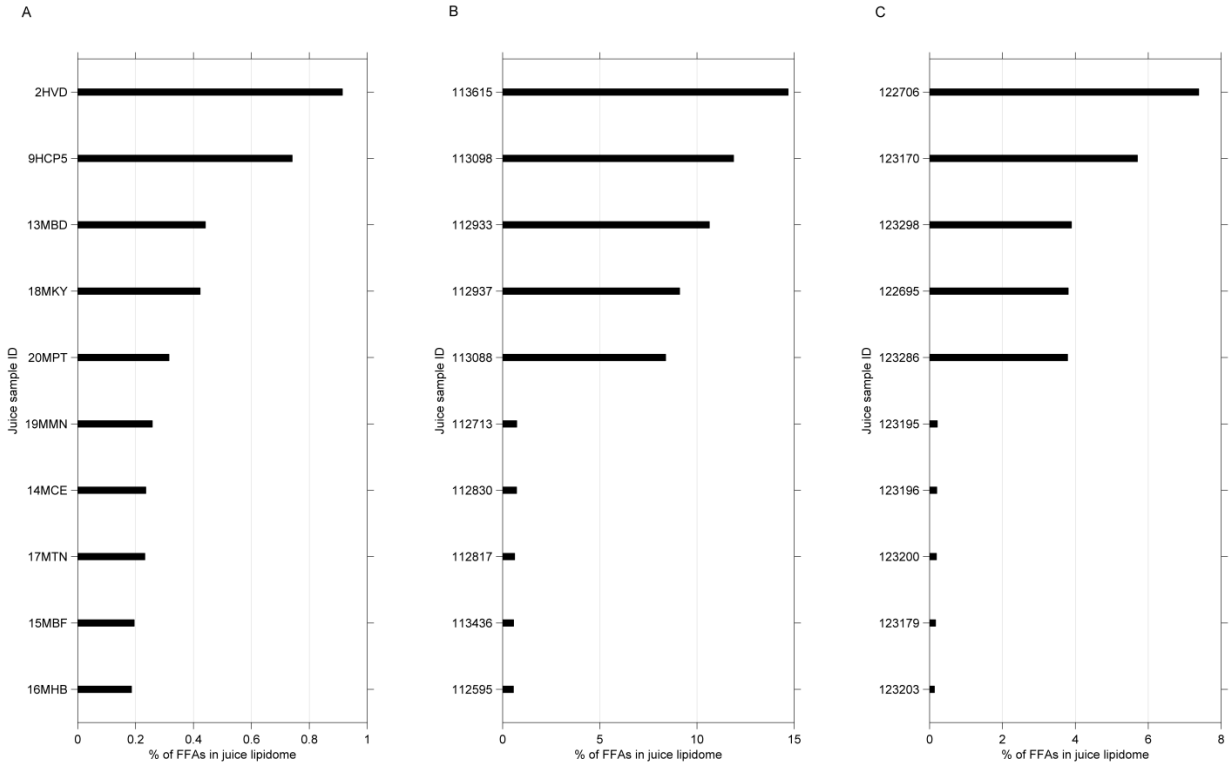

Figure 4.1. Percentage of free fatty acids over total fatty acid content in (A) 2010, (B) 2011 and (C) 2012 grape juice samples. Values were calculated as the ratio between total free fatty acid composition of grape juice and total amount of lipid. Each bar graph demonstrates data for 10 juice samples (5 highest and 5 lowest values) for each year

4.4.4. Lipidomic profile of Sauvignon Blanc grape juice samples

A shotgun lipidomics approach was used to determine the profile of complex lipids in grape juice and the distribution of fatty acids in those lipid species. On the basis of high-resolution Orbitrap MS, 83 different lipid species covering 7 lipid classes were identified in the different juice samples (**Table 4.2 and Figures 4.2 and 4.3**). In total, 13 free fatty acids including 4 hydroxy fatty acids were identified using the shotgun lipidomic approach. Some free hydroxy fatty acids contained odd-numbered carbon chains with different level of unsaturation. The occurrence of odd-numbered fatty acids in natural plant lipids has been considered to be uncommon with their content contributing with less than 3% of total fatty acids in seeds. However, recent studies showed that odd-numbered FAs are minor components in almost all lipid found in nature (Rezanka *et al.*, 2009). Considering that the juice making process is non-sterile (Rezanka *et al.*, 2009) with frequent contamination by bacteria and fungi (e.g. yeasts and spores of mould), the source of odd-numbered fatty acids originates from bacteria present in the grape juice.

Three different ceramide species were identified in the grape juices which contained palmitic, stearic and linolenic acid moieties. Among the glycerophospholipids, only lysophosphocholines and phosphocholines were identified. All identified glycerophospholipids were composed of common fatty acids from myristic to eicosatrienoic acid. Identifications from the monoacylglycerol class comprised 7 different species with both even- and odd-numbered fatty acids, whilst di- and triacylglycerols contained common fatty acid moieties. Triacylglycerols were found to be the most diverse class, with 29 different molecular species identified. A comprehensive list of lipids and their fatty acid composition is found in **Table 4.2** and **Figures 4.2 and 4.3**. Interestingly, we were unable to detect lipids belonging to other classes such as phosphatidylserines, phosphatidic acids, phosphatidylinositols, phosphatidylglycerols, and sterols, which suggest that they might be in grape juice or at very low levels.

Table 4.2. Summary of the number of lipid species identified in SB juice lipid extracts. The list of lipid species was generated based on data analysis of MS and MS/MS information of analysed lipid extracts. Data extraction and lipid identification were conducted using Lipid Mass Spectrum Data Analysis software in conjunction with an in-house XCMS-based R script.

	2010	**2011**	**2012**
Lipid class	**Number of identified lipid species**		
FA[1]	10	11	13
Cer[2]	3	3	3
LPC[3]	8	8	8
PC[4]	14	14	13
MAG[5]	6	5	5
DAG[6]	7	9	9
TAG[7]	25	26	28
Total	73	76	79

[1]Fatty acids, [2]ceramides, [3]lysophosphocholines, [4]phosphocholines, [5]monoacylglycerides, [6]diacylglycerides, [7]triacylglycerides

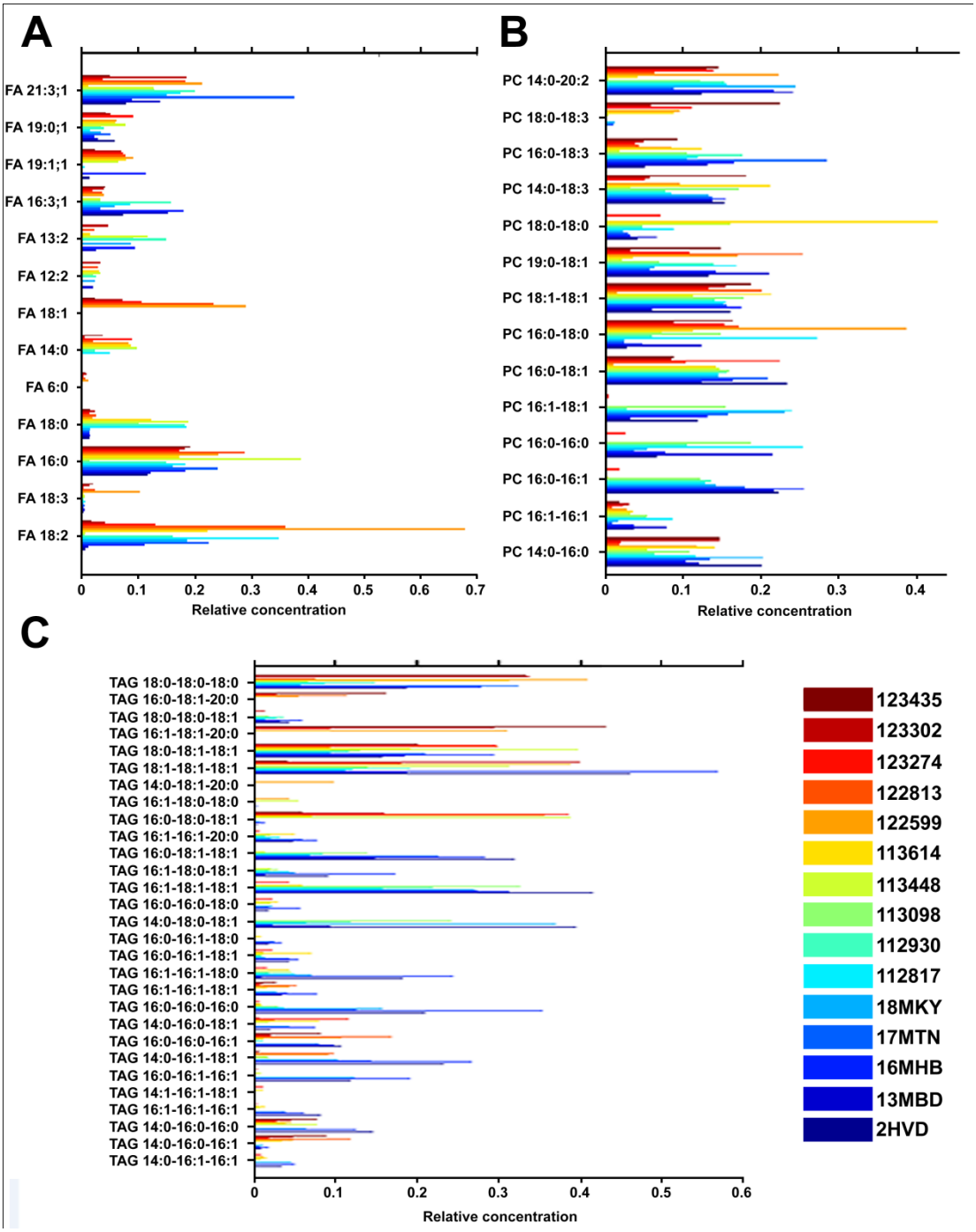

Figure 4.2. Molecular composition of (A) fatty acids (FA), (B) phosphocholines (PC), (C) triacylglycerides (TAG) species identified in grape juice. Each colour represents 15 different analysed grape juice samples

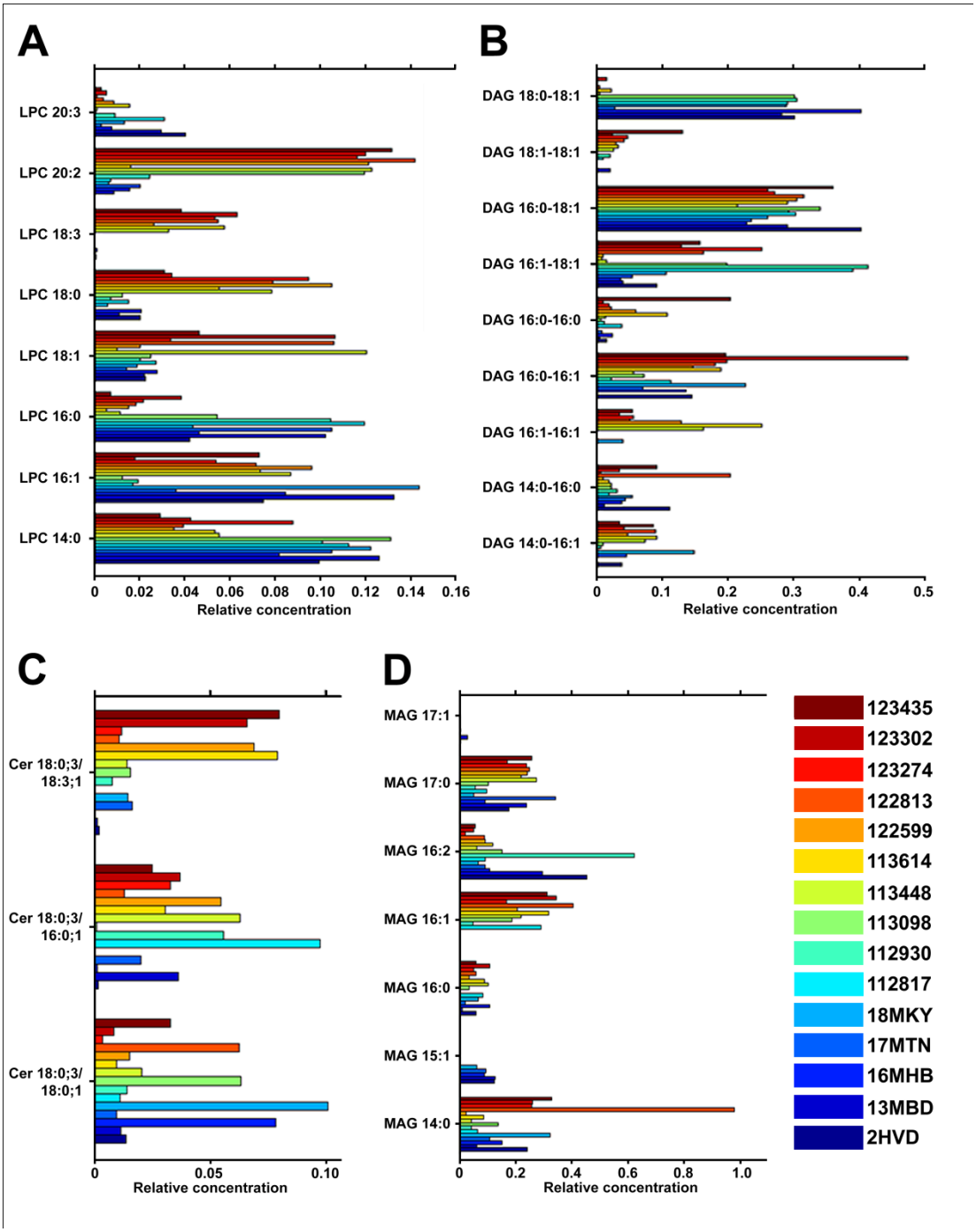

Figure 4.3. Molecular composition of (A) lysophosphocholines (LPC), (B) diacylglycerides (DAG), (C) ceramides, (D) monoacylglycerides (MAG) species identified in grape juice. Each colour represents 15 different analysed grape juice samples

Interestingly, the shotgun lipidomics approach revealed a high diversity of free fatty acids in grape juice, including saturated, monounsaturated and polyunsaturated hydroxy fatty acids. The GC-MS approach allowed for the identification and quantitation of common fatty acids based on an in-house FAME mass spectral library built using commercially available standards, whilst the hydroxy fatty acids remained unquantified.

4.4.5. Potential impact on Sauvignon Blanc wine fermentation

GC-MS analysis complemented with shotgun lipidomics approach revealed that grape juice contain a varieties of lipid molecules (**Table 4.2**). In this study, we observed many of free fatty acids in New Zealand Sauvignon Blanc grape juice that could potentially be uptaken by yeast cells during wine fermentation; 8 of which were unsaturated free fatty acids essential for yeast growth and viability under anaerobic conditions. However, most of the essential fatty acids for yeast anaerobic growth present in grape juice are in a biologically unavailable form for *S. cerevisiae*, a yeast unable to hydrolyse lipids extracellularly (Dyer *et al*., 2002).

Previous studies have found that traces of free fatty acids in the grape juice have a significant effect on the final aroma profile of wine (Pinu *et al*., 2014). The modern practice of wine making based on inoculating the grape juice with selected commercial strains of *Saccharomyces cerevisiae* (Ortiz *et al.*, 2013), impact negatively on the breakdown of complex lipids in the juice due to the inability of *S. cerevisiae* to produce extracellular lipases (Darvishi, 2012). During spontaneous wine fermentation, on the other hand, other yeast (e.g. *Candida* sp) (Zhang *et al*., 2010) and bacterial species start the fermentation alongside *S. cerevisiae* before being competed out. Majority of these contaminating microbial species are capable of producing extracellular lipases (Lock *et al.*, 2007), which could increase the amount of free fatty acids in grape juice to be further consumed by *S. cerevisiae*. The

same effect could be achieved by co-culture during wine fermentation using *S. cerevisiae* and a lipase-secreting yeast (e.g. *Candida utilis*, *Candida rugosa*, *Yarrowia lipolytica*).

It is obvious that the lipid content of grape juice is related to how the grape berries are processed to extract the juice. However, there is little information thus far regarding the lipid composition of grape berries available in peer-reviewed literature. We observed that lipid content varied significantly across different vintages. These differences in the concentration of free fatty acids and total lipids between juices could be related not only to seasonal variation but also to environmental conditions such as rainfall, sunlight hours, grape berry processing methods and grapevine metabolism (Pinu *et al*., 2014).

We identified free hydroxy fatty acids based on high resolution MS data. The origin of these fatty acids is unclear, however, studies have shown a significant physiological activity on yeast growth. Early studies indicated that hydroxy fatty acids can support cell growth anaerobcally similarly to some unsaturated fatty acids (Light *et al.*, 1962). Light *et al*. (1962) demonstrated that acetoxy fatty acids – acetylated products of uptaken hydroxy FAs exhibited growth activity equal to that of unsaturated fatty acids (UFAs), concluding that successful growth of yeast cells under hypoxic conditions can be achieved without supplementation of UFAs. Moreover, Pinu *et al*. (2014) reported the effect of linoleic acid on the production of aroma compounds in fermentation processes, so it is of a great interest to explore further the effect that other free fatty acids have on yeast metabolism and as a result, aroma profiles.

4.5. Conclusion

Based on the results of our comprehensive lipidome profiling of Sauvignon Blanc grape juice it can be concluded that grape juice contains a diverse range of lipid species with varying concentrations. However, the majority of lipids are present in form of complex lipids with a relatively small amount of free fatty acids (<15%). This information could potentially be used to explore ways to increase the amount of free fatty acids in grape juice medium through lypolitic activity of microbial enzymes and in turn supply the yeast demand for unsaturated fatty acids during wine fermentation. Our study is the first to detail the lipid profile of Sauvignon Blanc grape juice and we hypothesise that the potential liberation of intact fatty acids from grape juice medium may help improve fermentation performance and provide for the development of new wine styles.

4.6. Acknowledgments

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CHAPTER V

Effect of Fatty Acids on Sauvignon Blanc Fermentation

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This chapter presents preliminary results not submitted for publication

5.1. Abstract

3-mercaptohexanol (3MH), 3-mercaptohexyl acetate (3MHA) and 4-mercapto-4-methylpentan-2-one (4MMP) in New Zealand Sauvignon Blanc wine are regarded as key volatile thiols. Previously it was observed that traces of linoleic acid in grape juice can significantly alter the development of these volatile thiols and other aroma compounds in wine. In this study, we examined the effect of other fatty acids commonly found in grape juice, on the production of aroma compounds. Additionally, we performed juice manipulation experiments including supplementation of grape juice with acidic lipase from *Aspergillus niger* prior to fermentation and mixed fermentations with non-*Saccharomyces* lipase secreting yeasts (*Candida rugosa, Candida utilis* and *Yarrowia lipolytica*) in order to liberate fatty acids from complex lipids present in grape juice. The key findings presented in this study may lead to a new field of juice manipulation experiments employing the lipidome component of grape juice, which has not received much attention previously.

5.2. Introduction

Sauvignon Blanc (SB) is New Zealand's flagship wine style, recognised locally and internationally for its distinguishable tropical flavours. The recognised value of SB wine on an international level, its standings at wine competitions, and its increasing consumer demand, make SB New Zealand's most planted grape varietal. SB wine made up 85.5 % of New Zealand's wine exports, reaching over 80 countries, with a total wine export value upwards of $1.33 billion in 2014 – a figure which is predicted to grow in future (NZ Wine Annual Report, 2014). New Zealand Sauvignon Blanc wine has earned worldwide recognition mainly for its high levels of volatile thiols, such as 3-mercaptohexyl acetate (3MHA), 3-mercaptohexan-1-ol (3MH) and 4-mercapto-4-methylpentan-2-one (4MMP), which are extremely odorous molecules that contribute to passionfruit, grapefruit and box tree (cat's pee) aromas, respectively (Benkwitz *et al*, 2012). Despite the fact that trace amounts of 3MH are detected in grape juice (Capone *et al*, 2011), varietal thiols remain the products of yeast metabolism during alcoholic fermentation (Pinu *et al*, 2014). Potential biosynthetic pathways and putative precursors for volatile thiol production during fermentation have been studied extensively (Allen *et al*, 2011; Capone *et al*, 2011; Fedrizzi *et al*, 2009; Harsch *et al*, 2013; Schneider *et al*, 2006). Nevertheless, it was demonstrated that the concentrations of 3MH, 3MHA and 4MMP present low correlations with their cysteinylated (Cys-3MH, Cys-4MMP) and glutathionylated (GSH-3MH, GSH-4MMP) putative precursors in grape juice (Pinu *et al*, 2012; Roland *et al*, 2010).

Pinu *et al* (2014) proposed that low conversion efficiency of prospective precursors into volatile thiols could be limited by other regulatory metabolites in grape juice involved in the respective biochemical reactions. They also demonstrated how metabolomics, with its non-targeted approach, can be applied as a powerful tool for investigating the influence of individual juice metabolites on the development of volatile

thiols during alcoholic fermentation, through juice manipulation experiments. Pinu *et al* (2014) employed a comprehensive metabolomic analysis coupled to statistical analysis in order to shortlist grape juice metabolites that showed high correlation to volatile thiols in fermented wines. Interestingly, trace amounts of one particular fatty acid – linoleic acid – showed a significant influence on yeast metabolism and as a result on the production of aroma compounds, mainly through the reduction of the concentration of 3MHA and other acetate esters - indicating a strong effect on the acetylation processes. This finding clearly demonstrates that lipid metabolism could affect the biogenesis of varietal thiols. Our previous study (Chapter 4) reported that New Zealand SB grape juice has a high amount of lipids (up to 2.8 g/L) and its lipidome comprises close to a dozen free fatty acids in trace quantities, available for yeast fermentation in addition to a larger quantity of fatty acids present in the form of complex lipid molecules (e.g.; triglycerides, phospholipids, etc).

Saccharomyces cerevisiae can grow in the presence of a small range of essential organic and inorganic compounds, however, cell growth under the hypoxic fermentation conditions is limited without an exogenous source of unsaturated fatty acids (UFAs) and ergosterol (Varela *et al,* 2012). Anaerobic fermentation of juice with a depleted level of lipids governs a chain of morphological changes in *S. cerevisiae* cells resulting in oxidative stress on the yeast cells (Landolfo *et al,* 2010). Fatty acid biosynthesis is important for the maintenance of structural cell components (e.g. glycerophospholipids) which regulate the physical properties of membranes under metabolic stress (Quehenberger *et al,* 2010). *S. cerevisiae* is inefficient in acquiring complex lipids (i.e. triacylglycerols) from the extracellular medium making this yeast highly dependent on exogenous sources of unsaturated fatty acids (Dyer et al, 2002).

In this work we carried out a juice manipulation experiment using *S.cerevisiae* EC1118 wine strain and testing the direct supplementation of free fatty acid to grape

juice prior to fermentation, and increasing lipolytic activity in the grape juice by treatment with lipase or by co-culturing *S. cerevisiae* with different lipase secreting yeast species (*Candida rugosa, Candida utilis and Yarrowia lipolytica*) during wine making.

5.3. Experimental Section

5.3.1. Chemicals

All fatty acids, acidic lipase from *Aspergillus niger*, and dimethyl dicarbonate for juice chemical sterilisation, ethanol standard (99.5%) and ethyl acetate (99.8%) were purchased from Sigma-Aldrich (St. Louis, USA). Internal standard d_4-methanol was purchased from Cambridge Isotope Laboratories, Inc (Andover, USA). Internal standards for volatile thiol quantitation – d_2-3-mercaptohexan-1-ol (d_2-3MH), d_2-3-mercaptohexyl acetate (d_2-3MHA) and d_{10}-4-mercapto-4-methyl pentan-2-one (d_{10}-4MMP) and 4-methoxy-2-methyl-2-mercaptobutane (4M2M2MB) were synthesised at The University of Auckland, New Zealand. The derivatisation reagent ethyl propiolate and butylated hydroxyanisole were purchased from Aldrich (Castle Hill, NSW, Australia). Internal standards for quantitation of esters, higher alcohols and other aroma compounds – d_5-ethyl butanoate, d_5-ethyl hexanoate, d_5-ethyl octanoate were synthesised at Lincoln University, New Zealand; d_3-3-methylbutyl acetate, d_3-n-hexyl acetate, d_3-2-phenylethyl acetate, d_3-(±)-linalool, d_2-3-methyl-1-butyl-1,1-alcohol, d_{11}-n-hexyl-2,2,3,3,4,4,5,5,6,6,6 alcohol, d_5-2-phenyl alcohol were obtained from CDN ISOTOPES, Canada. 4-decanol (Lancaster, Pelham, NH, USA), DL-3-octanol (Acros Organics, Geel, Belgium) and 3,4-dimethylphenol (Aldrich, Milwaukee, WI, USA) were used for quantification of aroma compounds.

5.3.2. Microorganisms and media

Cultures of *S.cerevisiae* EC1118, *C. utilis* DSM70167, *C. rugosa* IFO0750 and *Y. lipolytica* ICPM 14995 were inoculated on YPD agar plates (Bacto Yeast Extract, 6 g/L; Bacto Peptone, 3 g/L; D-glucose, 10 g/L and Agar, 15 g/L; pH 5.5) and maintained at 28°C for 48 h. YPD broth medium (Bacto Yeast Extract, 6 g/L; Bacto Peptone, 3 g/L

and D-glucose, 10 g/L; pH 5.5) was used as pre-culture medium to produce biomass for further juice inoculation. Yeast cells were washed with sterile saline buffer before inoculation.

Sauvignon Blanc grape juice 2013 vintage from Square Block, Pernod Ricard winery (Marlborough, New Zealand) was used for all juice manipulation experiments. Grape juice was frozen at -20°C and transported frozen to the Metabolomics lab, at the University of Auckland, New Zealand. Defrosted juice was then chemically sterilised, as described in Pinu *et al* (2012) and kept at 4°C before inoculation. The oenological parameters of the grape juice were pH 3.21, Brix 19.5° and YAN 251 mg/L.

5.3.3. Free and total fatty acid quantification in grape juice

The quantification of free fatty acid (FFA) and total fatty acids was carried out in order to determine the amount of these lipids originally present in the grape juice to be used in the juice manipulation experiments.

1 mL of grape juice in triplicate was mixed with an aliquot of 5 mM nonadecanoic acid in chloroform solution, which was used as an internal standard. The juice sample was then freeze-dried using a BenchTop K manifold freeze dryer (VirTis, SP Scientific, Warminster, PA, USA) prior to chemical derivatisation. Freeze-dried samples were derivatised using methyl chloroformate (MCF) as described by Smart *et al* (2010), followed by GC-MS analysis. Quantification of total fatty acids (TFA) was performed using the method developed and described in Chapter 3.

GC-MS analysis was performed using Thermo Trace Ultra GC system coupled with a Thermo ISQ MS. The GC-MS parameters were set up as described by Smart *et al* (2010). Identification of fatty acids was performed using AMDIS software (National Institute of Standards and Technology, USA, http://chemdata.nist.gov/mass-spc/amdis/) and an in-house MS library of MCF standards. Either peak areas or peak heights could

be used for absolute quantification of fatty acids. However, due to partial co-elution of some fatty acids during chromatographic separation, peak ion heights were used in order to perform accurate quantification. An R-based package *MetabQ* (http://metabolomics.auckland.ac.nz/) was employed to extract the abundances of the fatty acids identified from the chromatograms. The quantification of the different fatty acids was performed using calibration curves of pure standards.

5.3.4. Validation of lipase activity under optimum conditions and in grape juice medium

Aspergillus niger lipase activity was tested under optimum conditions (i.e. 40°C and pH 7.4, 50 mM phosphate buffer) to confirm its enzymatic activity with trilinolein as substrate. 200 µL of 10 U/mL (50 mg/mL) of lipase was added to 50 mL of 50 mM phosphate buffer (pH 7.4) at 40°C under continuous stirring, followed by the addition of 30 mg of trilinolein dissolved in 100 µL of methanol. Samples were taken every 20 min for 100 min and the concentration of linoleic acid was determined by GC-MS analysis as described in 5.3.3. The concentration of linoleic acid produced was plotted against time.

Fermentation of grape juice was performed at low temperature and pH, conditions where enzymatic activity would likely be significantly reduced. In order to confirm lipase activity in this juice medium, lipase was added to 200 mL of grape juice at a concentration of 500 mg/L and kept at 15°C with moderate stirring for 48 hours under nitrogen gas. Every 8 hours a 1 ml aliquot of the juice sample was taken and the concentration of fatty acids released was determined by GC-MS as described in 5.3.3. The concentration of free fatty acids was plotted against time.

5.3.5. Validation of lypolitic activity produced by non-*Saccharomyces* yeasts in grape juice medium

In order to validate whether the chosen *Candida* species secreted lipases into grape juice medium under fermentation conditions, a microvinification experiment was carried out in 2 mL 96 deepwell plates equipped with a silicon mat (Thermo Fisher, USA). The silicon mat is designed as a one-way valve to release excessive pressure of CO_2 inside the well, whilst creating and maintaining anaerobic conditions **(Figure 5.1**).

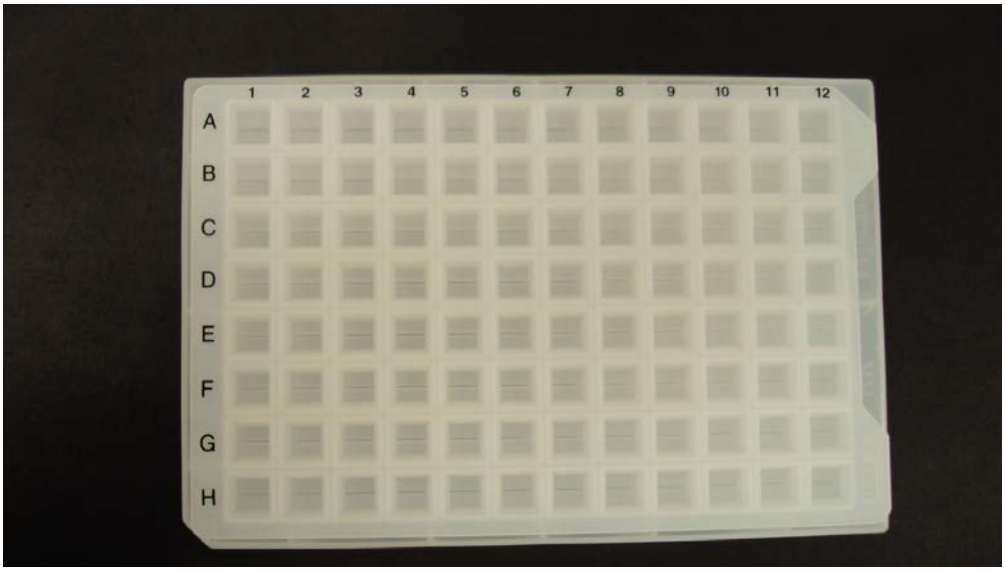

Figure 5.1. A 2 mL 96 deepwell plate equipped with a silicon mat. The silicon mat was used as a one-way valve to release the excessive pressure of CO_2 inside the well during fermentation

1 mL aliquot of sterilised grape juice was mixed with each *Candida* culture individually to a final cell concentration of $1x10^6$ cells/mL (hemocytometer). Each inoculum was transferred and distributed into individual 96 deepwell plates so each well contained 1 ml of inoculum. Plates then were covered with a silicon mat and left for incubation at 15°C and agitation at 100 rpm for 5 days. Each day a sample was collected through the silicon mat using a Pasteur glass pipette, cells were counted using a hemocytometer and the rest of the sample was filtered through a 0.22 μm membrane filter and the spent medium was stored for analysis at -20^{o}C. Spent medium was used for quantification of free and total fatty acid as described in 5.3.3. The reduction in concentration of complex lipids in the juice medium was used as an indicative parameter for lypolytic activity. Because liberated fatty acids could be metabolised by the yeast cells during their growth, their levels alone could not be safely used to assess lipolytic activity.

5.3.6. Juice manipulation experiment

5.3.6.1. Microvinification

All wines were fermented in 250-mL Erlenmeyer flasks at 15°C and agitated at 100 rpm according to the protocol described by Pinu *et al* (2012). Fermentation was carried out until daily weight loss of the flasks was less than 0.2 g for three or more consecutive days. After fermentation concluded, wines were harvested (Pinu *et al*, 2012) and transferred to sterile containers and stored at -20°C for further analysis.

5.3.6.2. Microvinification with individual fatty acid supplementation

Palmitic, oleic, linoleic and γ-linolenic acids were used in the juice manipulation experiment based on quantitative data (**Table 5.1**). Grape juice was supplemented with

palmitic, oleic, γ-linolenic and linoleic acids adjusted to three different concentrations (**Table 5.1**). After the addition of fatty acids, ferments were inoculated with *S. cerevisiae* to a final cell concentration of $1x10^6$ cells/mL, followed by microvinification. Control fermentations (n=3) were carried out without any lipid supplementation and were used as a reference.

5.3.6.3. Co-culture fermentations with non-Saccharomyces yeasts

Grape juice samples were inoculated with either *C. rugosa*, *C. utilis* or *Y. lipolytica* to a cell concentration of $9x10^5$ cells/mL and with *S. cerevisiae* to a cell concentration $1x10^5$ cells/mL, resulting in a total cell concentration of $1x10^6$ cells/mL (9:1 ratio), followed by microvinification. Control fermentations (n=3) were carried out with *S. cerevisiae* and were used as a reference.

5.3.6.4. Microvinification with lipase supplementation

Grape juice was supplemented with *A. niger* lipase in two different concentrations (500 and 1000 mg/L, n=3) followed by inoculation with *S. cerevisiae* (final cell concentration $1x10^6$ cells/mL) and microvinification. Control fermentations (n=3) were carried out without any lipase supplementation and were used as a reference.

5.3.7. Analysis of aroma compounds

5.3.7.1. Analysis of volatile thiols

Analysis of volatile thiols in fermented wines was performed as described by Herbst-Johnstone *et al* (2013). In brief, 50 ml of wine was mixed with internal standards (d_{10}-4MMP, 1-d_2-3MHA and 1-d_2-3MHA), followed by chemical derivatisation with ethyl propiolate. Thiol derivatives were then concentrated using an SPE column and

eluted with dichloromethane, followed by GC-MS analysis. GC-MS analysis was performed using an Agilent 6890N GC (Santa Clara, CA, USA) equipped with a 5973 mass selective detector. GC-MS parameters were set up as described in Herbst-Johnstone *et al* (2013). Data analysis was performed using MassHunter software (v. B.05.00) and thiols were quantified using corresponding calibration curves.

5.3.7.2 Analysis of esters, higher alcohols and other aroma compounds

Analysis of esters, higher alcohols and other aroma compounds was performed using HS-SPME/GC-MS as described by Herbst-Johnstone *et al* (2013b). 10 mL of wine was mixed with 3.5 g of sodium chloride and transferred to an Agilent 20-mL amber screw cap vial. The mixture of internal standards in methanol was added; each sample was purged briefly with argon gas and sealed with a screw cap. Samples were placed for agitation as described in the protocol used by Herbst-Johnstone *et al* (2013b) and left for further automated analysis. GC-MS analysis was carried out on Agilent 7890A GC System (Santa Clara, CA, USA) coupled to 5975C mass selective detector. Data analysis was performed using MassHunter software (v. B.05.00) and aroma compounds were quantified using calibration curves.

5.4. Results

5.4.1. Concentration of free and total fatty acids in grape juice medium

Using the method described in 5.3.3 we identified and quantified four free fatty acids in Sauvignon Blanc grape juice (2013 vintage) (**Table 5.1**). Only one saturated fatty acid was identified, whilst the other three were unsaturated fatty acids (UFAs) with different levels of unsaturation. Linoleic acid was the most abundant free fatty acid at a concentration of 18.68±1.11 mg/L. The total fatty acid profiling showed a higher diversity of fatty acids with a total of 12 different fatty acids (**Table 5.2**). It was observed that palmitic acid was the most abundant saturated fatty acid with an average concentration of 42.07±1.34 mg/L. Linoleic acid was found to be the most abundant unsaturated fatty acid with a concentration of 38.78±2.36 mg/L, with half of this amount presented in the form of free fatty acid. Based on quantitative data, the ratio between the total amount of free fatty acids and total lipid content in grape juice showed that only 17% of the lipidome consists of free fatty acids, whilst more than 80% of juice lipids are in a physiologically unavailable form for conventional *S. cerevisiae* fermentation (Dyer *et al*, 2002; Chapter 4).

Table 5.1. Free fatty acids detected in grape juice medium used for manipulation experiment (RSD% shown in brackets, n=3). Based on quantified data the juice was supplemented with each fatty acid individually in different concentrations

Fatty acid	Quantified amount, mg/L	Final concentration 1 (x2), mg/L	Final concentration 2 (x4), mg/L	Final concentration 3 (x6), mg/L
γ-Linolenic acid	1.85 (4.01)	4	8	12
Oleic acid	1.89 (2.78)	4	8	12
Palmitic acid	2.12 (3.24)	4	8	12
Linoleic acid	18.68 (5.94)	40	80	120
Total	***24.54***			

Table 5.2. Total fatty acid profiling of grape juice medium

Fatty acid	Quantified amount, mg/L	RSD, %
Arachidic acid	0.24	3.78
Decanoic acid	1.26	4.58
Dodecanoic acid	0.59	2.07
γ-Linolenic acid	2.58	5.78
Linoleic acid	38.78	6.09
Margaric acid	0.16	2.67
Myristic acid	22.47	4.17
Oleic acid	6.13	5.09
Palmitic acid	42.07	3.18
Palmitoleic acid	1.52	2.60
Pentadecanoic acid	0.17	4.87
Stearic acid	21.88	4.37
Total	***137.85***	

5.4.2. Validation of lipase activity

Testing the lipase activity under optimum conditions resulted in the liberation of linoleic acid from its substrate trilinolein at a rate of 1.9 mg/L.h^{-1} (**Figure 5.2**). However, we found that the activity of lipase supplemented to grape juice under fermentation conditions (15°C and pH 3.21) was reduced significantly as expected (**Figure 5.3**). Nevertheless, it was observed that a substantial level of lipid hydrolysis was still accomplished after 48 hours of incubation, confirming the liberation of free fatty acids from intact lipids.

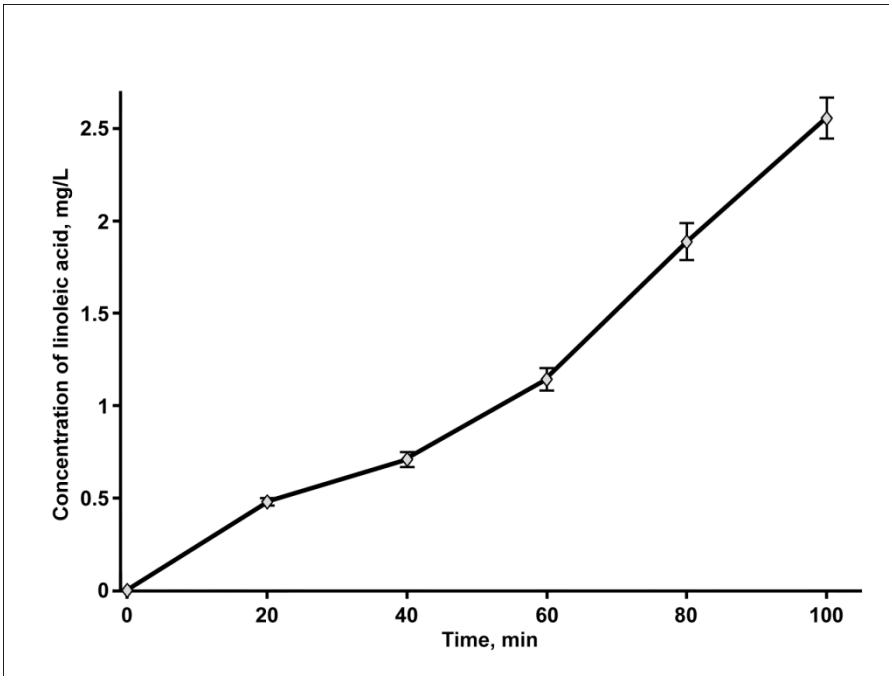

Figure 5.2. Liberation of linoleic acid from its substrate trilinolein by lipase under optimum conditions (40°C, pH 7.4). The average concentration of corresponding compounds of three replicates per conditions displayed

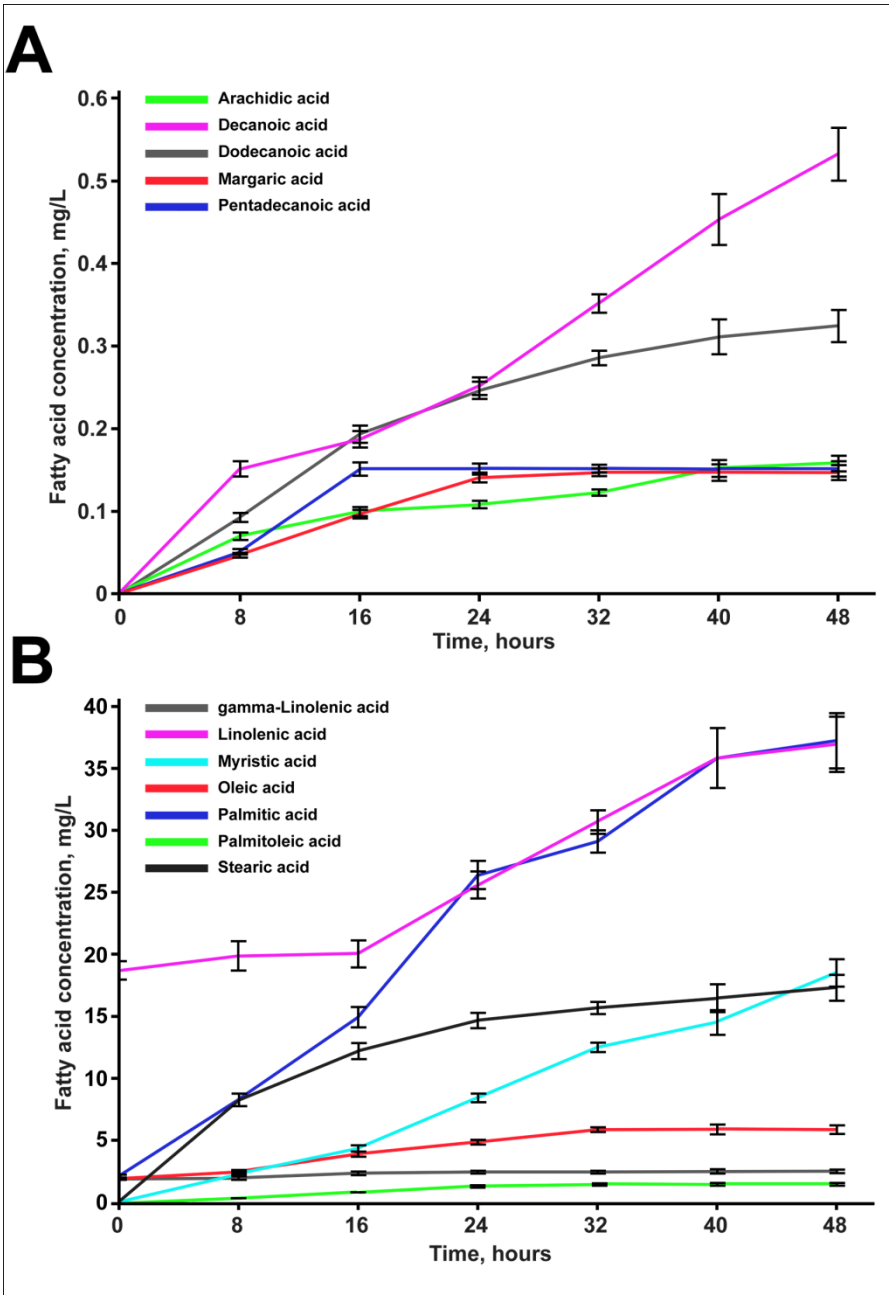

Figure 5.3. Liberation of fatty acids from intact lipids in Sauvignon Blanc grape juice by lipase under wine fermentation conditions (15°C, pH 3.21). The average concentration of corresponding compounds of three replicates per conditions displayed

5.4.3. Lypolytic activity of non-*Saccharomyces* yeasts in grape juice medium

Y. lipolytica showed the highest lipolytic activity when growing in grape juice medium under fermentation conditions, reducing the concentration of complex lipids 2-fold over 5 days (**Figure 5.4A**). Interestingly, grape juice appeared to be a suitable medium for *Y. lipolytica* fermentation in which yeasts started to grow exponentially after 3 days (**Figure 5.4B**). Grape juice medium, however, did not support the same degree of growth of *C. utilis* and *C. rugosa* (**Figure 5.4B**). After two days of fermentation both *C. utilis* and *C. rugosa* reached their highest growth rate followed by a decline in their cell populations. Nevertheless, both *C. utilis* and *C. rugosa* cultures presented lipolytic activity (**Figure 5.4A**), reducing the concentration of complex lipids over two days. With the decline of the cell populations we observed a simultaneous increase in the concentration of complex lipids in both inoculated grape juice ferments. We hypothesise that this may be due to yeast cell lysis after two days, where intracellular lipids could have been released into the extracellular medium (**Figure 5.4A**).

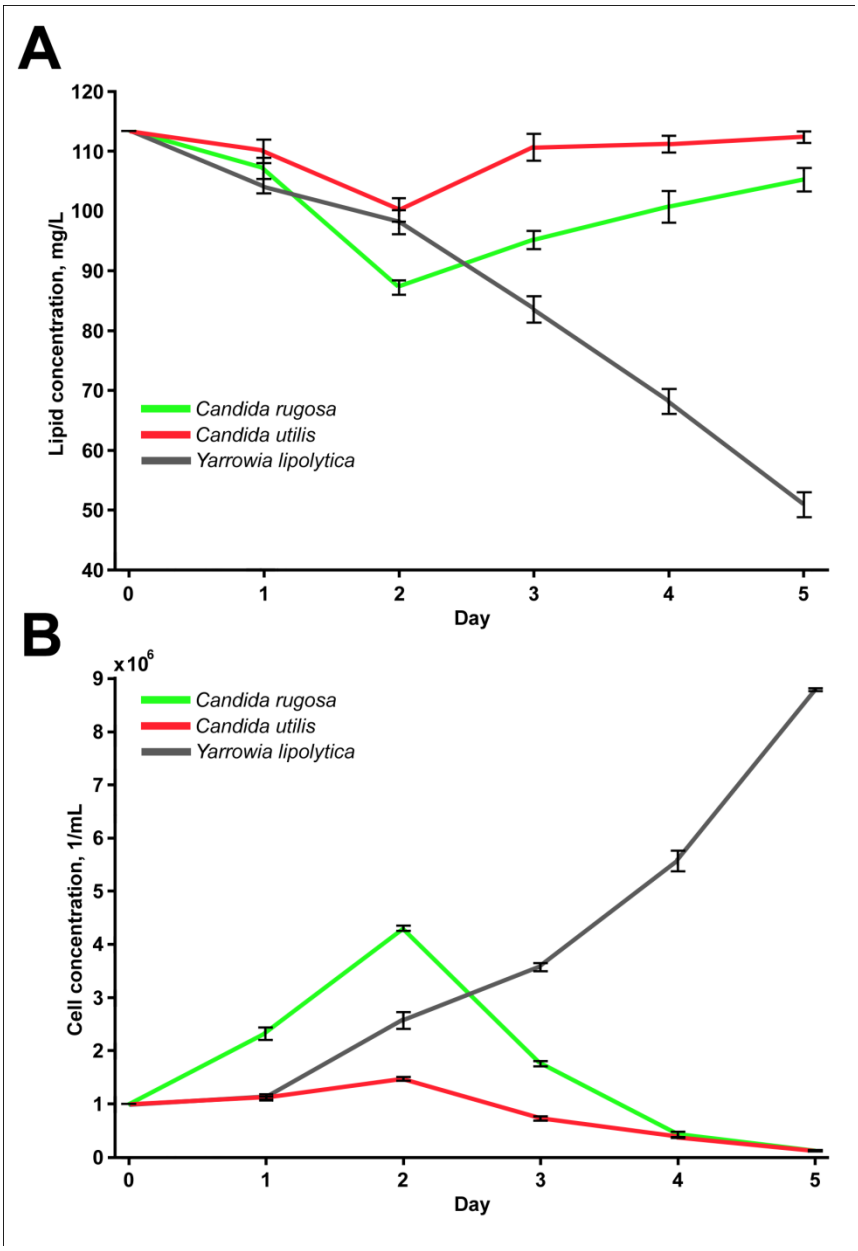

Figure 5.4. Change in total concentration of complex lipids in grape juice under lipolytic activity produced by *Candida utilis, Candida rugosa* and *Yarrowia lipolytica* (A) and growth curve of *Candida utilis, Candida rugosa* and *Yarrowia lipolytica* in Sauvignon Blanc grape juice under wine fermentation conditions. The average concentration of corresponding compounds of three replicates per conditions displayed

5.4.4. Impact of juice manipulation on volatile thiols

The level of 3MH (grapefruit aroma) was significantly affected only when lipase was supplemented to the grape juice, which induced a significant increase in 3MH concentration in the fermented wines (1.29-fold and 1.35-fold for 1x and 2x lipase concentration, respectively). Other conditions demonstrated insignificant variations in 3MH levels. Co-cultured fermentations and ferments supplemented with individual fatty acids did not affect the final level of 3MH in fermented wines (**Figure 5.5A**).

The acetylated product of 3MH – 3MHA (passionfruit aroma) – was observed to be significantly reduced in ferments supplemented with lipase and linoleic acid (**Figure 5.5B**). Oleic acid supplementation showed a significant impact on the 3MHA level only in two out of three conditions tested (4x and 6x). The strongest effect on 3MHA levels was observed for ferments supplemented with linoleic acid (6x). In this condition the concentration of 3MHA was reduced 33.2-fold in comparison to the levels observed in the control fermentation. Interestingly, the reduction of 3MHA in wines derived from grape juice supplemented with linoleic acid showed a strong concentration-response relationship – the higher the concentration of linoleic acid, the lower the level of 3MHA. A similar effect was observed for ferments supplemented with lipase. Lipase addition reduced the concentration of 3MHA 3.6-fold in 1x lipase condition and 8.2-fold in 2x lipase condition, respectively. Fermentation with 2x oleic acid supplementation did not show a significant change in 3MHA concentration, however, the addition of 4x and 6x oleic acid demonstrated a clear impact on 3MHA levels, where 4x oleic acid supplementation led to a more significant drop in 3MHA concentration (1.5-fold).

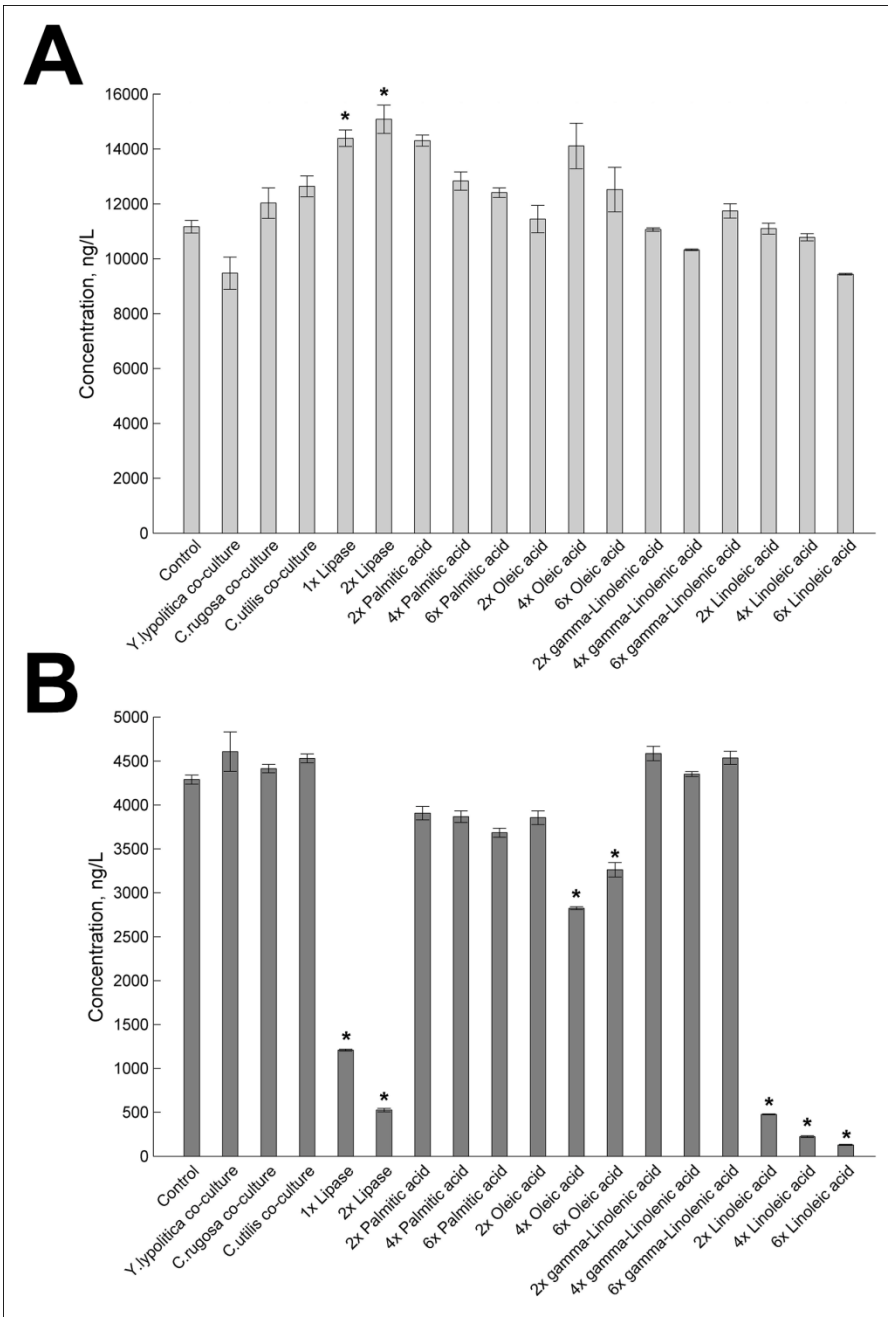

Figure 5.5. 3MH (A) and 3MHA (B) levels for fermentation conditions with fatty acid, lipase supplementation and mixed fermentations with non-*Saccharomyces* yeasts. The average concentration of corresponding compounds of three replicates per conditions displayed. Significant treatment effects (t-test, $p<0.05$) are marked with asterisk

Notably, it was observed that ferments supplemented with lipase experienced a reduction in 3MHA concentration and an increase in 3MH level, demonstrating the relationship of 3MH and 3MHA. The same trend was not observed in the linoleic acid supplementation experiment. This finding indicates that lipase supplementation may not be directly affecting the production and secretion of 3MH and 3MHA by *S.cerevisiae*, but may be regulating their ratio in the extracellular medium through enzymatic activity, whilst linoleic acid may have affected the production of these volatile thiols intracellularly by downregulating the acetylation processes in yeast cells (Pinu *et al*, 2014).

Based on the method we applied for the quantitation of volatile thiols we could not identify and quantify 4MMP (box tree, cat's pee) for any of the conditions tested, including the control. The employed method (Herbst-Johnstone *et al*, 2013) reported that the limit of 4MMP detection for white wines was 24.5 ng/L, indicating that the concentration in the fermented wines in our study was below this level.

5.4.5. Impact of juice manipulation on the production of other aroma compounds

Co-culture fermentations and experiments of the supplementation with lipase and different fatty acids to grape juice prior to fermentation resulted in substantial alterations in the overall development of aroma compounds in wines. The complete list of quantified aroma compounds and all the changes is provided in **Table 5.3** and **5.4**. For the control fermentation we managed to identify and quantify 36 aroma compounds.

All juice manipulation fermentations showed significant changes ($p<0.05$) in the development of a wide spectrum of aroma compounds including esters, norisoprenoids and terpenes, C_6 compounds, cinnamates and higher alcohols. Co-cultured fermentations with *C. rugosa, C. utilis* and *Y. lipolytica* resulted in an increase in the concentration of ethyl esters of medium chain fatty acids (ethyl decanoate, ethyl dodecanoate), 1-butanol,

and a general reduction in the level of norisoprenoids and terpenes, ethyl isovalerate and ethyl 2-methyl butanoate (**Table 5.3A**). Fermentations with lipase supplementation demonstrated a significant increase in the development of all ethyl esters and a significant reduction in the level of acetate esters (**Table 5.3B**).

The juice supplementation with palmitic acid significantly influenced the production of esters and some other aroma compounds (β-citronellol, *trans*-3-hexen-1-ol, ethyl dihydrocinnamate, *trans*-ethyl cinnamate and methionol) (**Table 5.4A**). The addition of oleic, γ-linolenic and linoleic acids had a similar effect on the development of aroma compounds (**Table 5.4C** and **5.4D**), significantly reducing the level of acetate esters. However, only γ-linolenic and linoleic acids caused a reduction of both acetate and ethyl esters in fermented wines.

Table 5.3. Aroma compound levels for (A) yeasts co-cultured fermentations and (B) fermentations supplemented with lipase. The average concentration ± standard deviation of corresponding compounds of three replicates per condition are displayed. Concentrations of all quantified aroma compounds for control *S.cerevisiae* fermentation are displayed, whilst for other conditions only significant changes (t-test, $p<0.05$) in aroma compound concentrations are presented. Arrow signs indicate an increase or decrease of concentration compared to the control condition.

A

Condition	Aroma compound	Concentration, µg/L	Condition	Aroma compound[1]	Concentration, µg/L
Control *S.cerevisiae*	***Esters***		Co-culture *C.rugosa*	***Esters***	
	Ethyl isobutyrate	17.2±1.4		Ethyl isobutyrate	9.5±0.5 ↓
	Ethyl butanoate	540.6±32.4		Ethyl 2-methyl butanoate	0.7±0.1 ↓
	Ethyl 2-methyl butanoate	1.8±0.5		Ethyl isovalerate	1.8±0.1 ↓
	Ethyl isovalerate	3.5±0.2		**Ethyl octanoate**	1025.1±32.3 ↑
	Ethyl hexanoate	1168.3±129.9		**Ethyl decanoate**	374.3±35.7 ↑
	Ethyl octanoate	731.7±115.7		**Ethyl dodecanoate**	95.0±13.3 ↑
	Ethyl decanoate	219.2±75.3		Isobutyl acetate	63.7±2.2 ↓
	Ethyl dodecanoate	49.9±18.1		**Hexyl acetate**	307.8±15.1 ↑
	Ethyl acetate	36627.8±2692.5		**β-phenylethyl acetate**	566.6±5.5 ↑
	Isobutyl acetate	74.2±5.7		**Methyl octanoate**	6.9±0.3 ↑
	Isoamyl acetate	4719.9±338.0		Diethyl succinate	355.6±1.1 ↓
	Hexyl acetate	251.3±22.0		Diethyl malate	5617.1±57.8 ↓
	cis-3-Hexenyl acetate	28.9±2.0		***Norisoprenoids and terpenes***	
	Ethyl phenylacetate	1.7±0.1		β-damascenone	3.3±0.2 ↓
	β-phenylethyl acetate	428.8±52.5		β-ionone	0.5±0.01 ↓
	Methyl octanoate	5.5±0.5		Linalool	2.9±0.1 ↓
	Diethyl succinate	365.8±4.8		(+)-terpinen-4-ol	1.3±0.1 ↓
	Diethyl malate	5829.8±83.4		β-citronellol	3.6±0.1 ↓
	Norisoprenoids and terpenes			***C6 compounds***	
	β-damascenone	4.4±0.4		**Hexanol**	2213.3±51.5 ↑
	β-ionone	0.5±0.01		*cis*-3-Hexen-1-ol	0.00 ↓
	cis/trans-Rose-oxide	0.3±0.01		***Cinnamates***	
	Linalool	3.7±0.3		**Ethyl dihydrocinnamate**	3.3±0.1 ↑
	(+)-terpinen-4-ol	121.0±44.1		***trans*-Ethyl cinnamate**	3.3±0.3 ↑

Table 5.3 (continued)

α-terpineol	3.1±0.6	***Alcohols***	
β-citronellol	5.3±1.0	**1-Butanol**	1825.4±136.0 ↑
C6 compounds			
Hexanol	1325.6±91.2		
trans-3-Hexen-1-ol	45.8±6.9		
cis-3-Hexen-1-ol	72.7±11.9		
Cinnamates			
Ethyl dihydrocinnamate	2.6±0.2		
trans-Ethyl cinnamate	1.3±0.1		
Alcohols			
Isobutanol	14929.7±870.4		
1-Butanol	1248.8±313.6		
Isoamyl alcohol	141034.7±3819.0		
Methionol	2319.5±344.7		
Benzyl alcohol	41.3±0.8		
Phenylethyl alcohol	21201.2±1569.0		

Condition	Aroma compound[1]	Concentration, µg/L	Condition	Aroma compound[1]	Concentration, µg/L
Co-culture *C. utilis*	***Esters***		Co-culture *Y.lipolytica*	***Esters***	
	Ethyl 2-methyl butanoate	0.9±0.01 ↓		Ethyl isobutyrate	7.2±0.3 ↓
	Ethyl isovalerate	2.1±0.02 ↓		Ethyl 2-methyl butanoate	0.8±0.02 ↓
	Ethyl hexanoate	1402.0±19.2 ↑		Ethyl isovalerate	1.9±0.1 ↓
	Ethyl octanoate	1209.6±25.9 ↑		**Ethyl hexanoate**	1666.5±118.8 ↑
	Ethyl decanoate	413.5±1.3 ↑		**Ethyl decanoate**	395.0±23.4 ↑
	Ethyl dodecanoate	100.4±3.8 ↑		**Ethyl dodecanoate**	94.4±7.3 ↑
	cis-3-Hexenyl acetate	19.9±1.7 ↓		**Isoamyl acetate**	6976.5±383.6 ↑
	β-phenylethyl acetate	563.1±12.6 ↑		**Hexyl acetate**	405.6±18.5 ↑
	Methyl octanoate	8.2±0.3 ↑		***cis*-3-Hexenyl acetate**	56.1±3.6 ↑
	Diethyl succinate	353.9±0.7 ↓		**β-phenylethyl acetate**	633.5±8.4 ↑
	Diethyl malate	5623.6±30.0 ↓		**Methyl octanoate**	8.2±0.4 ↑
	Norisoprenoids and terpenes			***Norisoprenoids and terpenes***	
	***cis*/*trans*-Rose-oxide**	0.3±0.01 ↑		(+)-terpinen-4-ol	1.7±0.11 ↓
	Linalool	2.7±0.1 ↓		***C6 compounds***	
	(+)-terpinen-4-ol	39.8±2.5 ↓		*cis*-3-Hexen-1-ol	0.00 ↓
	C6 compounds			***Alcohols***	

Table 5.3 (continued)

Hexanol	2009.3±74.8 ↑	**1-Butanol**	1975.6±145.9 ↑
***trans*-3-Hexen-1-ol**	77.0±2.8 ↑	Phenylethyl alcohol	18515.7±279.5 ↓
cis-3-Hexen-1-ol	0.00 ↓		
Cinnamates			
***trans*-Ethyl cinnamate**	3.4±0.03 ↑		
Alcohols			
Isobutanol	860.7±19.8 ↓		
1-Butanol	2192.9±129.7 ↑		

B

Condition	Aroma compound[1]	Concentration, µg/L	Condition	Aroma compound[1]	Concentration, µg/L
1x Lipase	***Esters***		2x Lipase	***Esters***	
	Ethyl isobutyrate	65.8±1.8 ↑		**Ethyl isobutyrate**	99.2±2.2 ↑
	Ethyl 2-methyl butanoate	27.6±0.2 ↑		**Ethyl 2-methyl butanoate**	43.0±2.9 ↑
	Ethyl isovalerate	9.2±0.1 ↑		**Ethyl isovalerate**	13.9±1.0 ↑
	Ethyl hexanoate	2509.0±178.6 ↑		**Ethyl hexanoate**	3050.0±160.7 ↑
	Ethyl octanoate	8917.7±562.3 ↑		**Ethyl octanoate**	10161.9±712.8 ↑
	Ethyl decanoate	4454.8±223.7 ↑		**Ethyl decanoate**	5121.4±209.2 ↑
	Ethyl dodecanoate	915.7±30.0 ↑		**Ethyl dodecanoate**	934.4±60.6 ↑
	Isobutyl acetate	100.2±0.4 ↑		Isoamyl acetate	1640.2±50.0 ↓
	Isoamyl acetate	2654.9±136.7 ↓		Hexyl acetate	69.9±4.2 ↓
	Hexyl acetate	123.0±14.1 ↓		*cis*-3-Hexenyl acetate	7.4±0.4 ↓
	cis-3-Hexenyl acetate	11.8±1.0 ↓		**Ethyl phenylacetate**	3.1±0.01 ↑
	Ethyl phenylacetate	2.6±0.01 ↑		β-phenylethyl acetate	120.5±2.7 ↓
	β-phenylethyl acetate	235.9±4.0 ↓		**Methyl octanoate**	51.6±6.9 ↑
	Methyl octanoate	42.6±2.0 ↑		**Diethyl succinate**	1503.1±102.7 ↑
	Diethyl succinate	797.0±59.1 ↑		***Norisoprenoids and terpenes***	
	Norisoprenoids and terpenes			***cis/trans*-Rose-oxide**	0.6±0.03 ↑
	***cis/trans*-Rose-oxide**	0.5±0.02 ↑		**Linalool**	5.0±0.1 ↑
	(+)-terpinen-4-ol	18.7±0.5 ↓		(+)-terpinen-4-ol	2.8±0.1 ↓
	β-citronellol	14.0±0.4 ↑		**α-terpineol**	5.8±0.1 ↑
	C6 compounds			**β-citronellol**	18.2±0.6 ↑
	Hexanol	2483.8±141.0 ↑		***C6 compounds***	
	trans-3-Hexen-1-ol	16.8±2.1 ↓		**Hexanol**	2744.4±42.9 ↑
	cis-3-Hexen-1-ol	0.0 ↓		*cis*-3-Hexen-1-ol	0.0 ↓
	Cinnamates			***Cinnamates***	

Table 5.3 (continued)

	***trans*-Ethyl cinnamate**	41.4±2.4 ↑	***trans*-Ethyl cinnamate**	45.9±2.9 ↑
	Alcohols		***Alcohols***	
	1-Butanol	1801.6±45.5 ↑	Benzyl alcohol	32.9±0.01 ↓
	Benzyl alcohol	32.9±0.02 ↓	Phenylethyl alcohol	7768.3±412.1 ↓
	Phenylethyl alcohol	8881.9±459.5 ↓		

[1] Compounds with a significant increase in concentration are shown in bold

Table 5.4. Aroma compound levels for fermentations supplemented with (A) palmitic acid, (B) oleic acid, (C) γ-linolenic acid and (D) linoleic acid. The average concentration ± standard deviation of corresponding compounds of three replicates per condition are displayed. Only significant treatment effects (t-test, $p<0.05$) are presented. Arrows indicate an increase or decrease of concentration compare to control condition

A

Aroma compound	Concentration of aroma compound in tested condition[2], µg/L		
	x2 Palmitic acid	x4 Palmitic acid	x6 Palmitic acid
Esters			
Ethyl 2-methyl butanoate	2.88±0.13 ↑	2.47±0.03 ↑	3.56±0.13 ↑
Ethyl isovalerate	6.42±0.28 ↑	5.99±0.11 ↑	6.43±0.41 ↑
Ethyl acetate	26172.0±332.0 ↓	26873.8±1504.9 ↓	30246.2±412.3 ↓
Isoamyl acetate	3325.4±39.5 ↓	3735.2±219.3 ↓	3858±59.5 ↓
Ethyl phenylacetate	2.00±0.02 ↑	1.89±0.03 ↑	1.94±0.01 ↑
β-phenylethyl acetate	296.8±6.3 ↓	265.57±18.0 ↓	291.2±7.2 ↓
Methyl octanoate	6.95±0.42 ↑	9.1±0.20 ↑	9.14±0.32 ↑
Diethyl malate	5909.7±21.3 ↑	6368.3±2.8 ↑	6066.0±0.11 ↑
Norisoprenoids and terpenes			
β-citronellol	8.11±0.08 ↑	7.53±0.13 ↑	7.67±0.27 ↑
C6 compounds			
trans-3-Hexen-1-ol	39.08±0.83 ↓	28.56±0.95 ↓	31.65±2.56 ↓
Cinnamates			
Ethyl dihydrocinnamate	1.92±0.03 ↓	1.76±0.07 ↓	2.01±0.13 ↓
trans-Ethyl cinnamate	0.48±0.11 ↓	0.39±0.06 ↓	0.41±0.10 ↓
Alcohols			
Methionol	1565.3±14.1 ↓	1524.6±125.6 ↓	1525.9±41.2 ↓

B

Aroma compound	Concentration of aroma compound in tested condition[2], µg/L		
	x2 Oleic acid	x4 Oleic acid	x6 Oleic acid
Esters			
Isoamyl acetate	3083.39±400.1 ↓	2985.72±61.7 ↓	3282.22±34.2 ↓
Hexyl acetate	179.65±33.0 ↓	152.76±9.9 ↓	200.85±4.7 ↓
cis-3-Hexenyl acetate	22.78±2.6 ↓	14.52±0.5 ↓	22.13±4.8 ↓
β-phenylethyl acetate	304.09±1.34 ↓	193.64±11.6 ↓	187.8±2.1 ↓
Norisoprenoids and terpenes			
β-ionone	0.55±0.01 ↑	0.56±0.01 ↑	0.55±0.01 ↑
Linalool	4.61±0.08 ↑	4.65±0.23 ↑	4.44±0.18 ↑
(+)-terpinen-4-ol	1.82±0.14 ↓	2.53±0.21 ↓	1.39±0.15 ↓

Table 5.4 (continued)

C6 compounds			
Hexanol	2098.8±18.3 ↑	2174.2±18.3 ↑	2329.0±148.6 ↑
Cinnamates			
trans-Ethyl cinnamate	0.8±0.1 ↓	0.33±0.06 ↓	5.64±0.35 ↑
Alcohols			
Isoamyl alcohol	159253.8±2166.4 ↑	160215.4±3573.6 ↑	158803.4±2315.0 ↑

C

Aroma compound	Concentration of aroma compound in tested condition*, µg/L		
	x2 γ-Linolenic acid	x4 γ-Linolenic acid	x6 γ-Linolenic acid
Esters			
Ethyl hexanoate	1419.04±3.12 ↑	1449.22±8.41 ↑	902.68±7.21 ↓
Ethyl octanoate	821.59±12.6 ↑	920.47±23.6 ↑	451.12±2.3 ↓
Ethyl decanoate	181.22±3.45 ↓	163.4±9.7 ↓	64.99±2.12 ↓
β-phenylethyl acetate	378.52±10.4 ↓	352.95±13.7 ↓	308.24±12.5 ↓
Methyl octanoate	6.59±0.12 ↑	9.33±0.25 ↑	3.82±0.11 ↓
Norisoprenoids and terpenes			
β-ionone	0.54±0.01 ↑	0.54±0.01 ↑	0.53±0.01 ↑
(+)-terpinen-4-ol	1.43±0.2 ↓	1.31±0.1 ↓	1.94±0.2 ↓
C6 compounds			
trans-3-Hexen-1-ol	48.57±0.87 ↑	30.52±1.9 ↓	20.28±0.43 ↓
Cinnamates			
Ethyl dihydrocinnamate	2.15±0.2 ↓	1.82±0.2 ↓	1.98±0.3 ↓
trans-Ethyl cinnamate	1.17±0.05 ↓	0.72±0.19 ↓	0.31±0.04 ↓
Alcohols			
Isobutanol	17884.24±61.04 ↑	15197.47±80.1 ↑	11892.52±98.3 ↓
1-Butanol	1058.39±20.8 ↓	898.68±60.9 ↓	659.48±9.24 ↓
Isoamyl alcohol	165623.2±1245.1 ↑	141762.6±4501.6 ↑	117323.7±1510.3 ↑
Methionol	2448.9±16.7 ↑	1715.1±13.5 ↓	1635.22±36.9 ↓
Phenylethyl alcohol	18019.4±40.9 ↓	16739.6±328.7 ↓	17697.1±66.0 ↓

D

Aroma compound	Concentration of aroma compound in tested condition*, µg/L		
	x2 Linoleic acid	x4 Linoleic acid	x6 Linoleic acid
Esters			
Ethyl butanoate	319.27±6.7 ↓	300.74±19.1 ↓	272.01±4.6 ↓
Ethyl hexanoate	697.58±24.3 ↓	620.04±28.1 ↓	541.53±28.4 ↓
Ethyl octanoate	481.33±17.9 ↓	437.27±43.0 ↓	503.5±15.1 ↓
Ethyl dodecanoate	242.59±8.5 ↑	133.3±4.9 ↑	171.09±17.8 ↑
Isobutyl acetate	33.55±0.2 ↓	38.27±0.8 ↓	37.8±2.9 ↓
Isoamyl acetate	1160.7±11.6 ↓	1102.52±24.4 ↓	1004.75±93.9 ↓
Hexyl acetate	58.39±0.14 ↓	37.58±3.01 ↓	38.84±9.12 ↓

Table 5.4 (continued)

cis-3-Hexenyl acetate	6.25±0.16 ↓	5.05±0.04 ↓	4.88±0.5 ↓
Ethyl phenylacetate	1.97±0.01 ↑	2.17±0.04 ↑	2.10±0.08 ↑
β-phenylethyl acetate	92.91±3.2 ↓	102.03±2.5 ↓	101.2±8.8 ↓
Norisoprenoids and terpenes			
β-damascenone	3.7±0.3 ↓	2.28±0.1 ↓	1.71±0.3 ↓
β-ionone	0.47±0.01 ↓	0.43±0.01 ↓	0.41±0.01 ↓
(+)-terpinen-4-ol	1.74±0.1 ↓	3.16±0.5 ↓	5.22±0.6 ↓
α-terpineol	2.69±0.06 ↓	1.53±0.06 ↓	1.92±0.02 ↓
β-citronellol	11.91±0.03 ↑	9.66±0.31 ↑	9.21±0.9 ↑
C6 compounds			
Hexanol	3601.0±80.6 ↑	4187.0±83.2 ↑	7164.4±48.5 ↑
Cinnamates			
Ethyl dihydrocinnamate	3.34±0.05 ↑	4.27±0.01 ↑	3.89±0.14 ↑
***trans*-Ethyl cinnamate**	14.17±1.3 ↑	12.43±1.4 ↑	12.28±2.1 ↑
Alcohols			
Isobutanol	19518.4±823.6 ↑	28074.1±1975.7 ↑	23587.5±563.3 ↑
Isoamyl alcohol	236905.9±4744.6 ↑	250431.9±4700.9 ↑	231674.6±3340.8 ↑
Benzyl alcohol	40.75±0.5 ↓	37.42±0.2 ↓	36.65±0.23 ↓

[2] Compounds with significant increase in concentration are shown in bold

5.5. Discussion

In this study the effect of New Zealand Sauvignon Blanc grape juice manipulation on the development of volatile thiols 3MH and 3MHA (passion fruit and grapefruit aroma respectively) and other aroma compounds was investigated using three different manipulation approaches. Firstly, supplementation with palmitic, oleic, γ-linolenic, and linoleic acids; secondly, with *A. niger* acidic lipase supplementation; and thirdly, using mixed fermentations with lipase secreting yeasts *C. rugosa*, *C. utilis* and *Y. lipolytica*. The current study is the first of its kind to investigate the effect of individual fatty acids supplemented to the grape juice prior the fermentation and the combined effect of free fatty acids liberated from complex lipids in juice by means of lipolytic activity. Results demonstrated a strong effect of both the free fatty acid supplementation to grape juice prior to fermentation, as well as the fatty acid changes that were generated *in situ* from the lipase addition and co-cultured fermentation experiments on the aroma profiles of the wines.

Oleic, linoleic and γ-linolenic acids are unsaturated fatty acids which are essential for cell viability, growth and fermentation activity and cannot be synthesised by *S.cerevisiae* under anaerobic fermentation conditions (Landolfo *et al*, 2010). In this study it was observed that the addition of these fatty acids to grape juice prior to fermentation resulted in an effect on aroma compound development in wine, but to varying degrees – confirming the fact they have different physiological activity, as was mentioned by Landolfo *et al* (2010). Fujii *et al* (1997) described that the acquisition of only unsaturated fatty acids by *S. cerevisiae* represses *ATF1* and *ATF2* gene expression regulating the acetylation processes within the cell resulting in the reduction of 3MHA concentration. In this study we did not observe any significant increase in the 3MH level in the ferments from juice manipulation experiments with fatty acid supplementation. We observed that linoleic acid was shown to have one of the strongest effects on wine

aroma. Supplementation with oleic acid caused a significant reduction in 3MHA levels, and the 3MH levels remained unchanged. Oleic acid demonstrated a lesser effect on biochemical processes regulating biosynthesis of 3MHA when compared to linoleic acid, however, this could be explained by the amount of fatty acid supplemented in the juice manipulation experiment (**Table 5.1**). Palmitic acid did not alter the 3MH or 3MHA levels produced during fermentation, which is in agreement with the findings of Fujii *et al* (1997) demonstrating no effect of saturated fatty acids on *ATF1* and *ATF2* gene expression.

The addition of acidic lipase to the grape juice medium prior the fermentation increased the levels of 3MH and reduced the levels of 3MHA in the fermented wines. Reduction in 3MHA levels may be a consequence of the liberation of unsaturated fatty acids (mainly linoleic acid (**Table 5.2**)) from intact lipids in the grape juice medium as it was demonstrated in 3.2. With exhaustive lipolysis of complex lipids with lipase, the concentration of linoleic acid reached 40 mg/L showing a similar effect to the fermentation containing 2x linoleic acid. However, the addition of lipase increased 3MH levels also. We assume that non-specific enzymatic activity might be responsible for converting 3MHA into 3MH in the extracellular medium as 3MHA is the esterified product of 3MH. It is conclusive that the addition of unsaturated fatty acids prior to fermentation, or the generation of them from intact lipids through enzymatic activity had a strong effect on 3MHA levels through the modulation of unsaturated fatty acid levels in grape juice. Our findings also suggest that there is no relationship between fatty acid supplementation and 3MH production; instead lipolytic activity was found to be the only factor that increasing the level of 3MH in fermented wine.

Co-culture fermentations with non-*Saccharomyces* yeasts showed no significant changes in either 3MH or 3MHA concentration. In this study it was demonstrated that these yeasts are able to secrete lipases, however, we speculate that the absence of any

effect can be explained by the potential metabolism of liberated unsaturated fatty acids by non-*Saccharomyces* yeasts at the beginning of fermentation since they were inoculated into juice samples in excess (9:1, non-*Saccharomyces* : *S.cerevisiae*) and require UFAs for adaptation to stressful conditions (Suutari *et al*, 1997; Arthur *et al*, 1976).

Juice manipulation experiments with unsaturated fatty acid supplementation showed similar results on the development of other aroma compounds in fermented wines – a significant reduction in the biosynthesis of esters and an increased production of higher alcohols and C_6 compounds (isoamyl alcohol, isobutanol, hexanol). Esters are the products of alcohol esterification with activated acyl-CoA catalysed intracellularly by alcohol acetyltransferase (Cordente *et al*, 2007; Lambrechts *et al*, 2000). The observed reduction in the level of esters could have been driven by the same mechanism as was described above for 3MHA development. Palmitic acid, however, had a significant effect on ethyl acetate and isoamyl acetate production reducing their concentration in wine.

While the addition of acidic lipases in our juice manipulation experiments also reduced the production of acetate esters, we observed a significant increase in the production of ethyl esters. Lipase with its broad enzymatic activity is able to generate a pool of free fatty acids liberated from intact lipids (**Table 5.2**). Because the biosynthesis of acetate and ethyl esters is regulated by different genes (*ATF1, ATF2* for acetate esters, and *EEB1, EHT1* for ethyl esters) (Pretorius *et al*, 2012; Saerens *et al*, 2008), liberated UFAs could reduce the production of acetate esters by suppressing the expression of *ATF* genes, while liberated and secreted saturated medium chain fatty acids play the role of substrates in ethyl ester production as was observed by Saerens *et al* (2008). Co-culture fermentations with non-*Saccharomyces* yeasts had a mixed effect on the production of esters and other aroma compounds in fermented wines, however, the

common feature for these conditions was an increase in ethyl octanoate, ethyl decanoate and ethyl dodecanoate levels. This observation may be related to an increase in corresponding fatty acids in grape juice medium by means of lipolytic activity, or simple secretion of these fatty acids by yeasts followed by their esterification by *S.cerevisiae*.

5.6. Conclusion

This study was exploratory in nature – with an aim to determine the effect of free fatty acids normally found in trace amounts in Sauvignon Blanc grape juice, on the development of volatile thiols and other aroma compounds. We confirmed that linoleic acid reduced the level of 3MHA and acetate esters in fermented wines. However, we also observed a reduction in the concentration of ethyl esters and a significant increase in the production of ethanol. Oleic acid was another fatty acid that we found to have an effect on 3MHA levels. Supplementation of grape juice prior to fermentation with other fatty acids did not alter the level of volatile thiols, but had a mixed effect on the development of other aroma compounds. Lipase supplementation had a similar effect to those observed for the addition of linoleic acid, and could possibly be explained by the liberation of free fatty acids from the pool of complex lipids. Co-culture fermentations led to moderate alterations in the final aroma profile of wine but did not result in dramatic changes in the level of any individual aroma compound. In this study we demonstrated that the level of wine aroma compounds, and in turn the aroma profile of wines could be manipulated by the addition of merely trace amounts of free fatty acids to the grape juice. Moreover, the observed effect of enzyme supplementation and fermentation with non-*Saccharomyces* yeast species demonstrates the potential of such manipulation to be used for further explorative work in Sauvignon Blanc wine aroma manipulation, with opportunities for subsequent translation into the industry setting.

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CHAPTER VI

Overall discussion and Future remarks

6.1. Overall discussion

The main aim of my thesis was to improve our capabilities for accurate quantification of metabolites in biological samples from a metabolomics prospective - pursuing metabolome analysis on a global, untargeted and unbiased scale. The developed strategies then were applied for analysis of Sauvignon Blanc grape juice and wine in order to study the effect of juice metabolome on the development of volatile thiols and other aroma compounds in wine through influence on *S.cerevisiae* metabolism under fermentation conditions. The importance of applying a metabolomics approach in this study is that it provided a wider overview on metabolome complexity and greater possibilities in the understanding of biochemical processes in living organisms, which served as an extremely powerful hypothesis generation tool.

My first study described a novel method for accurate quantification of metabolites employing gas chromatography mass spectrometry (GC-MS) as the major analytical platform, utilising methyl chloroformate (MCF) derivatisation for chemical modification of polar metabolites, and the isotope-coded derivatisation (ICD) strategy in order to generate internal standards for absolute metabolite quantification without the use of calibration curves. The synthesis of deuterium labelled methyl chloroformate (d-MCF) allowed me to develop a successful quantitative method. For the method development I used a series of different internal standards in order to generate absolute concentration values. Thus, I used alanine-d_4 to correct the losses of metabolites for the sample preparation step, deuterium labelled metabolites after d-MCF derivatisation in order to evaluate and correct the matrix effect of a biological sample, and dibutyl phthalate (DBP) as a mass reference standard to calculate the metabolite response factors (RF). The major novelty of this method was the ability to quantify metabolites in biological samples without the need to build calibration curves for each metabolite at every sample batch. The method covered a broad range of polar metabolites (i.e. amino- and organic

acids, amines, fatty acids, phenolic compounds), and, the most important, accurately calculating the absolute concentration values (i.e. mg/L) of identified metabolites. However, the drawback of this method is that only metabolites present in the mass spectral library with pre-calculated RFs can be quantified without calibration curves, which currently consist of 155 different metabolites. Nonetheless, this method showed high reproducibility, accuracy, and a wide quantification range (200 fold) for most of the amino- and organic acids in different biological samples (i.e. animal tissues, body fluids, microbial cell extracts and fruit juices with high sugar content). A group of 'problematic' metabolites (e.g. sulphur-containing metabolites (methionine, cysteine), phosphate-containing metabolites (phosphoenolpyruvate) and unstable metabolites (glutamine) that required additional correction steps in the concentration calculation process was identified. I observed that the response factors for metabolites analysed using different GC-MS systems (e.g. Agilent™ GC-MS and Thermo™ GC-MS) varied and could not be used as a universal set. I also observed that RFs potentially had a correlation with the composition of the corresponding metabolite, showing an increment function. Thus, I hypothesised that based on different analytical parameters (i.e. retention time, Kovats retention index, polarity of GC stationary phase, etc) it is possible theoretically to calculate the response factors, making the described approach quantitative for all chromatographically detectable and identified metabolites without having to calculate RFs experimentally for each analyte in the MS library. The described quantitative approach was based on complex mathematical calculations and was found to be extremely laborious when performed manually. For that reasons, my colleagues and I developed the package *MetabQ*, written in R-platform for a fast and automated data extraction and calculations of metabolite concentration values. This software is free, available on-line and user friendly. A brief user manual for *MetabQ* installation and use is enclosed in this thesis.

In my second study, I adapted a previously developed methodology for the analysis of the hydrophobic part of the metabolome – fatty acids. Thus, I developed and validated the method for absolute quantification of free fatty acid and total fatty acid (through saponification step of complex lipids) composition of different biological samples using MCF chemical modification followed by GC-MS analysis. The main benefit of this part of work was that fatty acid analysis as a separate approach is now possible to be performed using the same methodology described for absolute quantification of metabolites (MCF derivatisation, GC-MS program and MS library).

The developed methods were then applied to the analysis of more than 250 grape juices and wines from four different vintages (2010-2013) that resulted in the generation of an immense amount of data serving as a valuable databank for hypothesis generation and is now part of a grape juice index database maintained by Plant & Food Research Limited

Based on the finding observed by Dr. Farhana Pinu indicating a great impact of trace amounts of linoleic acid in grape juice on yeast metabolism and the development of volatile thiols and other aroma compounds during the wine fermentation, I mainly focused my further work on characterising the lipidome part of grape juice hypothesising that other lipids found in juice could have a strong impact on yeast metabolism, affecting the production of aroma compounds in fermented wines. Based on the results generated, I showed that the total concentration of lipid component of grape juice could be as high as 2.9 g/L, however, the majority of the lipidome (>85%) is present in the form of complex lipids which cannot be acquired by yeasts during fermentation. Despite the fact that *S.cerevisiae* cannot synthesise unsaturated fatty acids under anaerobic fermentation conditions, these fatty acids must be present in grape juice as they are essential for successful growth of yeasts in fermentation process. These observations led to the idea to explore ways to increase the amount of free fatty acids in

grape juice medium through lipolytic activity for further juice manipulation experiments. Also, I decided to use a more global approach – shotgun lipidomics – to study the lipid fingerprint of grape juice in order to find other possible lipid molecules that could have strong effect on the fermentation process but yet not detected by GC-MS. Thus, in my third study I employed shotgun lipidomics utilising direct infusion high resolution mass spectrometry (Orbitrap™), resulted in the identification of 83 different lipid species covering 7 lipid classes, including glycerolipids, phospholipids, ceramides and physiologically active hydroxyl fatty acids. Also, I observed that four free fatty acids (palmitic, oleic, linoleic and γ-linolenic acids) were the most abundant among identified free fatty acids in Sauvignon Blanc grape juice.

Based on quantitative data of free and total fatty acids in grape juice and the fore mentioned hypothesis, for my fourth study I performed juice manipulation experiments in order to confirm the finding observed by Dr. Farhana Pinu and validate my hypothesis regarding the effect of fatty acids on yeast metabolism and development of volatile thiols and other aroma compound during wine fermentation. Three different manipulation approaches were applied. Firstly, with the supplementation of palmitic, oleic, γ-linolenic, and linoleic acids; secondly, with *A.niger* acidic lipase supplementation; and thirdly, using mixed fermentations with lipase secreting yeasts *C.rugosa, C.utilis* and *Y.lipolytica*. This juice manipulation study was the first of its kind to investigate the effect of fatty acids individually supplemented to the grape juice prior to fermentation and the effect of fatty acids liberated from complex lipids in juice by means of lipolytic activity. The results from this manipulation study demonstrated a strong effect of both the free fatty acid supplementation to grape juice prior to fermentation, as well as the fatty acid changes that were generated *in situ* from the lipase addition and co-cultured fermentation experiments, on the aroma profiles of the wines. I observed that the addition of these fatty acids to grape juice prior to fermentation

affected the aroma compounds in wine, but to varying degrees. It is known that the acquisition of essential unsaturated fatty acids by *S.cerevisiae* represses *ATF1* and *ATF2* gene expression regulating the acetylation processes within the cell, resulting in the reduction of 3MHA concentration. However, I did not observe any significant increase in 3MH (direct precursor for 3MHA) in the juice manipulation experiments with fatty acid supplementation. I confirmed that linoleic acid was shown to have one of the strongest effects on wine aroma. Also, oleic acid supplementation caused a significant reduction in 3MHA levels, and the 3MH levels remained unchanged. Palmitic acid did not alter the 3MH or 3MHA levels produced during fermentation. Through juice manipulation experiments with unsaturated fatty acid supplementation I observed similar effects on the development of other aroma compounds in wines – a significant reduction in the biosynthesis of esters and an increase in the production of higher alcohols and C_6 compounds (isoamyl alcohol, isobutanol, hexanol), that could potentially be explained by the repression of the same *ATF1* and *ATF2* genes. The juice manipulation experiment with the addition of acidic lipase demonstrated similar results to those supplemented with unsaturated fatty acids – a reduction in 3MHA levels. However, I observed an increase of 3MH that could be explained by enzymatic activity. Also, I noted that lipase supplementation increased the production of some ethyl esters. The co-culture fermentations with non-*Saccharomyces* yeasts did not show dramatic changes in overall aroma profile of fermented wines. These findings could be explained by the hypothesis that the unsaturated fatty acids released into the juice medium under enzymatic activity of non-*Saccharomyces* yeasts could be acquired, thus, depleting the level of essential fatty acids in grape juice.

6.2. Main conclusions

The main conclusions of my PhD project are described below:

1. The method for absolute and accurate quantification of polar metabolites employing GC-MS platform and ICD strategy was developed. This calibration curve-free method was applied for accurate metabolite quantification in different biological samples, including Sauvignon Blanc grape juice and wine.

2. The data extraction and data processing were automated by creating a free and user-friendly software *MetabQ*. The main benefit of this software is fast data processing, producing spreadsheets of data with absolute concentration values ready for further data analysis.

3. The method for quantitative analysis of fatty acids was developed and adapted for GC-MS analysis utilising MCF chemical derivatisation which allowed to expand the number of metabolites that could be analysed utilising a single method.

4. Analysis of grape juice revealed the great complexity of its lipidome. 83 different lipid species were identified using high resolution mass spectrometry method. It was observed that >85% of juice lipidome consist of complex lipids (glycerolipids, phospholipids, ceramides, etc.). Hydroxy fatty acids were found to be a part of juice lipidome.

5. Based on juice manipulation experiment results, linoleic acid has shown the strongest effect on level of 3MHA and other aroma compounds, whilst oleic and γ-linolenic acids had minor effect. Palmitic acid showed significant effect on some esters and other aroma compounds.

6. Juice manipulation experiment with supplementation of acidic lipase from *A.niger* has shown a strong effect on development of majority of aroma compounds increasing the concentration of 3MH and ethyl esters and reducing 3MHA and acetate esters.

7. Fermentations with non-*Saccharomyces* yeasts did not affect the development of aroma compounds.

6.3. Future remarks

My study was focused on method development for absolute metabolite quantification in biological samples using GC-MS as an analytical platform. In principle, the developed method does not have a limitation on the number of metabolites which could be quantified as long as the metabolite response factor is determined prior the analysis. To date, our mass spectral library contains 155 different metabolites that could be accurately quantified; however, this number can be significantly improved. This could be achieved through analysis of purchased metabolite standards or participation in metabolomics standard initiative programmes (http://msi-workgroups.sourceforge.net/) that could provide with standard metabolite mixtures. It should be taken into account that building up and maintenance of an in-house mass spectral library is extremely laborious task that requires significant dedication.

In Chapter 2 I discussed that the developed approach could be truly untargeted if response factors of metabolites could be determined theoretically using other analytical parameters such as retention time and retention index, polarity of the column stationary phase, boiling point of analyte, structural composition of analyte, mosaic and bond increments, etc. There are several studies describing the relationship between the metabolite response factor and mentioned analytical parameters, however, there are no studies in peer-reviewed literature that characterise multi-parameter equation for accurate calculation of metabolite response factors. In my opinion, research work in this area of theoretical analytical chemistry should be continued in order to allow for quantification of all chromatographically detectable metabolites that would make the described quantitative approach universal.

My juice manipulation experiments clearly showed the strong effect of unsaturated fatty acids supplemented to the grape juice prior to fermentation on yeast metabolism and development of volatile thiols and other aroma compounds. Additional

metabolomics experiments with supplementation of fully ^{13}C-labelled unsaturated fatty acids are necessary to elucidate the metabolic fate of these fatty acids on *Saccharomyces cerevisiae* metabolism. These experiments should be performed in minimum media in order to eliminate possible effects from other components of the juice. Both metabolite analysis and shotgun lipidomics strategy should be employed in order to reconstruct the metabolic network and track the distribution and flux of ^{13}C. GC-MS analysis of intracellular free and total fatty acids could provide key information regarding lipid metabolism in tested conditions, whilst shotgun lipidomics will expand our knowledge in processes of utilisation of supplemented fatty acids. This series of experiments would help to define a mechanism of influence of these lipids on the development of volatile thiols and other aroma compounds in wine.

Shotgun lipidomics approach revealed the complexity of grape juice lipidome showing the presence of a variety of fatty acids including hydroxyl fatty acids. In Chapter 4 I discussed the physiological role of these fatty acids on yeast metabolism describing their close relation to unsaturated fatty acids. The absolute quantification of these hydroxy fatty acids would be of a great importance. There are no studies showing the effect of these fatty acids on development of volatile thiols in fermented wine, thus, this insight would help to better understand *S.cerevisiae* lipid metabolism through a set of new juice manipulation experiments in order to determine the effect of this unknown part of juice lipidome on wine aroma.

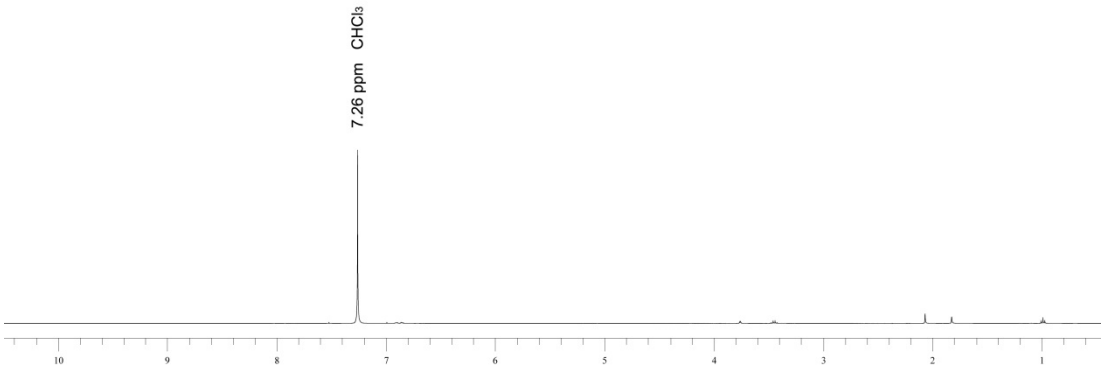
Juice manipulation experiment with *A.niger* lipase supplementation has demonstrated interesting results showing an increased level of 3MH, ethyl esters and reduced level of 3MHA that, overall, has given a distinctive aroma to fermented wine. Other concentrations of this acidic lipase as well as lipases from other microorganisms should be tested because these fermentation conditions could become a valuable tool for

wine makers in order to customize the modulation of volatile thiols and other aroma compounds in fermented wines.

APPENDICES

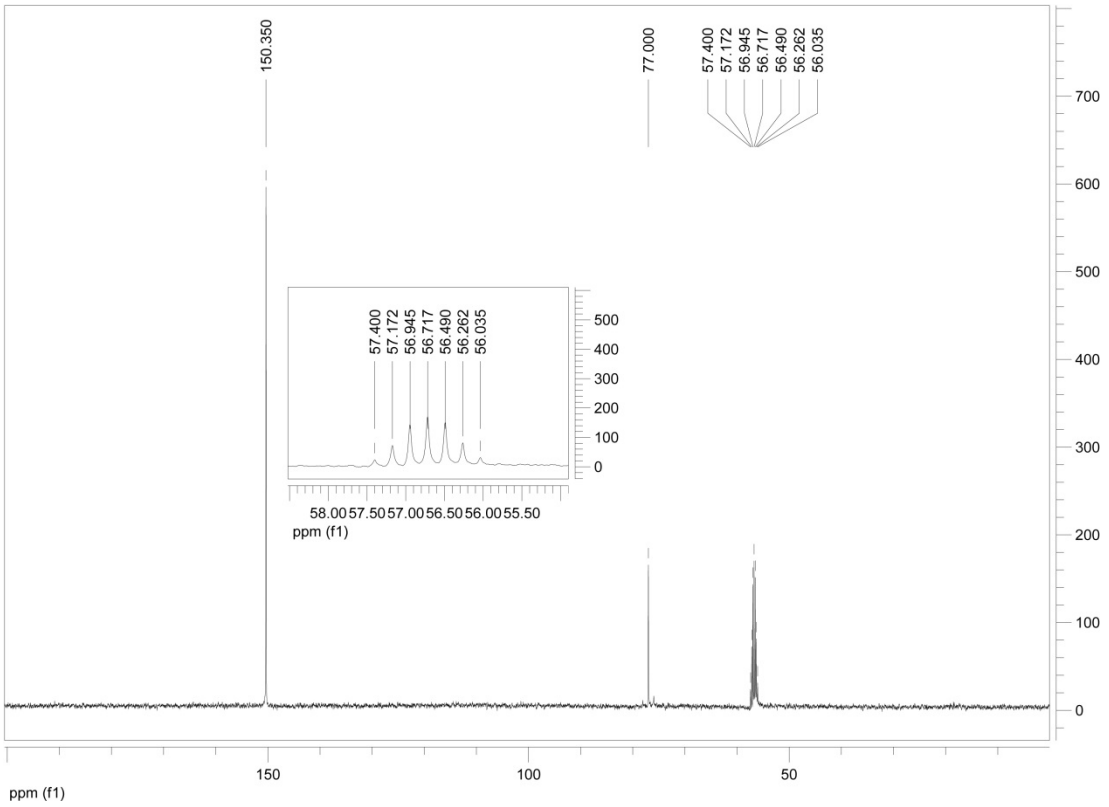
Appendix 2.1

^{1}H NMR spectrum of d_3-MCF (600 MHz)



Appendix 2.2

^{13}C NMR spectrum of d_3-MCF (600 MHz)



Appendix 2.3

Instructions for *MetabQ* installation and use

Introduction

MetabQ is a package in R software created for automated data processing of raw GC-MS data files performing data extraction and calculation of absolute metabolite values. The package processes NetCDF files employing AMDIS mass spectral libraries and reports, generating data spreadsheet with absolute concentration values of metabolites. Functions of *MetabQ* package provide high flexibility in correcting parameters of analysis and graphical representation of ion chromatograms.

Developed by Yuri Zubenko, Vladimir Obolonkin and Sergey Tumanov.
Maintenance: Yuri Zubenko (8dark8@gmail.com), Sergey Tumanov (stum447@aucklanduni.ac.nz)

1. Installations

1.1. Software to be installed before using *MetabQ*

1. AMDIS
2. R software and packages (*xcms, tcltk2, scatterplot3d*)
3. ChemStation (Agilent) or
4. Xcalibur (Thermo Fisher Scientific)

1.1.1. How to install AMDIS

a. Download AMDIS software from http://chemdata.nist.gov/mass-spc/amdis/downloads/
b. Click "**AMDIS32_V2.71.exe**".
c. AMDIS will install automatically.

> *Note: You can download the latest version of AMDIS from* http://chemdata.nist.gov/mass-spc/amdis/downloads/

1.1.2. How to install R

a. Download R software from http://cran.r-project.org/bin/windows/base/
b. Click "**R- 3.1.1-win.exe**".
c. R will install automatically.

> *Note, MetabQ_1.0 was built under R 3.1.0, but you can download the latest version from* http://www.r-project.org/

1.2. How to install packages required for *MetabQ* in R

a. Open R software
b. To install *xcms* package type the following code in console

```
source("http://bioconductor.org/biocLite.R")
biocLite("xcms")
```

c. To install *tcltk2* package, on the menu bar, click "**Packages**" → select "**Install package(s)…**" → choose the country from a pop-up window (e.g. New Zealand), click OK and find *tcltk2* package in the list.
d. To install *scatterplot3d* package please follow the procedure as for *tcltk2*
e. To check if you have all the required packages please type the code in console:

```
library (xcms) (press "Enter")

library (tcltk) (press "Enter")

library(scatterplot3d) (press "Enter")
```

If no ERROR messages appear – proceed with *MetabQ* installation

1.3. How to install *MetabQ* in R

a. Install *MetabQ* by typing the following codes in the R console. Please **don't forget** to provide the path to the file

install.packages("C:/Users/...../MetabQ_1.0.tar.gz",type="source",report=NULL)

b. To check if *MetabQ* packages was installed please type the code in console:

library (MetabQ) (press "Enter")

2. Preparing documents to run *MetabQ*

2.1. The following documents are required to run *MetabQ*

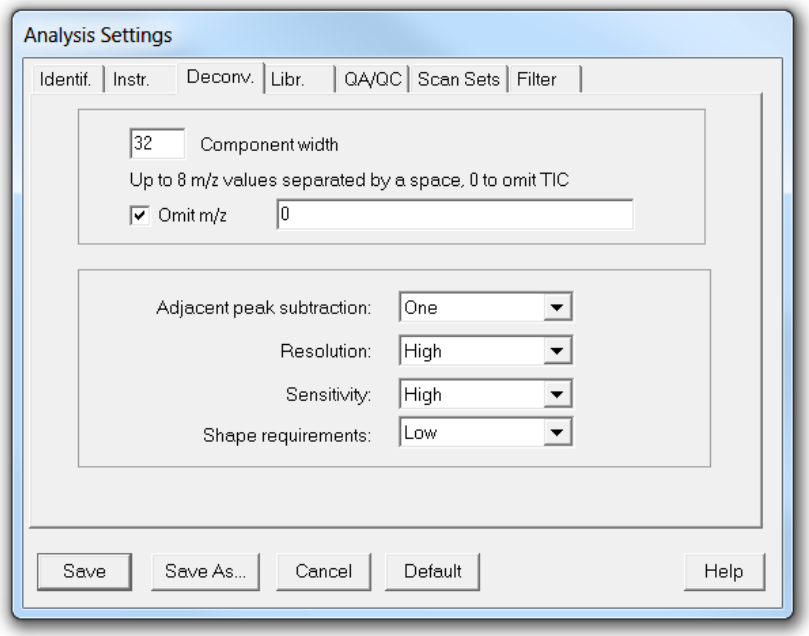
1) AMDIS batch report(s) (in a text file)
2) CDF files organising in their conditional folders
3) MetabQ.settings.csv file

Note: All the above documents are located in a single folder

2.1.1. How to create an AMDIS batch report

2.1.1.1. Setup AMDIS analysis settings

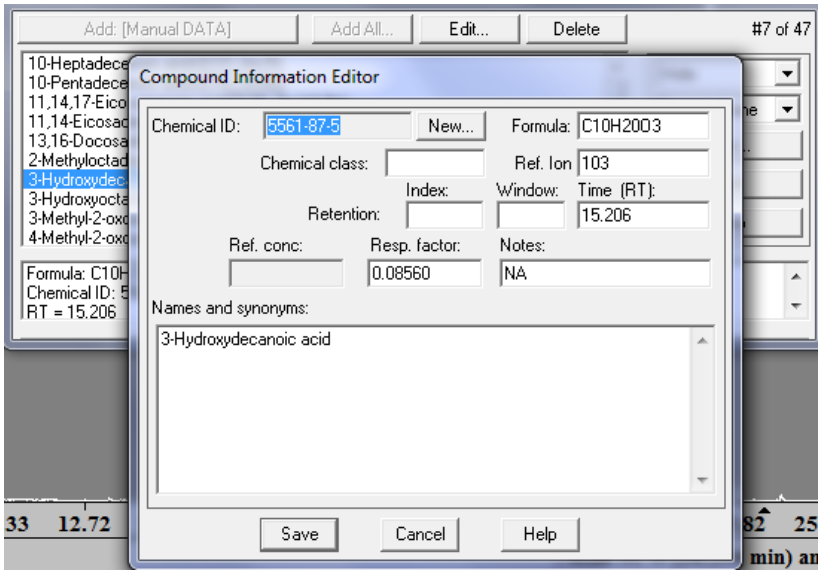
a. On the menu bar, click "**Analyse**" → select "**Settings…**" → under "**Identif.**" tab bar set "**75**" for Minimum match factor and **remove tick** for "**Multiple identifications per compound**".
b. → Under "**Deconv.**" tab bar, suggests the following settings:



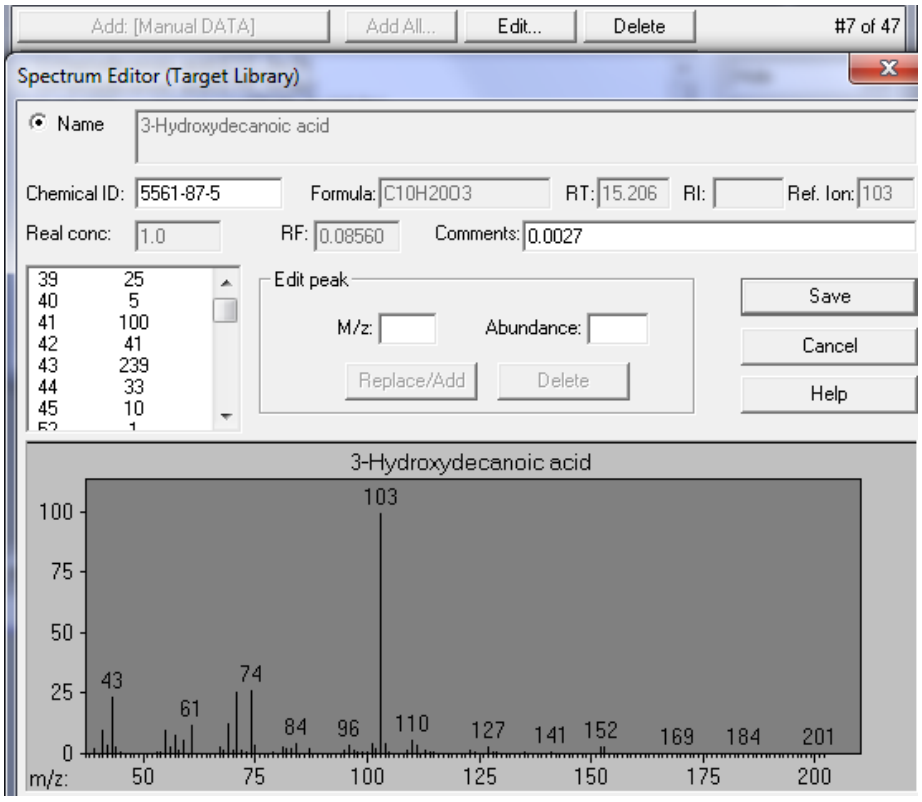
c. → Under "**Libr.**" tab bar, select "**Target Compounds Library**" → click "**Target Compounds Library**" button → select required ***.msl** metabolite library

2.1.1.2. Set or change metabolite response factor and y-intercept

a. To perform absolute metabolite quantitation, AMDIS metabolite library must contain the information about response factor and y-intercept for each metabolite. To set the response factor or change it, on the menu bar, click "**Library**" → select "**Build One Library...**" choose the metabolite and click "**Edit**" → "**Compound**" and insert/change the response factor value. After that click "**Save**":



b. To set the y-intercept or change it, on the menu bar, click "**Library**" → select "**Build One Library...**" choose the metabolite and click "**Edit**" → "**Spectrum**" and insert/change y-intercept value in **Comments** field. After that click "**Save**":

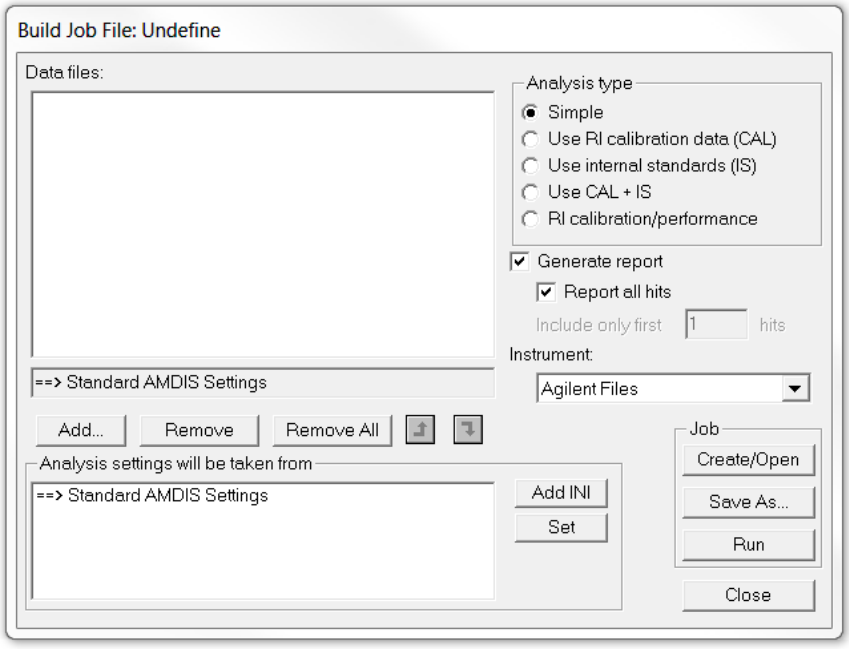


Note: Please make sure that AMDIS library contains metabolite response factors and y-intercepts in order to perform absolute quantitation.

Note: Please verify the RF and y-intercept values as they are not universal for all GC-MS machines

2.1.1.3. To generate AMDIS batch report

a. On the menu bar, click "**File**" → click "**Batch Job**" → click "**Create and Run Job…**"
b. Make sure "**Simple**" is Analysis Type and tick "**Generate report**" (see figure below).
c. Click "**Save As…"** to choose where to save the report.
d. Click "**Add…**" to input raw GC-MS files.
e. Click "**Run**" to generate an AMDIS batch report.

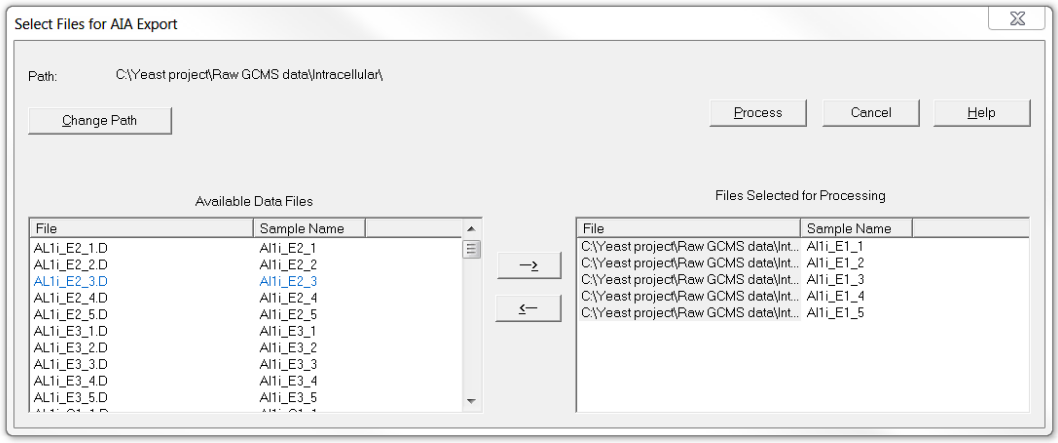


2.1.2. How to generate CDF files?

2.1.2.1. Generating CDF files from Agilent raw GC-MS data

a. Open ChemoStation.
b. On the left window select the folder containing raw GC-MS data.

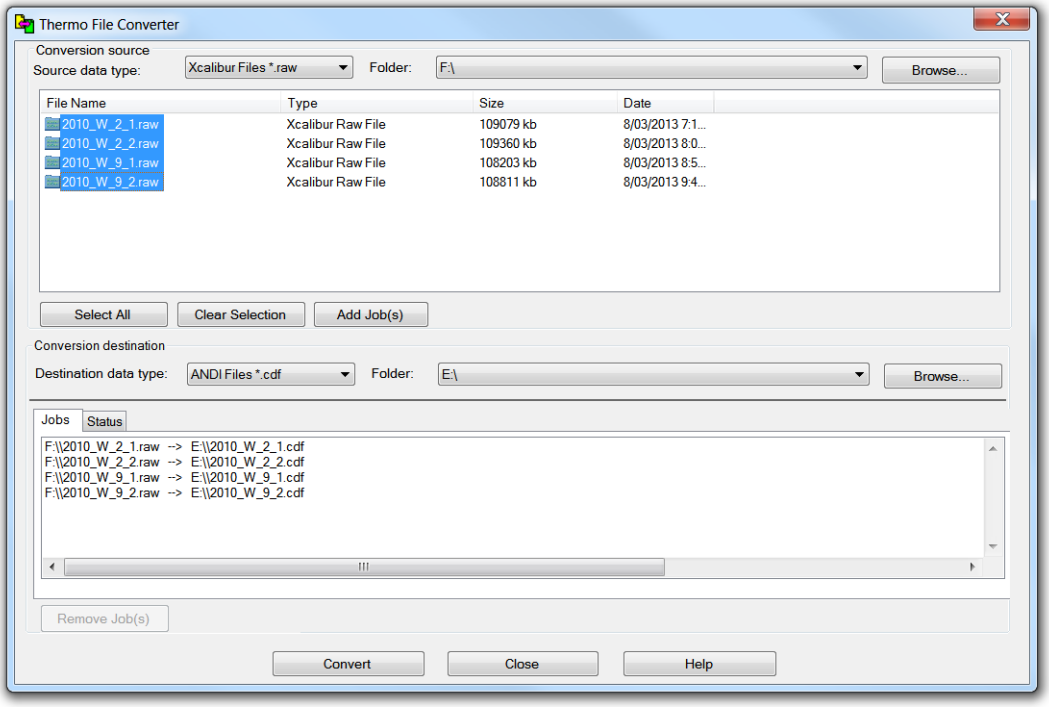
c. In the menu bar, click "**File**" → choose "**Export data to AIA format…**"→ select "**Create New Directory**" → select a location where all CDF file saved.
d. Afterward, a window will appear → input files by choose files in left window and click right arrow head button (see figure below).



e. Click "Process" button to generate CDF files

2.1.2.2. Generating CDF files from Thermo raw GC-MS data

a. Use search from Window start menu to find "**xconvert**".
b. Choose "**Xcalibur Files*.raw**" as the source data types and browse the location of raw thermo GC-MS data.
c. Choose "**ANDI Files*.cdf**" as the destination data type and browse the folder where cdf files are saved (see figure below).
d. Select raw thermo files on the top window
e. Click "**add job(s)**" button
f. Click "**Convert**" button and raw thermo GC-MS data will convert to cdf files.
g. Change the file extension from ".cdf" to ".CDF", otherwise metab won't work.



2.1.3. MetabQ.settings.csv file

This file will be used by *MetabQ* package and contains the information in order to calculate the absolute metabolite values in the initial sample. Values of only those parameters that are presented in the table below should be changed by user:

Parameter	**Value**	**Description**
Extention	CDF	Check the case type of cdf-filesas R is case sensitive (CDF of cdf)
Int standard	d_4-Alanine	Spell the internal standard exactly as it in the AMDIS library
Concentration (d_4-Alanine)	10	Concentration of used internal standard, mM
Concentration (DBP)	1	Concentration of used mass reference internal standard, mM
Sample volume, uL	40	The aliquot of a sample used for analysis
Extract volume, uL	400	The aliquot of chloroform used for extraction of metabolites during

		sample derivatization process
Sample aliquot, uL	100	The aliquot of MCF derivatized sample taken for analysis
Pooled sample aliquot, uL	50	The aliquot of d-MCF derivatized pooled sample taken for analysis
DBP aliquot, uL	20	The aliquot of mass reference internal standard taken for analysis
a	0.0353	Response factor of d_4-Alanine
b	0.0348	Y-intercept of d_4-Alanine

3. Run *MetabQ*

Make sure all the required documents are placed in a single folder (Read: 3. Prepare documents to run METAB)

a. Open R
b. Load the *MetabQ* package by typing:

```
library(MetabQ)
```

c. Start *MetabQ* by typing:

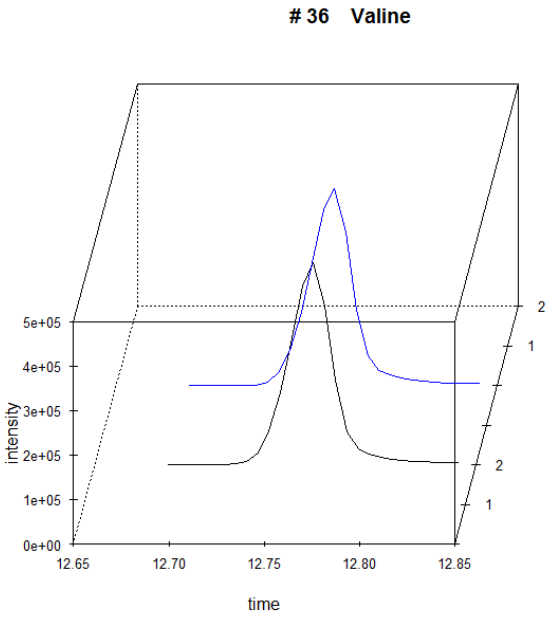
```
settings()
```

d. A pop-up window will appear for your to browse the folder where all the required documents are located
e. A pop-up window will appear for your to browse the folder with AMDIS libraries (*.msl) and select the AMDIS MSL library
f. This step will generate “folder name”_lib.csv file which contains all the analytical parameters required for further data extraction step.
g. Start the next function by typing :

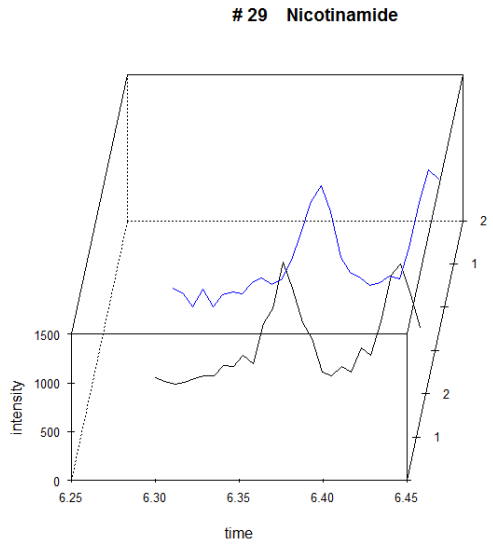
```
relative()
```

h. The result of this step is “folder name”_auto.csv file containing the extracted abundances of detected metabolites. This list includes both non- and

deuterated metabolite derivatives. Also *.png files are generated for each identified metabolite:



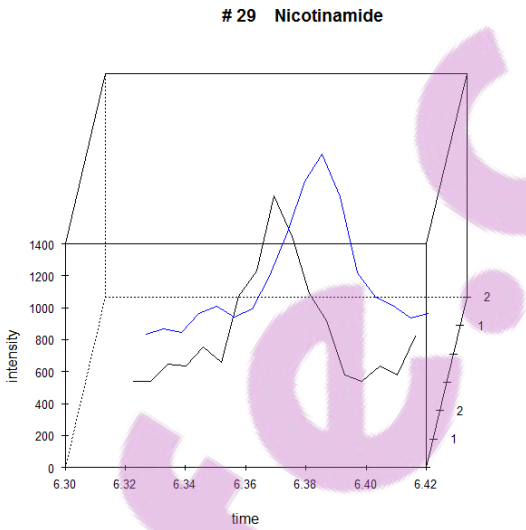
i. Please check all metabolite.png files by means of “One metabolite – one peak” rule (as on figure above for valine). In case there are more than one peak occurred in selected time window (e.g. nicotinamide), the time window should be changed by using *correct()* function.



The retention time for nicotinamide is 6.35 min, however, within set time window 6.25-6.45 min (6.35±0.1 min) two peaks occurred. In order to extract the abundance of correct peak the time window should be changed to 6.30-6.40 min (6.35±0.05 min) by typing the following in console:

```
correct(29,0.05,0.05)
```

This function re-draws the figure according to settings (29-metabolite #, 0.05 and 0.05 – time deviation, min). After this step, “folder name”_correct.csv file was generated with corrected abundances. This step can be run as many times as required and each time the _correct.csv file would be overwritten.



j. After all the corrections have been made, the final function will calculate the absolute values for identified metabolites. To start the function type in console:

```
quant()
```

This function will generate the “folder name”_mg_per_L.csv file containing absolute metabolite concentration values in mg/L.

Appendix 2.4

Analytical characteristics of metabolites used to perform quantitative method on Agilent GC-MS instrument

Metabolite	RI	RF	Y-intercept	Quantification range, mM	m/z
10-Heptadecenoic acid	1055	0.1046	-0.0384	0.02-4	55
10-Pentadecenoic acid	1043	0.1001	-0.0402	0.02-4	55
11,14,17-Eicosatrienoic acid	1068	0.0863	0.0238	0.01-2	79
11,14-Eicosadienoic acid	1067	0.0916	0.0288	0.01-2	67
13,16-Docosadienoic acid	1077	0.0801	-0.0388	0.01-2	67
1-Aminocyclopropane-1-carboxylic acid	1025	0.0362	0.0232	0.01-1	141
1-Phenylethanol	1014	0.0281	0.0136	0.01-1	107
2,3-Butanediol	1001	0.0405	-0.0157	0.01-1.5	45
2,4-Diaminobutyric acid	1055	0.0422	-0.0374	0.02-4	114
2,6-Diaminopimelic acid	1076	0.0464	-0.0413	0.05-10	200
2-Aminoadipic acid	1046	0.0327	-0.0261	0.05-10	114
2-Aminophenylacetic acid	1040	0.0504	-0.0424	0.02-4	164
2-Hydroxybutyric acid	1016	0.0428	0.0087	0.05-10	117
2-Hydroxycinnamic acid	1050	0.056	-0.0196	0.05-10	161
2-Hydroxyisobutyric acid	1012	0.0595	-0.0189	0.02-4	73
2-Isopropylmalic acid	1023	0.0563	0.0009	0.05-10	145
2-Methyloctadecanoic acid	1060	0.0887	-0.0231	0.02-4	88
2-Oxoadipic acid	1028	0.0456	0.0237	0.01-2	129
2-Oxobutyric acid	1001	0.0443	-0.038	0.05-10	57
Glutaric acid	1015	0.0215	-0.0344	0.05-10	100
Glutathione	1045	0.0214	0.0286	0.05-1.5	142
Glyceric acid	1020	0.0102	-0.0183	0.01-1	119
Glycerol	1016	0.0146	0.0187	0.01-1	61
Glycine	1019	0.0652	-0.0428	0.05-10	88
Glyoxylic acid	1018	0.0105	0.0269	0.01-1	75
Gondoic acid	1067	0.069	0.0051	0.02-4	55
Heneicosanoic acid	1073	0.1167	-0.0253	0.01-2	74
Hexanoic acid	1002	0.1169	-0.0164	0.02-4	74
Hippuric acid	1050	0.0567	-0.0064	0.05-10	105
Histidine	1068	0.0106	0.0136	0.05-10	139
Homocysteine	1056	0.0133	0.0316	0.02-4	114
Indole-3-butyric acid	1065	0.028	-0.0258	0.01-1	130
Isocitric acid	1048	0.0281	-0.0231	0.05-10	129
Isocitric acid secondary peak	1047	0.0296	0.0272	0.05-10	129
Isoleucine	1027	0.0626	0.0302	0.05-10	115
Itaconic acid	1015	0.0376	-0.0216	0.05-10	127
Lactic acid	1012	0.0272	0.027	0.02-4	103
Leucine	1028	0.0827	-0.0154	0.05-10	144

Table continued

2-Oxoglutaric acid	1027	0.0246	0.0066	0.05-10	115	Levulinic acid	1011	0.0762	-0.0312	0.05-10	99
2-Oxovaleric acid	1006	0.0278	-0.0113	0.01-1.5	71	Lignoceric acid	1088	0.0762	-0.0323	0.01-2	74
2-Phosphoenolpyruvic acid	1029	0.0416	0.0033	0.05-1	109	Linoleic acid	1058	0.1115	0.0161	0.02-4	67
2-Phosphoglyceric acid	1036	0.0455	-0.0241	0.01-1	169	Lysine	1065	0.0628	0.0034	0.05-10	142
3,5-Diiodo-L-tyrosine	1019	0.0374	0.0139	0.01-1	429	Malic acid	1019	0.036	0.0029	0.05-10	103
3-Hydroxybenzoic acid	1037	0.0345	0.0204	0.05-10	135	Malonic acid	1007	0.0265	-0.0416	0.05-10	101
3-Hydroxydecanoic acid	1031	0.0629	0.0054	0.05-10	103	Margaric acid	1055	0.1426	0.0131	0.02-4	74
3-Hydroxyoctanoic acid	1022	0.0964	0.03	0.02-4	103	Methionine	1040	0.0389	-0.0038	0.08-2.8	147
3-Hydroxypropionic acid	1025	0.0686	-0.0431	0.05-10	87	Myristic acid	1038	0.1179	-0.0077	0.02-4	74
3-Methyl-2-oxopentanoic acid	1008	0.0799	0.0121	0.05-10	57	Myristoleic acid	1038	0.0374	0.0037	0.01-2	55
3-Oxoadipic acid	1028	0.0782	-0.0371	0.05-10	69	N-Acetylcysteine	1050	0.0541	-0.0199	0.02-4	176
4-Aminobenzoic acid	1056	0.0162	-0.0301	0.05-10	178	N-Acetylglutamic acid	1043	0.0172	0.0074	0.02-4	116
4-Aminobutyric acid	1029	0.1038	-0.0406	0.05-10	102	N-alpha-Acetyllysine	1074	0.0214	-0.0262	0.01-1	129
4-Hydroxycinnamic acid	1053	0.1137	-0.0378	0.02-4	161	Nicotinamide	1002	0.0138	-0.0346	0.1-5	57
4-Hydroxyphenylacetic acid	1043	0.1108	0.0022	0.05-10	121	Nicotinic acid	1017	0.0343	-0.025	0.01-1	137
4-Hydroxyphenylethanol	1043	0.1117	-0.0214	0.02-4	121	Nonadecanoic acid	1063	0.1143	-0.0397	0.02-4	74
4-Methyl-2-oxopentanoic acid	1008	0.0831	-0.0079	0.02-4	85	Norvaline	1025	0.0794	-0.0301	0.05-10	130
5-Hydroxy-L-lysine	1078	0.0952	-0.0367	0.05-10	101	O-Acetylserine	1033	0.085	-0.0344	0.01-2	100
5-OH-methyl-2-furaldehyde	1028	0.1158	0.0122	0.01-2	168	Octanoic acid	1012	0.1195	0.0264	0.02-4	74
5-Methoxytryptophan	1109	0.0178	-0.0267	0.05-10	160	Oleic acid	1058	0.0816	-0.0024	0.02-4	55
5-Oxotetrahydrofuran-2-carboxylic acid	1028	0.0998	-0.0146	0.01-2	85	Ornithine	1061	0.0339	0.0137	0.05-10	128
9-Heptadecenoic acid	1055	0.0858	-0.0264	0.02-4	55	Oxalic acid	1002	0.0462	-0.021	0.05-10	59

Table continued

Adipic acid	1021	0.1165	0.0265	0.05-10	114	Oxaloacetic acid	1014	0.0084	0.0251	0.01-1	101
Adrenic acid	1076	0.0795	-0.0429	0.01-2	79	Palmitic acid	1050	0.1079	-0.0096	0.02-4	74
Alanine	1018	0.0782	-0.0322	0.05-10	102	Palmitoleic acid	1049	0.0701	0.0108	0.02-4	55
Anthranilic acid	1029	0.1	-0.0219	0.05-10	146	para-Toluic acid	1019	0.0571	0.0251	0.05-10	119
Arachidic acid	1068	0.1044	-0.0099	0.01-2	74	Pentadecanoic acid	1043	0.1217	-0.0249	0.02-4	74
Arachidonic acid	1066	0.0875	-0.0241	0.02-4	79	Phenylalanine	1046	0.0509	-0.0224	0.05-10	162
Asparagine	1036	0.1117	-0.0094	0.01-1	127	Pimelic acid	1027	0.033	-0.0221	0.05-10	115
Aspartic acid	1034	0.0935	-0.0114	0.05-10	160	Proline	1030	0.0998	0.0299	0.05-10	128
Azelaic acid	1035	0.0355	0.0134	0.05-10	185	Putrescine	1051	0.0168	-0.0343	0.05-10	88
Behenic acid	1077	0.0958	-0.0119	0.01-2	74	Pyroglutamic acid	1035	0.0649	-0.0032	0.01-1	84
Benzoic acid	1013	0.0248	0.0192	0.05-10	105	Pyruvic acid	1003	0.0143	-0.0062	0.02-2	89
bishomo-γ-Linolenic acid	1066	0.0914	0.0009	0.02-4	79	Quinic acid	1035	0.0082	-0.0077	0.05-10	191
Caffeine	1057	0.1137	0.0213	0.02-4	194	Salicylic acid	1036	0.0334	0.0162	0.05-10	135
cis-4-Hydroxyproline	1045	0.0409	-0.0269	0.02-4	144	Sebacic acid	1039	0.0713	0.0091	0.05-10	199
cis-Aconitic acid	1032	0.0212	-0.0303	0.05-10	153	Serine	1038	0.0152	-0.0271	0.05-10	100
cis-Vaccenic acid	1058	0.0959	0.0125	0.02-4	55	Sinapic acid	1076	0.0124	0.0163	0.05-10	296
Citraconic acid	1015	0.055	-0.0206	0.05-10	127	Stearic acid	1059	0.1191	-0.0394	0.02-4	74
Citramalic acid	1017	0.1064	-0.0301	0.05-10	117	Suberic acid	1031	0.0573	-0.0385	0.05-10	129
Citric acid	1034	0.0578	-0.004	0.05-10	143	Succinic acid	1012	0.0599	0.0107	0.05-10	115
Citric acid secondary peak	1033	0.0493	0.029	0.05-10	101	Syringic acid	1057	0.0522	-0.011	0.05-10	211
Creatinine	1036	0.0956	0.0082	0.05-10	202	Tartaric acid	1047	0.0439	0.0174	0.05-10	59
Cystathionine	1092	0.009	-0.0121	0.1-3.5	160	Threonine	1032	0.0159	0.0255	0.05-10	115
Cysteine	1046	0.0136	-0.0133	0.07-2.5	192	trans-4-Hydroxyproline	1039	0.0413	-0.0415	0.05-10	216
Decanoic acid	1022	0.1212	-0.0093	0.02-4	74	trans-Cinnamic acid	1028	0.0699	0.0044	0.05-10	162
DHA	1076	0.0682	-0.0022	0.01-2	79	Tricosanoic acid	1082	0.0942	-0.0185	0.01-2	74
Dipicolinic acid	1040	0.1158	-0.0291	0.05-10	137	Tridecanoic acid	1034	0.1213	0.0022	0.01-2	74
Dodecanoic acid	1030	0.1197	0.0161	0.01-2	74	Tryptophan	1087	0.0763	0.019	0.05-10	130
DPA	1076	0.0602	-0.0307	0.01-2	79	Tyrosine	1073	0.0348	0.0278	0.05-10	236
EDTA	1069	0.0274	-0.024	0.05-10	174	Undecanoic acid	1026	0.1839	-0.0393	0.01-2	74
EPA	1067	0.0889	0.0009	0.01-2	79	Valine	1023	0.0937	-0.0127	0.05-10	130
Erucic acid	1076	0.0711	0.0308	0.01-2	55	Vanillic acid	1048	0.0151	0.0286	0.05-10	165

Table continued

Ferulic acid	1063	0.04	0.0031	0.05-10	222	α-Linolenic acid	1058	0.1019	-0.0116	0.02-4	79
Fumaric acid	1012	0.0604	-0.0031	0.05-10	113	β-Alanine	1023	0.039	-0.0188	0.05-10	88
Glutamic acid	1040	0.0342	-0.0042	0.05-10	174	γ-Linolenic acid	1059	0.0961	0.0237	0.02-4	79
Glutamine	1063	0.0079	0.009	0.5-7.5	84						

RI, Kovats retention index; RF, response factor; m/z, quantifier ion

Appendix 2.5

List of the most common metabolites quantified in biological samples used for method validation (%RSD shown in brackets, n=3). Results were presented as a mean value for (A) rat plasma, (B) rat urine and (C) rat liver extract together with published literature values for these metabolite concentrations

A

Metabolites	Concentration, mg/L	Concentration reported[1,2], mg/L
Alanine	25.52 (1.28)	37.6, 42.6
Asparagine	13.97 (4.15)	11.8, 12.7
Aspartic acid	2.64 (4.85)	1.0, 1.1
Glutamic acid	7.74 (1.20)	7.6, 9.0
Glutamine	38.47 (12.76)	58.6, 61.2
Glycine	21.07 (3.86)	20.3, 22.1
Histidine	14.41 (2.74)	10.2, 11.3
Isoleucine	8.31 (1.73)	15.2, 17.8
Leucine	26.54 (2.13)	29.0, 33.2
Lysine	42.78 (3.78)	59.1, 64.8
Ornithine	7.95 (1.95)	7.7, 8.4
Phenylalanine	10.87 (2.78)	12.3, 14.3
Serine	15.84 (5.21)	20.4, 25.0
Threonine	33.08 (11.26)	34.8, 40.6
Tryptophan	6.50 (6.18)	15.4, 18.7
Tyrosine	17.01 (10.46)	16.6, 17.2
Valine	22.78 (6.78)	28.6, 35.5

B

Metabolites	Concentration, mg/L	Concentration reported[3,4], mg/L
Alanine	6.76 (3.27)	1.36, 50.78
Asparagine	3.92 (0.79)	0.67
Aspartic acid	2.01 (1.62)	0.22, 0.67
Citric acid	4.27 (4.12)	0.37, 5.76

Glutamic acid	10.81 (1.17)	2.22, 125.06
Glycine	3.51 (4.60)	2.28
Hippuric acid	8.24 (3.05)	0.54, 14.33
Histidine	7.13 (1.57)	2.27, 15.52
Isoleucine	10.18 (5.91)	0.29, 9.18
Leucine	2.04 (3.20)	0.40, 2.62
Lysine	6.08 (3.63)	13.98, 38.01
Ornithine	6.75 (2.36)	0.91, 6.61
Phenylalanine	nd	0.41, 1.65
Proline	3.76 (4.58)	0.68, 10.36
Serine	nd	0.66, 12.61
Threonine	12.40 (1.85)	5.96, 52.41
Tryptophan	4.43 (0.25)	0.08, 1.02
Tyrosine	5.25 (1.07)	5.91, 5.44
Valine	1.10 (1.76)	3.14, 4.69

C

Metabolites	Concentration, mg/g tissue
Amino acids	
Alanine	40.85 (2.99)
Aspartic acid	7.31 (6.32)
Glutamic acid	28.23 (0.82)
Glycine	2.74 (3.58)
Histidine	83.24 (0.38)
Isoleucine	3.70 (3.95)
Leucine	9.41 (0.07)
Lysine	17.62 (5.12)
Ornithine	17.36 (0.67)
Proline	4.72 (0.97)
Serine	14.19 (4.27)
Threonine	25.99 (9.47)
Tryptophan	5.63 (1.61)

Tyrosine	22.02 (4.54)
Valine	5.15 (1.41)
Fatty acids	
Arachidonic acid	49.64 (7.21)
DHA	43.23 (1.79)
EPA	47.59 (7.09)
Linoleic acid	23.54 (7.52)
Myristic acid	1.56 (2.27)
Oleic acid	37.27 (3.14)
Palmitic acid	50.06 (1.64)
Palmitoleic acid	2.39 (8.02)
Stearic acid	51.08 (6.20)

nd, not detected

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[3]Wang, C., Zhu, H., Pi, Z., Song, F., Liu, Z., Liu, S. (2013). Classification of type 2 diabetes rats based on urine amino acids metabolic profiling by liquid chromatography coupled with tandem mass spectrometry. *Journal of Chromatography B* **935**: 26-31.

[4]Tang, X., Gu, Y., Nie, J., Fan, S., Wang, C. (2014). Quantification of amino acids in rat urine by solid-phase extraction and liquid chromatography/electrospray tandem mass spectrometry: application to radiation injury rat model. *Journal of Liquid Chromatography and Related Technologies* **37**: 951-973.

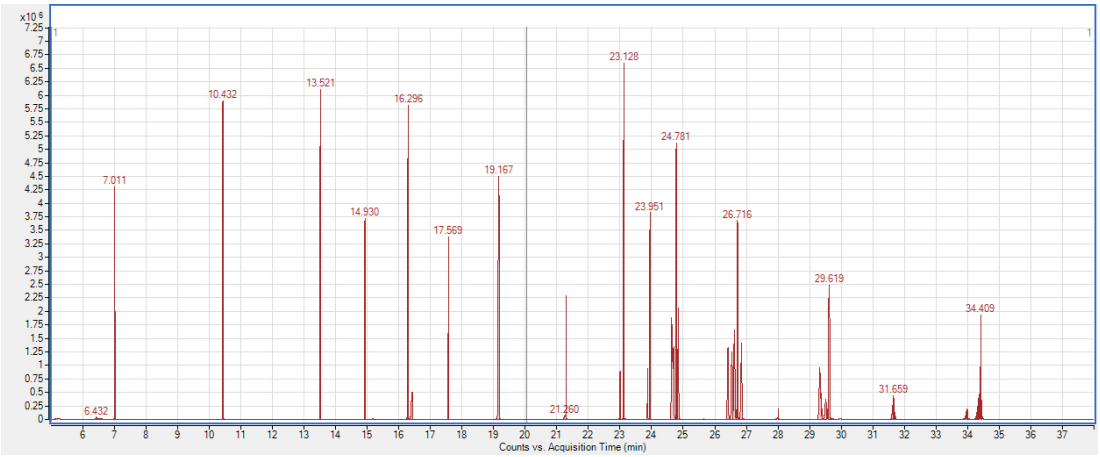
Appendix 3.1

Analytical characteristics of the fatty acid methyl esters detected by GC-MS

Common name	Chain length	Retention time (min)	Limit of detection (µM)	Range of quantification (mM)	Coefficient R^2
Decanoic acid	10:0	12.199	3.19	0.05-5	0.9916
Lauric acid	12:0	14.935	2.97	0.05-5	0.9907
Myristic acid	14:0	17.458	1.21	0.03-4	0.9934
Myristoleic acid	14:1n5	17.397	2.84	0.01-1	0.9927
Palmitic acid	16:0	21.093	1.69	0.05-5	0.9967
Palmitoleic acid	16:1n7	20.744	3.74	0.01-1	0.9957
Stearic acid	18:0	24.004	8.72	0.05-5	0.9989
Oleic acid	18:1n9	23.798	5.02	0.05-5	0.9937
Linoleic acid	18:2n6	23.848	4.62	0.05-5	0.9957
α-Linolenic acid	18:3n3	23.723	4.01	0.01-1	0.9945
γ-Linolenic acid	18:3n6	24.018	2.46	0.01-2	0.9964
Arachidic acid	20:0	26.807	6.01	0.01-2	0.9994
Eicosenoic acid	20:1n9	26.493	2.31	0.01-1	0.9927
Eicosadienoic acid	20:2n6	26.597	1.74	0.01-1	0.9921
Bishomo- γ - Linolenic acid	20:3n6	26.429	2.42	0.01-1	0.9999
Arachidonic acid	20:4n6	26.206	2.74	0.01-1	0.9990
Eicosapentaenoic acid	20:5n3	26.496	2.37	0.01-1	0.9998
Docosanoic acid	22:0	29.821	3.02	0.01-1	0.9982
Docosapentaenoic acid	22:5n3	29.597	2.74	0.01-1	0.9967
Docosahexaenoic acid	22:6n3	29.436	3.71	0.01-1	0.9909
Lignoceric acid	24:0	33.455	2.09	0.01-1	0.9993
Nervonic acid	24:1n9	33.072	1.49	0.01-1	0.9982
Tridecanoic acid	13:0	16.190	1.00	0.001-0.5	0.9911
Nonadecanoic acid	19:0	25.219	1.00	0.001-0.5	0.9902

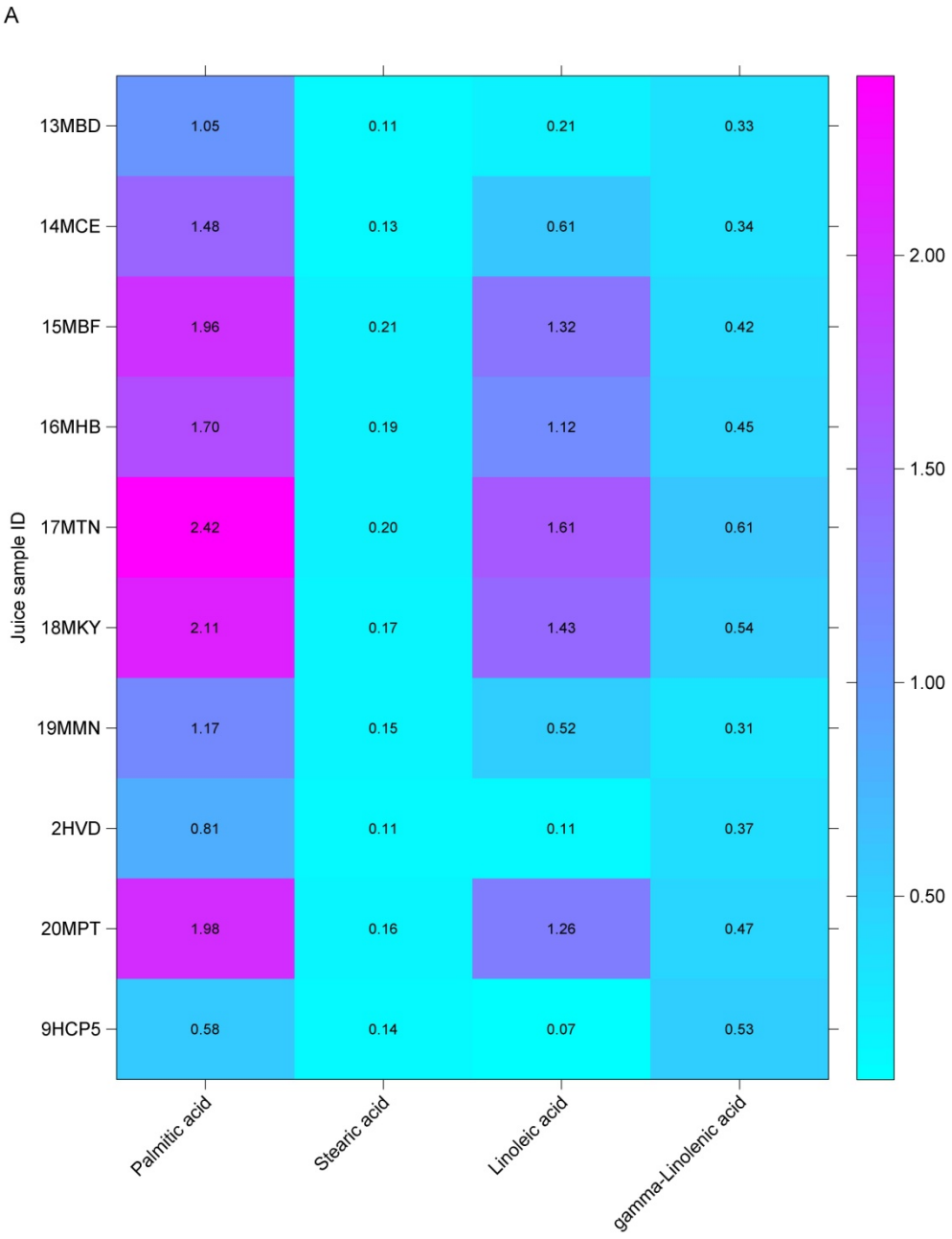
Appendix 3.2

Total ion current chromatogram of human serum lipid extract

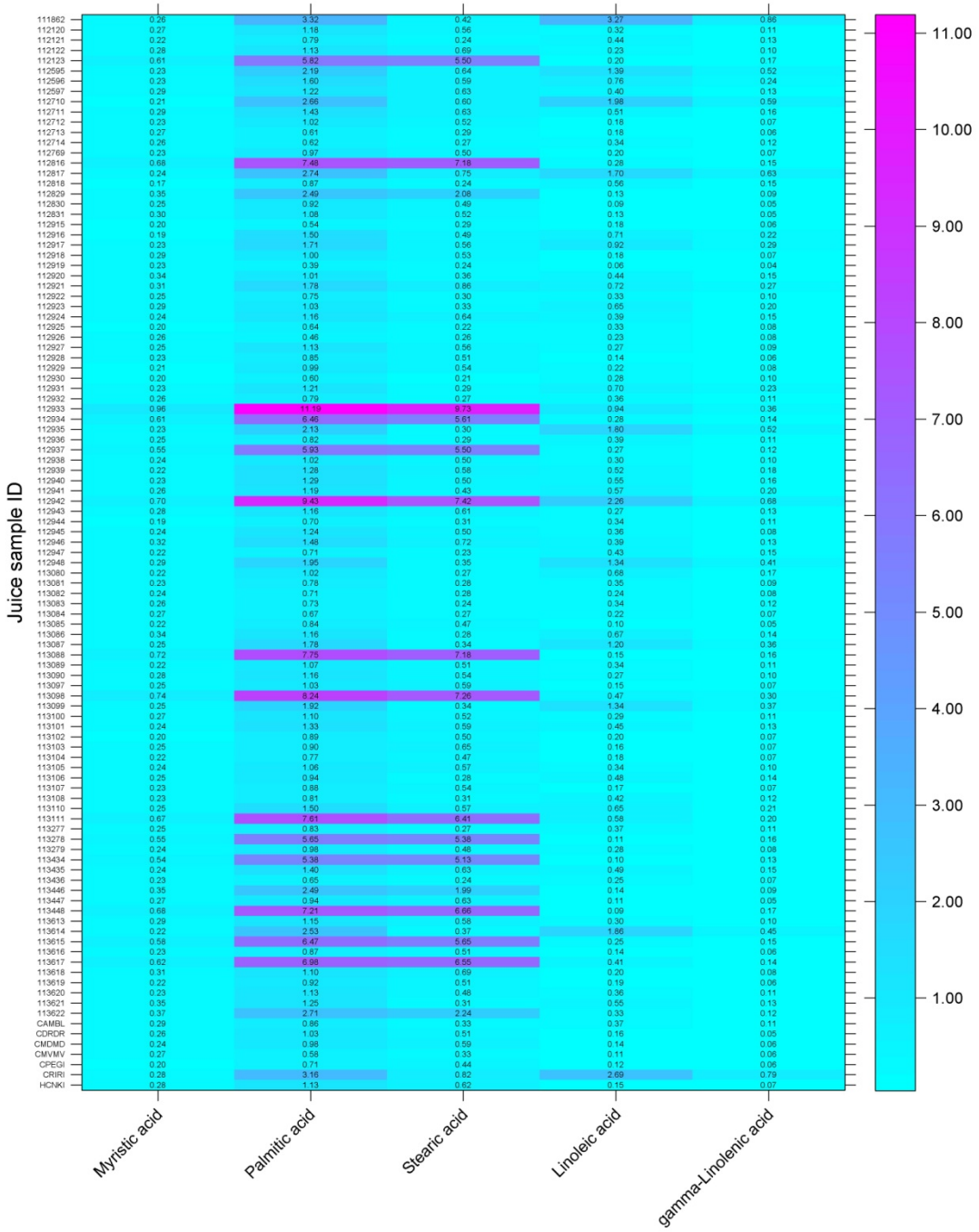


Appendix 4.1

Absolute concentrations (mg/L) of identified free fatty acids in (A) 2010, (B) 2011 and (C) 2012 grape juice samples by GC-MS



B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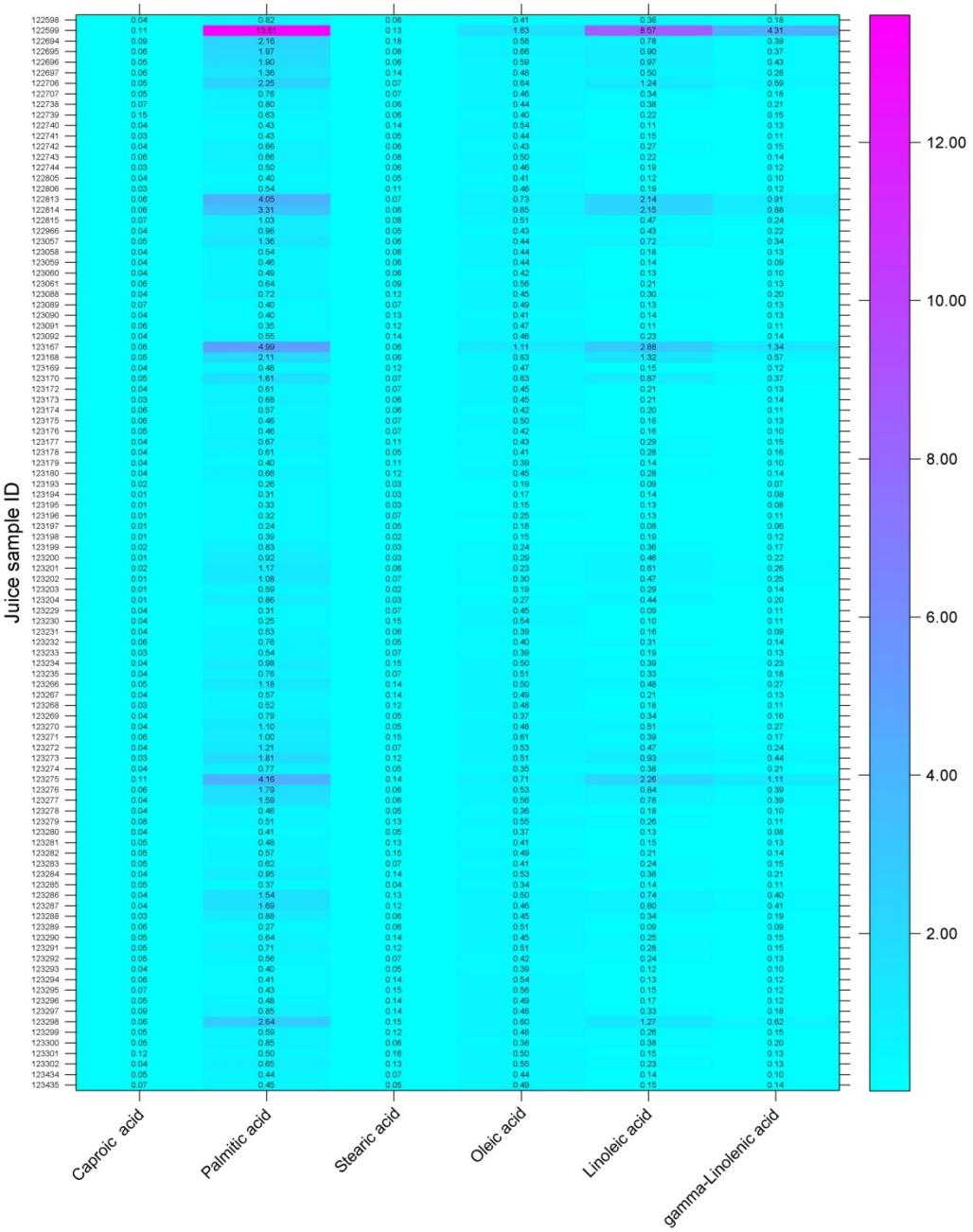
Myristic acid
Palmitic acid
Stearic acid
Linoleic acid
gamma-Linolenic acid
11.00
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00

C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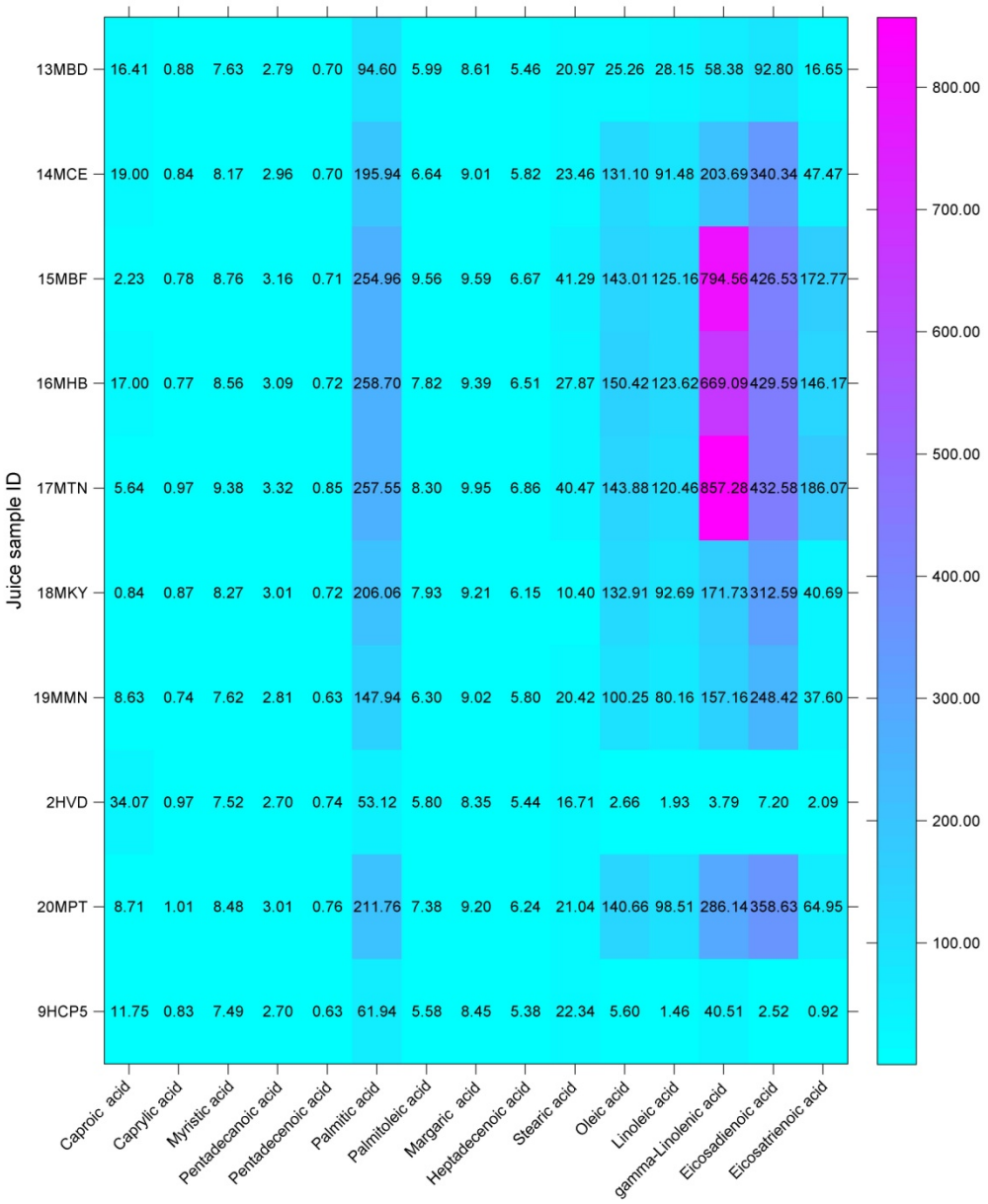
Caproic acid
Palmitic acid
Stearic acid
Oleic acid
Linoleic acid
gamma-Linolenic acid
12.00
10.00
8.00
6.00
4.00
2.00

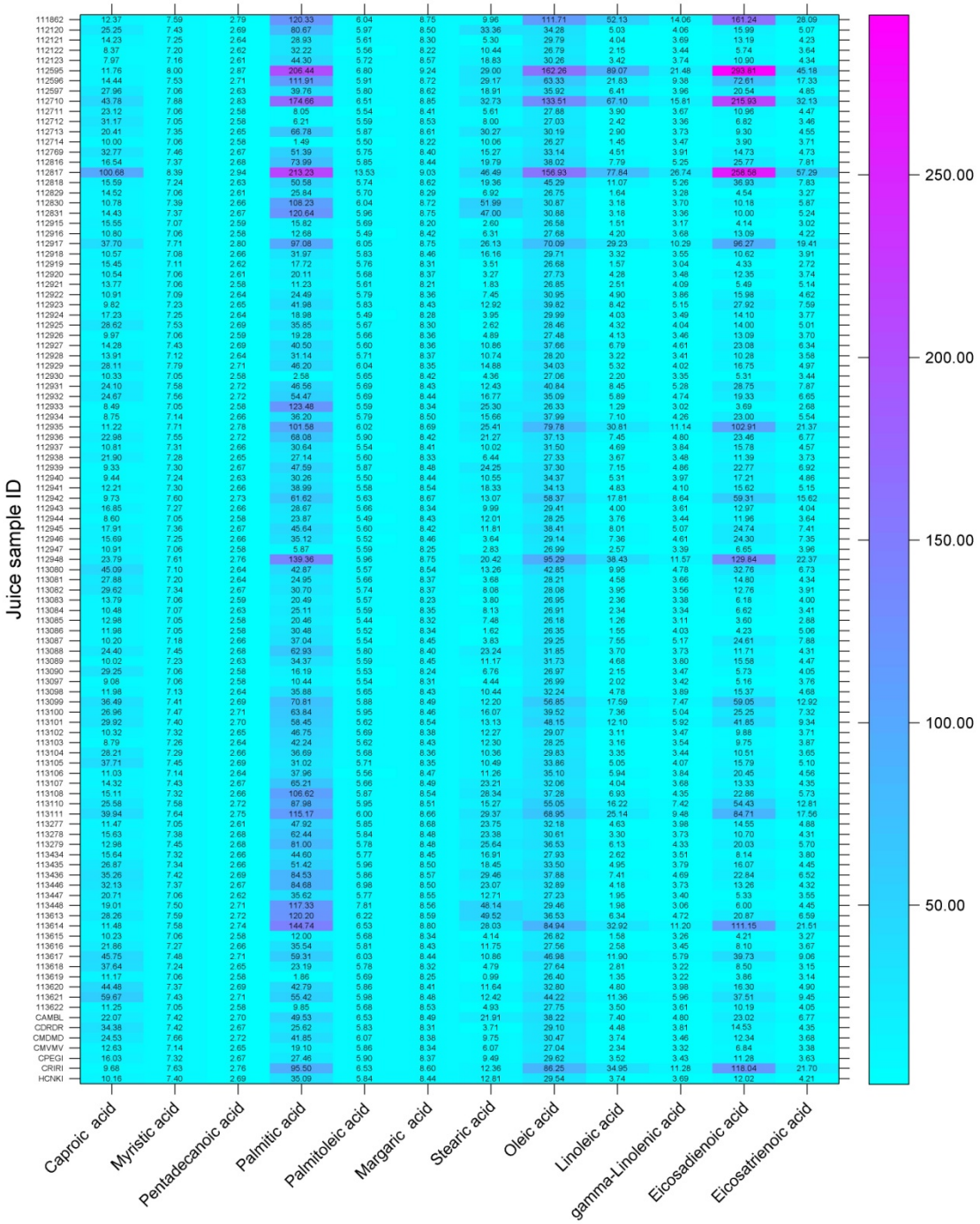
Appendix 4.2

Quantitative fatty acid profile of (A) 2010, (B) 2011 and (C) 2012 grape juice samples by GC-MS. Concentrations of identified fatty acids are in mg/L

A



B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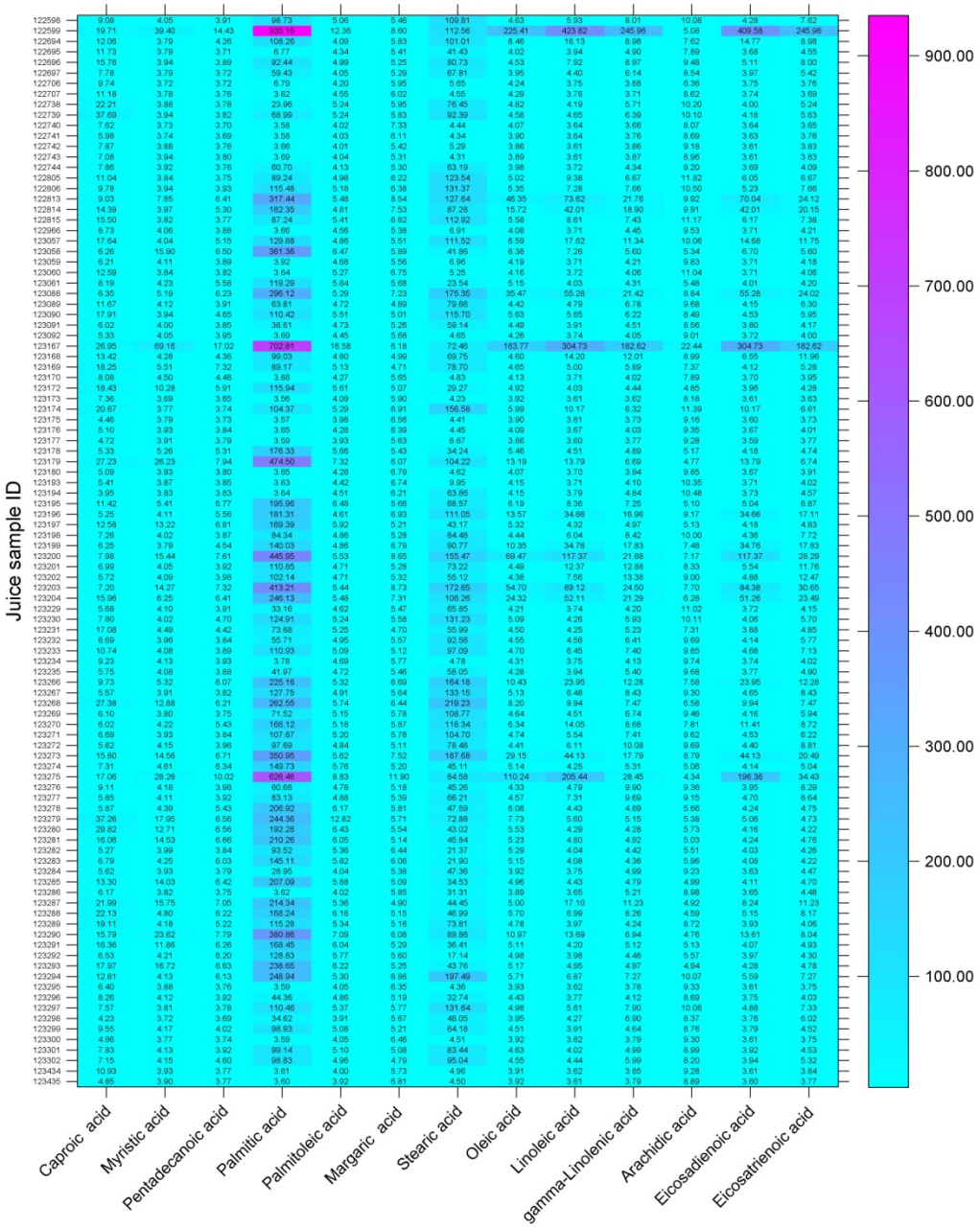
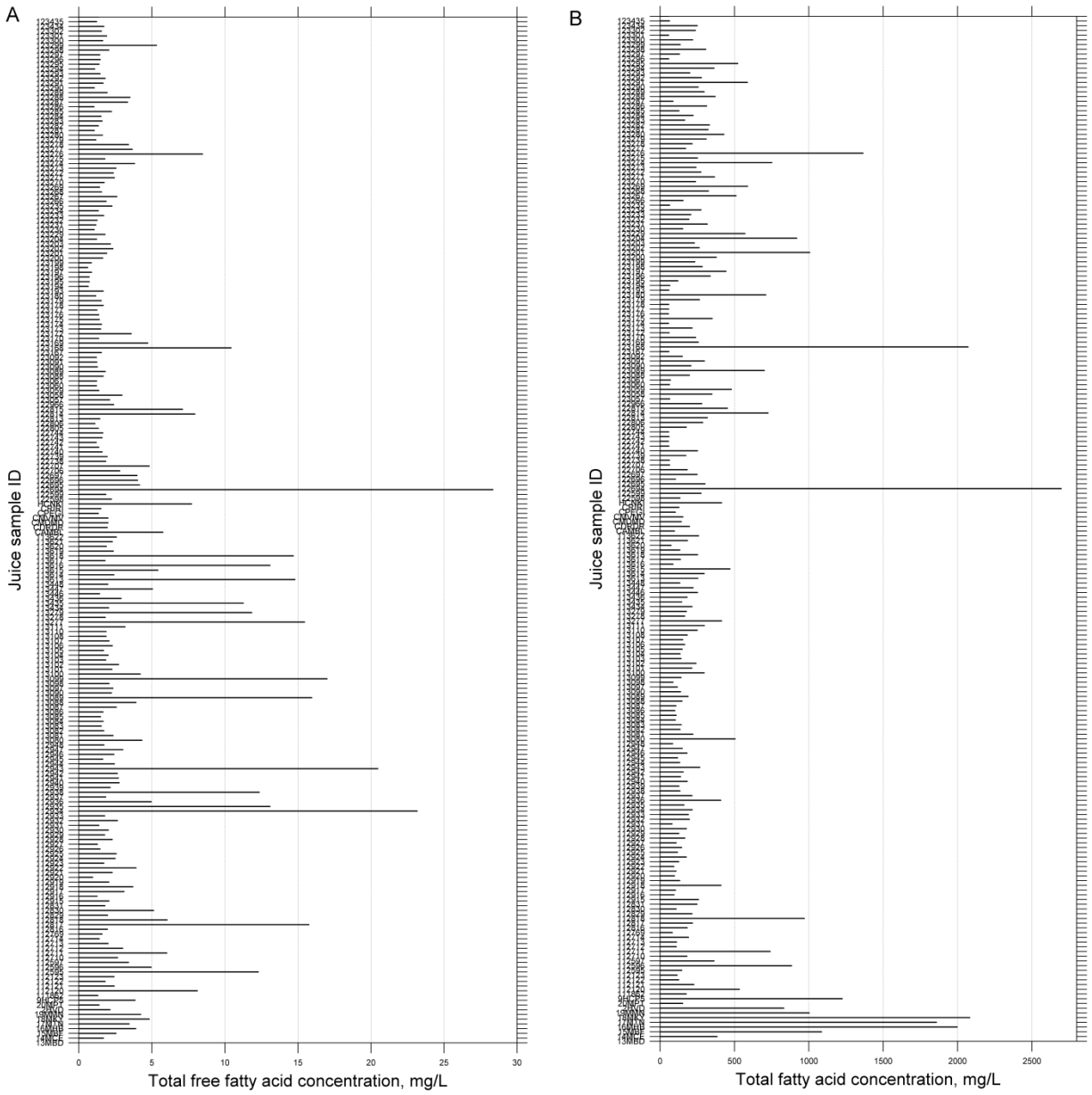
Myristic acid
Pentadecanoic acid
Palmitic acid
Palmitoleic acid
Margaric acid
Stearic acid
Oleic acid
Linoleic acid
gamma-Linolenic acid
Eicosadienoic acid
Eicosatrienoic acid
250.00
200.00
150.00
100.00
50.00

C


Juice sample ID
Caproic acid
Myristic acid
Pentadecanoic acid
Palmitic acid
Palmitoleic acid
Margaric acid
Stearic acid
Oleic acid
Linoleic acid
gamma-Linolenic acid
Arachidic acid
Eicosadienoic acid
Eicosatrienoic acid
900.00
800.00
700.00
600.00
500.00
400.00
300.00
200.00
100.00

Appendix 4.3

Summary of (A) free fatty acid content and (B) total lipid content of all 217 analysed SB grape juice samples. Values presented in milligrams of lipid per litre of grape juice



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