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A Gas Chromatography-Mass Spectrometry Method for the Determination of Fatty Acids and Sterols in Yeast and Grape Juice

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Abstract: Lipids are essential components of all living cells. In an oenological context, the supply of unsaturated lipids in grape juice allows the yeasts to grow and ferment, despite very low levels of oxygen. The current study proposes a systematic optimization procedure for the analysis of fatty acids and sterols relevant to the grape fermentation process, including both extracellular and intracellular (i.e., yeast cells) lipids. Even though it was extensive, the sample preparation yielded reproducible results for all compounds of interest. The stability of the analyzed compounds was also tested to offer some implementation flexibility for the extensive procedure. The performance parameters (i.e., selectivity, linearity, limit of detection and quantitation, accuracy, and precision) indicated that the method was suitable for future practical implementation. The proof of concept also suggests that the list of compounds of interest can be expanded if additional peaks are identified. Given the large variation in concentrations, the dilution of the matrix needs to be carefully considered in order to ensure that the lipids of interest are still within the dynamic range and not below the limit of detection and/or quantification.

Keywords: lipids; fatty acids; sterols; grape juice; yeast; gas chromatography-mass spectrometry

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1. Introduction

Lipids are defined as hydrophobic molecules that are soluble in organic solvents and can be classified according to their molecular structure as well hydrolysis products/chemical backbone. Most commonly, lipids are divided into eight categories, namely fatty acids, sterols, glycolipids, glycerophospholipids, polyketides, sphingolipids, prenol lipids and saccharolipids [1]. Furthermore, lipids can be regarded as simple (sterols, fatty acids, etc.) or complex (glycerophospholipids, glycolipids); the former yields, at most, two products after hydrolysis, and the latter results in three or more products [2,3].

These hydrophobic compounds are found in microorganisms [4,5], plants [6], and animals [7]. Lipid functions are related to energy storage [8], organelle structure maintenance [9], and enzyme activation or signaling [10]. Furthermore, lipids are an integral part of the plasma membrane, which serves as a semi-impermeable bilayer that ensures compartmentalization and facilitates the transport of metabolites, and other biochemical processes necessary for cellular functioning [11]. Changes in the plasma membrane phospholipid, sterol, and fatty acid content are necessary for cellular viability and survival in stressful and dynamic environmental conditions [12–14]. In yeast, the synthesis of fatty acids is a complex process mediated by a variety of enzymes with acetyl-CoA as the main building block [15]. Ergosterol is the main sterol produced in yeasts and, similarly to fatty

acids, this sterol is synthesized with acetyl-CoA as the main substrate [16]. While the production of saturated fatty acids in the yeast cell can occur in the absence of oxygen, the biosynthesis of unsaturated fatty acids [17] and ergosterol is oxygen dependent [18]. Thus, the presence of unsaturated acids and sterols in the surrounding environment is necessary for yeast growth in oxygen-limiting conditions such as those occurring during the alcoholic fermentation of grape must [19].

In addition to influencing yeast growth, the presence of unsaturated fatty acids and sterols in the plasma membrane is also linked to ethanol stress resistance during vinification [20–22]. Moreover, these lipids were observed to have an effect on the production of aroma compounds such as ethyl esters, acetate esters, and higher alcohols, which play a key role in wine quality [13,20,23].

Therefore, the analysis of free fatty acids and sterols in grape juice provides essential information to understand and predict yeast performance as well as aroma production during fermentation, which allows for a better characterization of the yeast lipid metabolism. The Bligh and Dyer method [24] as well as the Folch method [25] are popular protocols used to extract fatty acids from a variety of matrices using a chloroform:methanol solution. In oenology, modified extraction protocols based on the Folch or Bligh and Dyer methods have been described for the extraction and profiling of fatty acids from Sauvignon Blanc grape juice [26] and red wine [27]. Furthermore, fatty acids could also be extracted from Koshu and Pinot Noir juice samples using methanol, 2,6-di-*tert*-butyl-*p*-cresol and formic acid [28]. Free fatty acids and sterols were also extracted from Chardonnay grape musts using ethanol:water [29], but this method requires large sample and solvent volumes.

The analysis of yeast-derived fatty acids and sterols is also necessary to evaluate changes in the cell composition as fermentation progresses. Numerous studies focused on the lipid composition of yeasts have been published and the extraction protocols used are also mainly based on the Folch or the Bligh and Dyer methods [30,31]. However, complex or combined lipids such as phospholipids and steryl esters in yeasts have to be hydrolyzed to liberate free fatty acids or sterols using an acid (such as HCl) or a base (such as KOH). This process can happen either before or after lipid extraction [29,32–34].

Fatty acids and sterols are not naturally volatile and require derivatization to increase their volatility, stability at high temperatures, and ionization yield for MS detection. These compounds can be derivatized using silylation and alkylation [35]. The silylation of fatty acids or sterols occurs at high temperatures (around 100 °C) via the use N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) in pyridine or N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) with trimethyl chlorosilane yielding TMS derivatives [29,36–38]. Fatty acid methyl esters (FAMEs) resulting from alkylation using acids or bases and acetyl chloride sodium methoxide (CH_3NaO) can also be analyzed via MS [35,39–42].

The available literature reports on specific lipid classes present in yeasts, grape juice and wine extracted and analyzed using different methods. For example, fatty acids were analyzed using gas chromatography-mass spectrometry (GC-MS) [26,43] and gas chromatography-flame ionization detection (GC-FID) [44]. In another study, fatty acids extracted from Koshu and Pinot Noir berries were detected using gas chromatography coupled with tandem mass spectrometry (GC-MS/MS) whereas glycerolipids were analyzed using liquid chromatography-mass spectrometry (LC-MS) [28]. Recently, liquid chromatography tandem-mass spectrometry (LC-MS/MS) was used to analyze fatty acids in grape skin and seeds [45]. The fatty acids' profile was determined quantitatively by GC-MS and qualitatively by Fourier transform infrared-attenuated total reflection (FTIR-ATR) in a study focused on the recovery of bioactive compounds in grape waste (in this case, seeds) [46]. Sterols in the grape matrix can also be analyzed using GC-MS [47] and GC-FID [29,39,48]. Even when both sterols and fatty acid were extracted from yeast using the same protocol and instrumental method (GC-FID), the proposed protocol was long and required large volumes of sample and of solvent [29].

In this context, the aim of the current study was to optimize a fatty acid and sterol extraction protocol in a synthetic medium mimicking grape juice (synthetic grape juice

medium or SGM) and a synthetic medium used to cultivate yeasts prior fermentation (yeast nutrient base or YNB), as well as yeast using minimal sample and solvent volume. In addition, the optimization of a GC-MS method that allows the analysis of more than one lipid class in one single run was performed.

2. Materials and Methods

2.1. Optimization Design

It was important to first optimize an extraction method in a synthetic medium in order to monitor the uptake or potential release of free fatty acids and sterols by yeasts during alcoholic fermentation. The protocol was optimized in a YNB medium used for growing yeast cultures and SGM used for alcoholic fermentation.

Even though the analysis follows the steps extraction–derivatization–separation–detection, the optimization process was carried out in stages following a different progression, taking into account the information available from the literature, instrumental limitations (e.g., MS dynamic range), and the intended applications (Table 1). Additional relevant aspects were related to identifying other possible compounds of interest when using yeast or juice as original matrix, the number of samples that could be processed at once, and the stability of the extract. The method performance was tested before applying it to a number of samples as proof of concept.

Table 1. Method optimization strategy.

Stage	Aim	Sample Type	Matrix/Solvent	Parameters Tested	Observations
1	GC-MS optimization	Standard mixtures	Chloroform	Separation MS parameters MS dynamic range	Derivatization well documented in the literature
2	Extraction optimization	Standard mixtures	YNB medium	Volume (sample, solvent, aliquot)	GC-MS optimized in stage 1
			Yeast (one strain)	Pellet size, solvent volume, aliquot	Any additional compounds of interest?
			SGM	Volume (sample, solvent, aliquot)	Any additional compounds of interest?
3	Method testing	Standard mixtures	YNB medium	Performance parameters	Reference, non-interfering matrix
			Yeast (various strains)		-
			SGM		-
4	Applications	Real samples from fermentation experiments			

2.2. Chemicals and Standard Solutions

Nonadecanoic acid (Internal Standard, IS), cholesterol (IS), myristic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, linolenic acid, β-sitosterol, ergosterol, squalene, chloroform, hexane, methanol, pyridine, NO-bis(trimethylsilyl)trifluoroacetamide (BSTFA), with trimethylchlorosilane (TMCS) were purchased from Sigma (Western Cape, South Africa).

A 100 mg/L lipid stock mixture was prepared (using the above-mentioned lipids) in degassed anhydrous chloroform and stored at 4 °C. This stock mixture was used to prepare a standard curve with lipid mixtures at the following concentrations: 0.5, 1, 2, 5, 10, 20 and 50 mg/L. Following this, the lipid mixtures were stored at −20 °C until they were used. The lipid mixtures used in this study are based on the concentration of free fatty acids and sterols found in grape juice [26,43]. Lipids were extracted in SGM which was comprised of 100 g/L glucose, 100 g/L fructose, 2.5 g/L tartaric acid, 3 g/L malic acid, 0.2 g/L citric acid, 1.14 g/L potassium phosphate dibasic, 1.23 g/L magnesium sulfate heptahydrate, 0.44 g/L calcium chloride dihydrate, supplemented with vitamin, trace element and nitrogen stocks [49,50]. Furthermore, lipids were extracted from a YNB

medium (Difco, Le Pont-de-Claix, France) supplemented with 20 g/L sugars and nitrogen stock used for the preparation of SGM.

An SGM medium was autoclaved prior to performing lipid extractions, whereas the YNB medium was filter-sterilized. Next, lipid stock mixtures were added to the SGM and YNB media at the aforementioned concentrations.

2.3. Sample Preparation—Extraction

Synthetic grape juice-like and YNB media (Figure 1): The sample (2.5 mL) was spiked with IS (100 µL of 100 mg/L each IS in chloroform) and thoroughly mixed. Next, 2.5 mL chloroform:methanol (1:2) was added to the sample and vortexed for 3 min. This was followed by the addition of 1 mL chloroform and a minute-long vortex. Finally, 1 mL distilled water was added to the sample and vortexed for a minute. The mixture was centrifuged at 3000 rpm for 3 min, and the lower organic layer was collected (approx. 1.5 mL) and transferred into a glass vial. Excess water was removed by the addition of anhydrous sodium sulphate ($NaSO_4$). The extract (100 µL) was diluted in chloroform (400 µL) to ensure that the concentration of lipids injected into the GC-MS was within the dynamic range and a 100 µL aliquot was dried under nitrogen before derivatization.

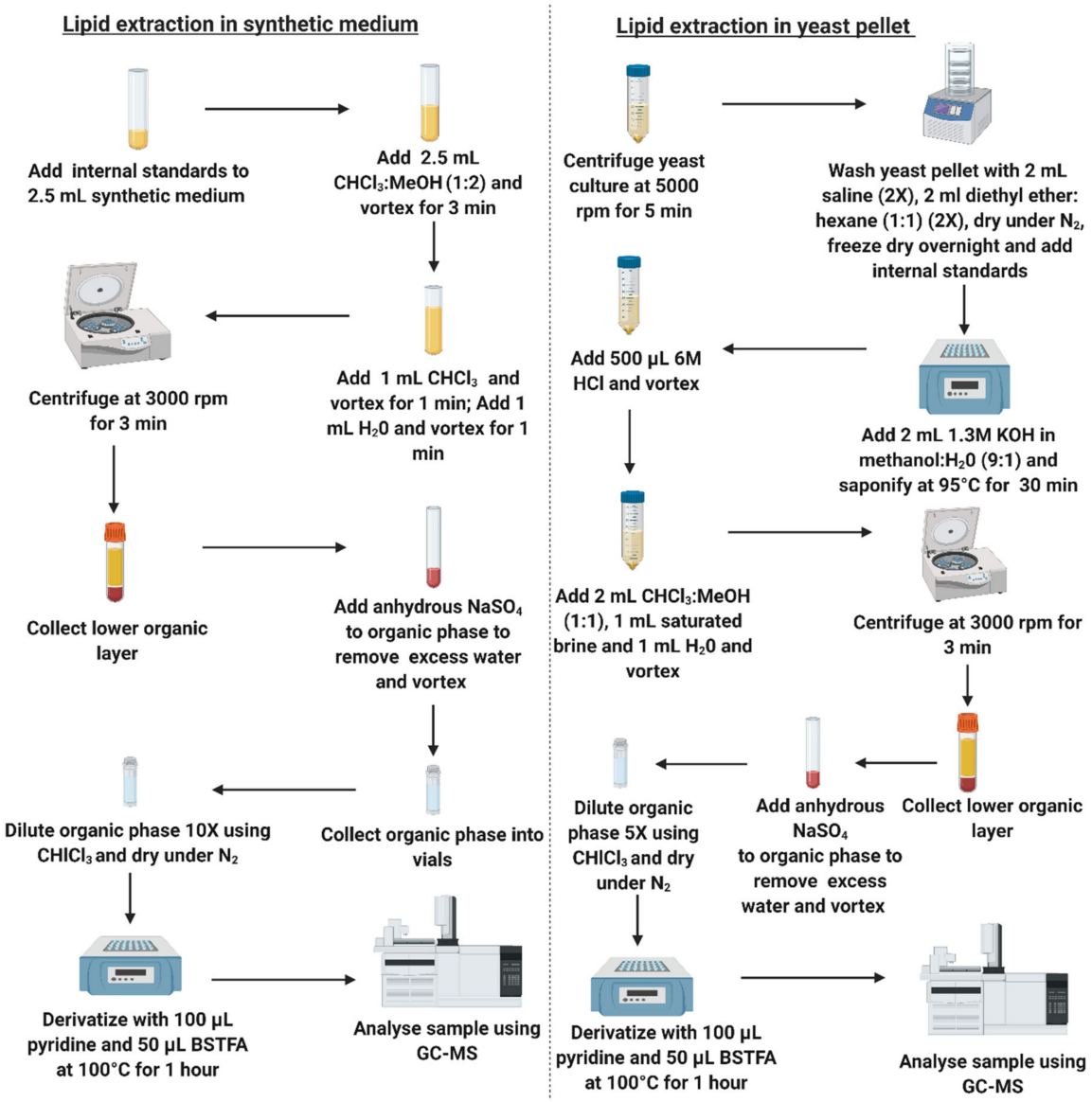


Figure 1. Sample preparation workflow. Created with BioRender.com.

Yeast (Figure 1): Prior to the lipid analyses, *Metschnikowia pulcherrima* (Flavia™ MP346), *Torulaspora delbrueckii* (Biodiva™ TD291) *Saccharomyces cerevisiae* (Lalvin® EC1118) *S. cerevisiae* K1M from Lallemand Inc (Montreal, QC, Canada) and *Kluyveromyces marxianus* (IWBT Y885) from the yeast culture collection of the South African Grape and Wine Research Institute, Stellenbosch University, South Africa, were cultured in a YNB medium (with no lipids) under semi-aerobic conditions. Samples (10 mL) were harvested at log phase to yield approx. 100 mg dried pellet, and centrifuged at 5000 rpm. The pellet was washed with 2 mL saline (9%NaCl in distilled water) twice, and then with a diethyl ether: hexane (1:1) solution twice. The pellet was then dried under nitrogen to evaporate the solvents (approximately 20 min) and freeze-dried overnight. Next, the pellet was ground and the sample weighed (target mass approx. 50 mg), 100 µL of 100 mg/L each IS in chloroform was added and the sample was saponified as described by Tumanov et al. [26]. This step was necessary to neutralize the saponification reaction products and ensure maximal extractions of lipids.

The extraction started with the addition of 2 mL chloroform:methanol (1:1) mixture, followed by 1 mL chloroform and 1 mL brine(saturated NaCl in distilled water) to the saponified sample. The mixture was vortexed and centrifuged as previously described for the synthetic juice. The rest of the protocol was identical to the one for synthetic media.

2.4. Sample Preparation—Derivatization

The derivatization was carried out according to Delfini and Cocito [29] with some modifications. In brief, to the dried extract obtained from the previous step (Figure 1), 100 µL of anhydrous pyridine and 50 µL BSTFA/TMCS (99/1 v/v) were added. The derivatization mixture was kept at 100 °C for 1 h before analysis by GC-MS.

2.5. Instrumental Parameters

GC-MS analysis was performed with a 7890B GC (Agilent, Palo Alto, CA, USA), equipped with a 5977B single quadrupole mass detector (Agilent, Palo Alto, CA, USA) and a PAL RSI 85 autosampler (CTC Analytics AG, Zwingen, Switzerland). Chromatographic separation was performed on an Agilent HP-5MS-UI capillary column (30 m × 0.25 mm × 0.25 µm, Agilent, Palo Alto, CA, USA). The initial oven temperature was 100 °C held for 2 min, then ramped up to 180 °C at 15 °C/min. The temperature was subsequently ramped up to 250 °C at a rate of 5 °C/min and held for 3 min, followed by 20 °C/min to 320 °C and held for 12 min. Total run time was 40 min.

Liquid sample injection was performed in the GC inlet port with the temperature maintained at 280 °C; the 1 µL injection was conducted in splitless mode with the split flow set to 30 mL/min for 2 min. A 870 µL universal low pressure drop, an ultra-inert liner with glass wool was used (Agilent 5190-2295, Palo Alto, CA, USA). Helium was the carrier gas and the flow rate was set to 1.0 mL/min (constant flow). Data were acquired in SIM mode with the solvent delay set at 4.5 min. The ionizing voltage was set to 70 eV. MS source and quad temperatures were maintained at 230 and 150 °C, respectively. Data analysis was performed using MassHunter qualitative (B.07.00) and quantitative (B.07.01) workstation software.

The method was first conducted in scan mode to allow for structural comparison using the NIST library. Compounds of interest identified based on the comparison of RT and mass spectra to authentic standards. Additional minor lipids resulting from the analysis of matrices other than the synthetic ones were tentatively identified but not included in the quantification at this stage. Quantification was performed only for the compounds for which authentic standards were available, and was carried out using internal standard calibration. The results were normalized to the volume of juice and mass of the pellet, respectively.

2.6. Performance Parameters

The performance of the proposed method was tested using a number of qualitative and quantitative parameters [51]. In addition to the yeast strain used during optimization (*S. cerevisiae* EC1118), another three species were included at this stage (*T. delbrueckii*, *K. marxianus*, and *M. pulcherrima*). Selectivity was evaluated based on the separation (retention times) and MS spectra using standard mixture spiked into the juice and yeast matrices. The linearity was tested using internal calibrations based on peak area and peak height at seven concentrations of the compounds of interest (0.50–50 mg/L) in the minimal media. LOD (S/N = 3) and LOQ (S/N = 10) were calculated based on the linearity. MS dynamic range also had to be tested due to the concentration and high ionization of the BSTFA-derivatives. The accuracy of the method was evaluated through recovery tests (direct injection *vs* recovered amount after sample processing) at 1 mg/L and 10 mg/L (n = 4); for calculations, a blank subtraction was performed. Precision was determined through repeatability for the extraction (for juice n = 4 and for yeast n = 3, the pellet size being the main limitation in this case), derivatization (n = 4), and instrumental characteristics (retention time and response, n = 3). Carryover was also evaluated by running an IS blank after the highest calibration level (50 mg/L).

Due to the length of the procedure, the stability of the yeast extracts was also determined in triplicate (Figure 2). Firstly, extracts were dried, derivatized, and injected on the same day (control); the samples were left on the sample tray for two days at room temperature and then injected. In the second scenario, the extract was dried, derivatized and stored at −20 °C for two days and then injected. In a third case, a 100 µL aliquot of extract was stored at −20 °C for two days, then dried, derivatized and injected. Lastly, the dried sample was stored at −20 °C for two days and then analyzed.

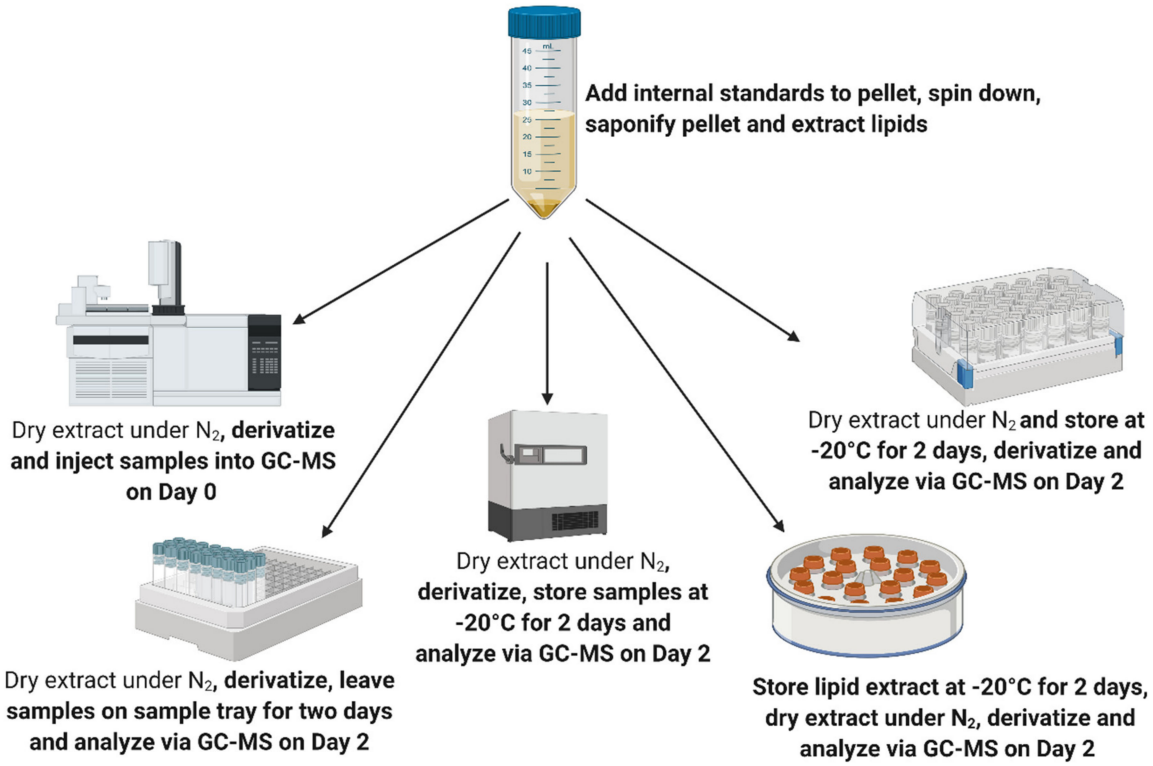


Figure 2. Stability tests. Created with BioRender.com.

2.7. Samples

Freeze cultures were streaked onto yeast peptone dextrose (YPD) agar (Merck, Gauteng, South Africa) plates and incubated at 30 °C for 48 h. A single colony was inoculated into 5 mL YNB medium with 20 g/L sugar for 24 h. A 1 mL culture was then transferred into 100 mL YNB and incubated until the yeast populations reached late exponential growth under semi aerobic conditions. Yeast cultures were harvested, centrifuged at 5000 rpm for 5 min, washed with saline (0.9% NaCl), and centrifuged again. The resulting yeast pellet was inoculated into 70 mL SGM containing no lipids (control) or 50 mg/L linoleic acid, and incubated at 25 °C without agitation under semi-aerobic conditions. Fermentation progress was monitored via carbon dioxide production. Samples were harvested when fermentations became stuck, sluggish or complete depending on yeast strain (this will be termed "end of alcoholic fermentation" in this study) for lipid analyses in the yeast pellet and supernatant.

3. Results

3.1. GC-MS Optimization

The separation of the analytes of interest was first performed using a standard mixture. The eight fatty acids (including nonadecanoic acid as an internal standard) and four sterols (including cholesterol as internal standard) were separated in less than 30 min as TMS-derivatives (Table 2). The MS parameters were subsequently optimized for selected ion monitoring mode and the ions were used further for the quantification of the compounds (Table 2).

Table 2. Retention times for fatty acids and sterols detected as TMS derivatives. The quantifier and qualifier ions are for the TMS-derivative of each compound. Nonadecanoic acid and cholesterol were used as IS for the fatty acids and sterols, respectively.

Lipid Name (Detected as TMS Derivative)	Lipid Name in Abbreviated Form (TMS Derivative)	Compound Molecular Weight (Underivatized)	Retention Time (Min)	Quantifier Ion (*m*/*z*)	Qualifier Ion (*m*/*z*)
Myristic acid	C14:0	228.37	11.28	285.2	300.3
Palmitoleic acid	C16:1	254.41	13.72	311.1	129.0
Palmitic acid	C16:0	256.4	14.00	313.3	328.3
Linoleic acid	C18:2	280.45	16.49	73.1	337.3
Oleic acid	C18:1	282.47	16.58	73.1	339.3
Linolenic acid	C18:3	278.43	16.61	75.1	335.2
Stearic acid	C18:0	284.48	16.97	341.3	297.3
Nonadecanoic acid (IS)	C19:0	298.5	18.47	117.0	370.3
Squalene	$C_{30}H_{50}$	414.71	25.98	117.0	355.3
Cholesterol (IS)	$C_{27}H_{46}O$	386.65	28.33	129.1	458.4
Ergosterol	$C_{28}H_{44}O$	396.65	28.88	253.2	363.3
β-Sitosterol	$C_{29}H_{50}O$	414.71	29.61	129.1	396.4

The instrumental method used was adapted from Smart et al. [52]. Co-elution was observed for TMS derivatives of oleic acid (C18:1) and linolenic acid (C18:3). Chromatographic separation was first attempted for the critical pair C18:1 and C18:3, but no resolution was achieved (Figure 3). The unique ions were identified for the compounds except for the co-eluting peaks. Due to their structural similarity, corresponding fragmentation patterns are also similar. In this case, the 335.2 and 339.3 m/z fragments were used for linolenic and oleic acid TMS derivatives, respectively, as these are unique ions.

The standard mixture was also spiked into the synthetic juice and the extraction was performed according to the literature [26]. During this stage, it was observed that the signal from the detector reached saturation at medium concentrations of the lipids. This prompted an evaluation of the dynamic range of the MS in response to the high signal observed and, at the same time, a modification of the original extraction protocol

to include a dilution step. The dynamic range was evaluated using peak area and height. The peak area displayed a linear correlation from 750 ppb to 40 mg/L, whereas the peak height displayed linearity from 750 ppb to 50 mg/L for some compounds. These values are important because they need to be taken into consideration for sample preparation optimization and routine analysis.

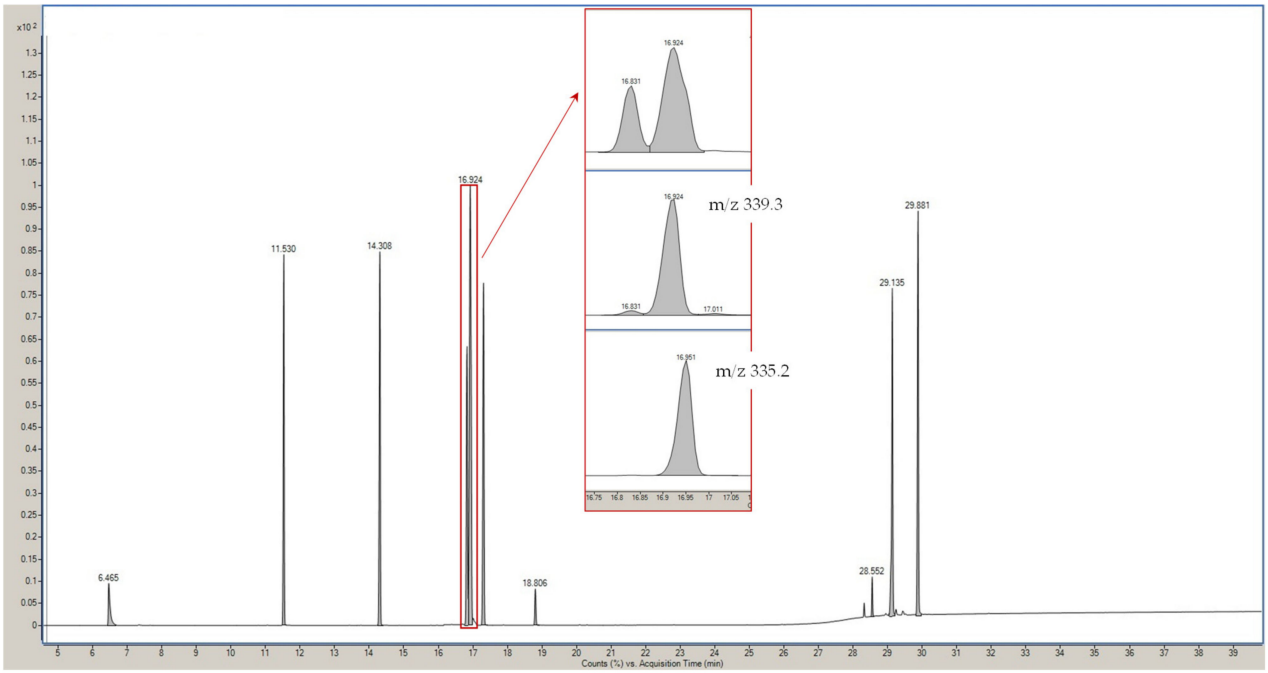


Figure 3. Extracted ion chromatogram (EIC, SIM mode) of the standard mixture. The insert is a zoom-in of the critical pair area (top) with the oleic acid peak in SIM mode (m/z 339.3) and linolenic acid peak in SIM mode (m/z 335.2). The critical pair zone is marked with the red rectangle.

3.2. Extraction Optimization

The extraction of free fatty acids and sterols was based on a study performed by Tumanov et al. [26], with some modifications. A freshly prepared synthetic medium (2.5 mL) was used instead of freeze-drying because it was time-consuming and the sticky caramel-like residue proved difficult to handle (for example, weighing out different sample masses). In addition, as previously mentioned, an additional dilution step was incorporated into the extraction protocol in order to bring the concentration of the lipids into the dynamic range of the MS.

Fatty acids and sterols predominantly exist in bound form (steryl esters, triacylglycerols, phospholipids, etc.). Therefore, it was necessary to liberate the lipids of interest before extraction through saponification [24,52]. Before taking this step, the yeast pellet was first washed with saline and then with a diethyl ether:hexane (1:1) mixture. Even though this was an additional step to include in an already multi-step procedure, it was considered necessary as residual compounds on the pellet can interfere with analyses when sensitive instruments (such as the GC-MS) are used.

For the yeast, hexane was initially added to the saponified material as the method was initially aimed at sterols (compounds with high lipophilicity). However, hexane alone did not separate the organic and aqueous phase in the sample. Three solvent extractions were evaluated: (1) hexane followed by chloroform, the extracts then combined; (2) chloroform followed by hexane, the extracts then combined; and (3) extraction with a chloroform/hexane mixture. Small differences between treatments were observed, and although the chloroform/hexane mix was best for sterols, it did not allow the optimal extraction of fatty acids. In the end, a different mixture, consisting of chloroform/methanol was used. In this case, the main modification of the method compared to literature [24,52]

was the change from a 1/2 to a 1/1 for the saponified sample. The addition of brine was also considered necessary, as a clear separation between the organic and aqueous phase was not observed without it.

The volume of sample harvested was adjusted based on the yeast growth stage. Three resulting pellet sizes were tested: 5, 50, and 200 mg. The lowest mass gave a low response in MS, while the highest resulted in a response outside the dynamic range of the detector, unless an additional dilution step was included. Most importantly, it was difficult to recover 200 mg from a 10 mL fermentation sample. The medium mass (50 mg) gave reasonable response in the MS and was easier to recover from the small volume fermentation.

3.3. Method Performance

The instrumental method was able to separate successfully the compounds of interest. Oleic acid (C18:1) and linolenic (C18:3) co-eluted, but could be separated in the MS at a level considered appropriate for this application (Table 2 and Figure 3). The figures of merit for the performance parameters showed that the method was appropriate for the purpose (Table 3), with values obtained for SGM generally being better than those for YNB in terms of recovery.

When testing instrumental variability, the response to repeated direct injections varied between 2.07 and 3.07%, with the exception of ergosterol, in which case the RSD was 3.98%. Retention times varied between 0.01 and 0.02% for all compounds.

As palmitoleic acid and squalene were added later based on observations from the analysis of yeasts other than *S. cerevisiae* EC1118, no stability data are available for these two compounds (Table 4). With the exception of β-sitosterol, all compounds were relatively stable regardless of the stage of the storage, as long as it was not in the autosampler tray. The β-sitosterol was unstable after 48 h both underivatized and derivatized, regardless of the storing conditions.

3.4. Application

As proof of concept, the fermentation performance of wine-relevant yeasts in SGM was tested in the absence (control) and presence of lipids (50 mg/L linoleic acid) under semi-aerobic conditions (Figure 4). Overall, the highest carbon dioxide production was observed in in *S. cerevisiae* EC1118 followed by *T. delbrueckii*, *K. marxianus* and *M. pulcherrima*. Interestingly, an increase in fermentation performance (carbon dioxide production) was only observed in *K. marxianus* and *S. cerevisiae*, whereas minimal differences were observed in other yeasts when linoleic acid was added to SGM (Figure 5).

Figures 5 and 6 show the lipid composition of the yeasts investigated in this study at the end of alcoholic fermentation and the corresponding fatty acid and sterol profiles. *M. pulcherrima* produced the highest relative amount of oleic, linoleic and linolenic acid during fermentations in SGM with no lipids. On the other hand, the highest amount of palmitoleic acid was detected in *K. marxianus* at the end of fermentation in SGM without the supplementation of lipids. No linoleic or linolenic acid were detected in *S. cerevisiae* control fermentations. However, linoleic acid was taken up in all yeasts but to varying degrees. Indeed, the highest uptake of linoleic acid was observed in *M. pulcherrima*, followed by *K. marxianus*, *T. delbrueckii* and *S. cerevisiae*. In terms of sterols, the highest ergosterol level was observed in *M. pulcherrima*, whereas high amounts of squalene were observed in *T. delbrueckii* and *S. cerevisiae*. Overall, all the yeast investigated in this study assimilated linoleic acid. Although selected fatty acids (including linoleic acid) were detected in the supernatant at the end of alcoholic fermentation, these compounds were below the limit of quantification (data not shown).

Table 3. Figures of merit for the method performance.

Analyte	Calibration Range (mg/L)	R2	LOD (µg/L)	LOQ (µg/L)	Recovery (%) at 1 mg/L	Recovery (%) at 10 mg/L	Extraction Repeatability (RSD %) at 1 mg/L	Extraction Repeatability (RSD %) at 10 mg/L
YNB								
Myristic acid	0.5–50	0.9996	4.3	14.2	70.7	93.58	12.24	6.72
Palmitoleic acid	0.5–50	0.9992	35.5	118.3	81.5	84.45	4.53	7.13
Palmitic acid	0.5–50	0.999	1.4	4.7	90.65	132.67	16.44	18.55
Linoleic acid	0.5–50	0.9997	15.1	50.2	103.59	97.96	4.27	4.82
Oleic acid	0.5–50	0.9994	37.2	124.0	95.43	97.99	9.83	6.74
Linolenic acid	0.5–50	0.9987	58.5	195.1	95.10	99.09	2.24	4.85
Stearic acid	0.5–50	0.9996	6.8	22.6	91.91	87.11	10.38	12.82
Squalene	0.5–50	1	62.0	206.5	119.56	103.03	7.59	2.88
Ergosterol	0.5–50	1	94.3	314.3	102.44	101.53	9.15	1.40
ß-sitosterol	0.5–50	0.9996	474.9	1583.0	132.54	112.94	2.77	1.94
SGM								
Myristic acid	0.5–50	0.9991	0.7	2.3	141.29	118.37	5.76	6.83
Palmitoleic acid	0.5–50	0.9987	3.0	10.0	105.29	117.35	4.07	8.18
Palmitic acid	0.5–50	0.9996	0.1	0.2	110.31	75.37	17.36	14.97
Linoleic acid	0.5–50	0.9986	11.8	39.3	96.53	111.63	5.20	6.25
Oleic acid	0.5–50	0.9977	35.0	116.5	104.78	113.56	10.12	9.74
Linolenic acid	0.5–50	0.9979	61.3	204.4	105.15	112.42	3.51	6.05
Stearic acid	0.5–50	0.9988	0.8	2.5	108.80	114.80	14.66	5.41
Squalene	0.5–50	0.9999	9.2	30.6	83.64	96.94	4.00	16.84
Ergosterol	0.5–50	1	12.2	40.7	97.61	107.36	4.73	8.76
ß-sitosterol	0.5–50	0.9991	66.8	222.6	75.45	97.68	4.19	8.57

Table 3. *Cont.*

Analyte	Calibration Range (mg/L)	R2	LOD (µg/L)	LOQ (µg/L)	Recovery (%) at 1 mg/L	Recovery (%) at 10 mg/L	Extraction Repeatability (RSD %) at 1 mg/L	Extraction Repeatability (RSD %) at 10 mg/L
Direct injection (Yeast)								
Myristic acid	0.2–50	0.9984	0.0	0.2	n/a	n/a	8.53	8.61
Palmitoleic acid	0.2–50	0.9984	0.1	0.3	n/a	n/a	– *	– *
Palmitic acid	0.2–50	0.9985	0.0	0.1	n/a	n/a	13.39	6.39
Linoleic acid	0.2–50	0.9989	4.3	14.4	n/a	n/a	6.52	6.59
Oleic acid	0.2–50	0.999	5.7	18.9	n/a	n/a	6.52	5.02
Linolenic acid	0.2–50	0.9994	4.8	15.9	n/a	n/a	3.13	5.89
Stearic acid	0.2–50	0.9982	0.1	0.3	n/a	n/a	5.63	5.24
Squalene	0.2–50	0.9995	0.7	2.3	n/a	n/a	– *	– *
Ergosterol	0.2–50	0.9962	0.3	1.1	n/a	n/a	8.83	1.56
ß-sitosterol	0.2–50	0.9994	1.8	5.9	n/a	n/a	13.42	6.42

n/a: not available as the calculations were based on comparison to direct injection; * Compounds included after initial testing.

Table 4. Figures of merit for the stability tests expressed in percentage compared to the control extraction, derivatization, and instrumental analysis.

Analyte	48 h Sample Tray	Extract Dried, Derivatised, Stored at −20 °C for 48 h	Extract Stored in Chloroform 48 h at −20 °C, Dried, Derivatised after 48 h	Extract Dried, Stored at −20 °C, Derivatised after 48 h
Myristic acid	10.34	2.97	4.61	5.07
Palmitoleic acid	-	-	-	-
Palmitic acid	10.46	3.46	6.44	6.00
Linoleic acid	3.93	0.82	19.16	0.35
Oleic acid	8.40	2.33	3.70	3.84
Linolenic acid	3.89	3.66	15.64	2.22
Stearic acid	13.94	9.28	11.67	10.50
Squalene	-	-	-	-
Ergosterol	3.43	11.09	11.89	12.26
ß-sitosterol	33.37	34.82	35.13	36.84

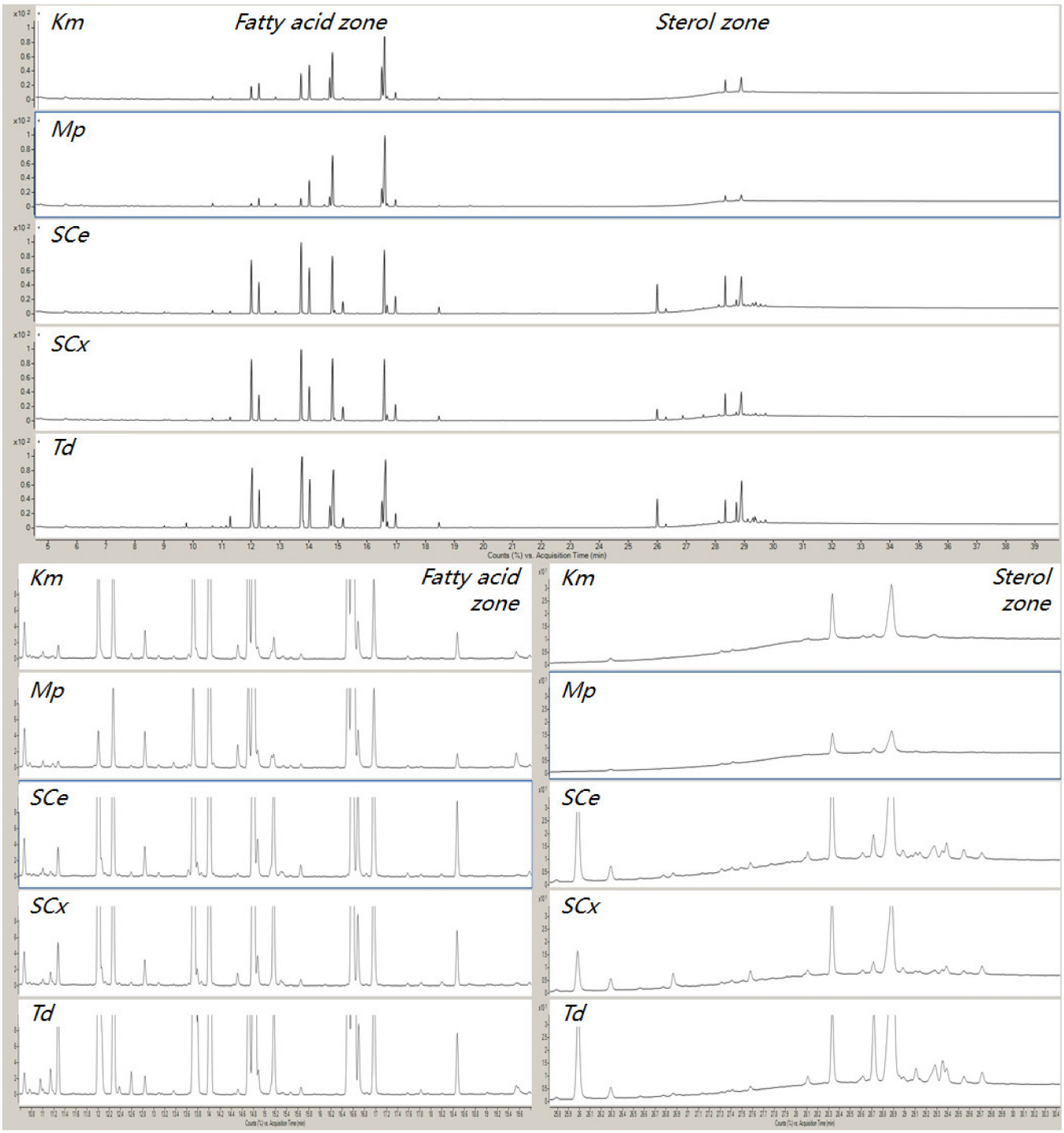


Figure 4. Yeastlipid profiles at log phasein YNB. Mp—*M. pulcherrima*; Km—*K. marxianus*; Td—*T. delbrueckii*; SCe—*S. cerevisiae* EC1118, ScX—*S. cerevisiae* K1M.

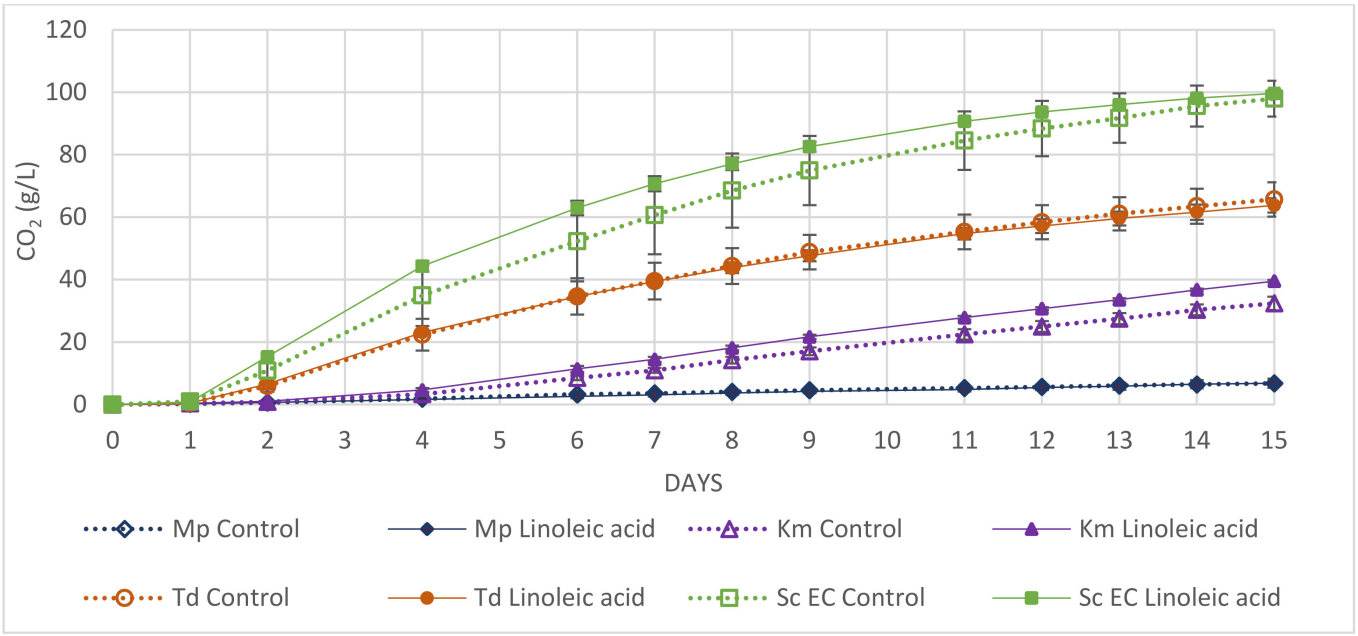
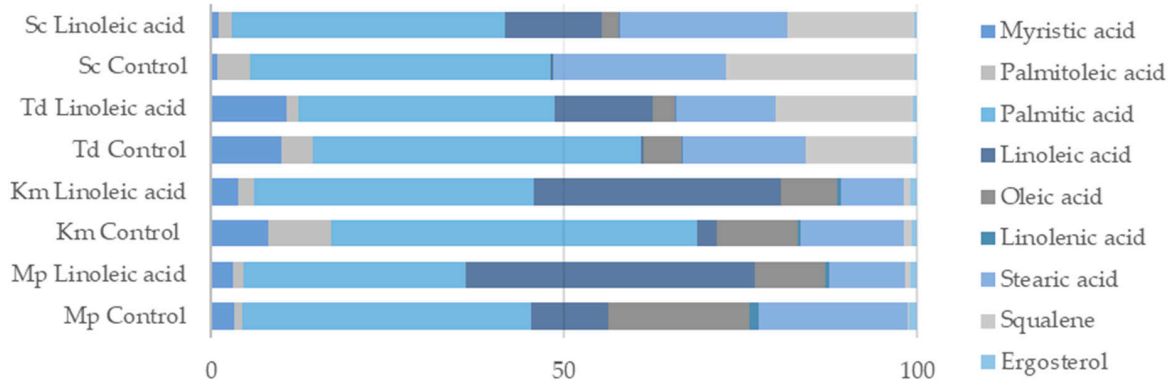


Figure 5. Carbon dioxide production during alcoholic fermentation in SGM supplemented with 0 (control) and 50 mg/L linoleic acid (linoleic acid). Mp—*M. pulcherrima*; Km—*K. marxianus*; Td—*T. delbrueckii*; Sc EC—*S. cerevisiae* EC1118.



	C14:0	C16:1	C16:0	C18:2	C18:1	C18:3	C18:0	Squalene	Ergosterol
Mp C	145 ± 4.55	40 ± 7.4	1742 ± 61.4	464 ± 37.2	850 ± 102.3	47.5 ± 2.9	899 ± 101.3	5.91 ± 1.12	48.33 ± 5.61
Mp LA	152.5 ± 29.7	67 ± 55	1567.5 ± 167.4	2264 ± 861.4	548 ± 189.5	30.3 ± 11.8	581 ± 161.9	32.6 ± 12.95	52.53 ± 17.50
Km C	205 ± 144.9	230 ± 162.9	1263 ± 896.9	75 ± 52.8	307 ± 217.3	8.04 ± 5.7	395 ± 279.9	24.6 ± 17.59	17.49 ± 12.51
Km LA	105 ± 17.3	63 ± 30.3	1083 ± 206.1	975 ± 257	221 ± 60.2	15.6 ± 4.8	249 ± 35.1	24.2 ± 2.59	24.42 ± 7.74
Td C	565 ± 67.7	249 ± 41.8	2637 ± 296	24.5 ± 7.9	293.5 ± 56.3	0.34 ± 0.1	999 ± 109.3	871 ± 246.4	33.85 ± 5.52
Td LA	652 ± 38.3	104 ± 7.6	2242 ± 212.4	854 ± 54.9	184 ± 22	1.68 ± 0.28	894 ± 143.7	1216 ± 281.3	31.31 ± 9.11
Sc C	62.5 ± 5.6	301 ± 52	2775 ± 181.2	3.7 ± 0.96	11.96 ± 4.56	0.40 ± 0.20	1617 ± 335.3	1719 ± 58.2	28.21 ± 3.71
Sc LA	75 ± 23.5	111 ± 25.1	2588 ± 790	878 ± 127.5	155 ± 17.2	1.37 ± 0.18	1599 ± 499.2	1177 ± 264.8	17.00 ± 4.79

Figure 6. Percentages (top—graph) and concentrations (bottom—table, expressed in µg/biomass) of individual lipids detected in yeasts at the end of alcoholic fermentation in SGM. Mp—*M. pulcherrima*; Km—*K. marxianus*; Td—*T. delbrueckii*; Sc—*S. cerevisiae* EC1118. Control (C)—SGM with no lipids; linoleic Acid (LA)—SGM supplemented with 50 mg/L linoleic acid.

4. Discussion

As lipids are one of the main classes of molecules considered 'building blocks' of all biological organisms, their analysis has been developed for a variety of applications. Even

though some authors have opted for the 'shotgun lipidomics' approach introduced by Han and Gross [53], many researchers still prefer the more classical approach of sample preparation, then separation, followed by detection. These methods have to take into account the nature of the compounds of interest, the matrix where the lipids are present, whether they are intra- or extra-cellular, the potential interferences from the matrix, etc. These methods tend to be adapted to suit the conditions of the experiment and the instrumentation available. With an increase in accessibility to higher performance equipment, a more general method could soon replace these various (albeit similar) methods. However, this general method will have to overcome a number of issues, such as the large variation in concentrations of the compounds of interest due to the variety of matrices tested, which in themselves can be challenging.

Since only what has been isolated can be analyzed, sample preparation plays a major role in the process of lipid analysis. Extracellular lipids are generally easier to isolate [26]; intracellular lipids require extensive and careful sample preparation to minimize unwanted loss of lipids [51]. One of the aspects considered relevant during the sample preparation optimization was the incorporation of pellet washes after fermentation. When, during the experiments, the media is spiked with FAS, only a certain percentage is absorbed by the yeast, resulting in residual FAS in solution [54]. Without the pellet washes, this could lead to misleading results as there is no distinction between FAS adsorbed by/absorbed on the yeast vs. residual FAS in the juice.

In the literature, most fatty acid analyses are based on a methylation derivatization (the derivatives are known as FAMEs; fatty acid methyl esters), followed by GC-MS or GC-FID [26,52,55,56]. This derivatization reaction is well-documented and its advantages and disadvantages have been previously discussed in the literature [52]. However, in the current study, the derivatization with BSTFA was preferred due to the stability of the resulting TMS-derivatives. As the sample preparation procedure is time consuming, especially in the case of the yeast samples, the stability of the compounds of interest (in their free or derivatized form) was paramount for the applicability of the method. Even though stability tests are not often included in method development/optimization, in this case it was considered essential. Looking at the results, the most reproducible results were for the TMS-derivatives stored at −20 °C until analysis followed by the dried extract stored at −20 °C, derivatization, and then immediate GC-MS analysis (Table 4). This can offer some flexibility in the practical application of the method, especially when a high number of yeast samples have to be processed.

One of the issues that required particular attention was the dynamic range of the MS fitting the range of concentration of the compounds of interest. For the calibration range tested, all calibrations were quadratic, and curve fit assistant (Agilent-based software) was used to identify best calibration based on R^2. As the value of the quadratic coefficient was small, if the calibration range was reduced by removing the two highest concentrations (50 and 20 mg/L points), the calibration would become linear. However, based on the experiments, it was decided to keep the quadratic calibrations in order to enable the quantification of a wider range of concentrations [55,57]. Dilution of the sample is another practical approach worth considering when the expected concentrations are approximatively known and/or when they are presumed to be high.

However, there is a disadvantage to the dilution approach. As the analysis of other yeast strains demonstrated, there were additional compounds possibly of relevance to the study of yeast metabolism. Some of these compounds were at high enough concentration to be included in the further testing of the method (i.e., palmitoleic acid and squalene), but others were present at levels that were too low to be included in the quantitation. By diluting the samples, the chance of detecting and possibly identifying and quantifying these low concentration compounds would be an issue. The natural variability of the matrix should already be considered at method development stages, especially in the case of biological samples and when there is not much information available in the literature with regard to the composition and/or concentrations. The application of a screening

method first is advisable, but not always possible. However, some targeted methods can also be used for screening purposes first when a new matrix is analyzed (in this study new yeast strains, but it can also be applied to grape juice or wine); retention indices from the literature would then have to be used for tentative identification before proceeding further.

In case the concentration ranges for compounds of interest varies greatly, and taking into account the dynamic range of the instrument, a combination of internal and standard addition calibration could be considered. A mixture of IS with the standards for the low concentration compounds would be added to the sample to fit into the calibration range with or without further dilution, based on the high level compounds already present. Even though not very practical for big sample sets, this approach might allow the quantification of minor lipids that are potentially of relevance for the yeast metabolism.

Even though the application included here was only a proof of concept, there were some interesting findings. *S. cerevisiae* is known to only produce monounsaturated fatty acids (oleic and palmitoleic acid) due to the absence of specific desaturases responsible for the biosynthesis of polyunsaturated acids, as confirmed in this study [58]. On the other hand, *K. marxianus* [31] and *T. delbrueckii* [59] are known to produce linoleic and linolenic acid with functions related to stress resistance, and this study confirmed the presence of linoleic acid and linolenic acid in all non-*Saccharomyces* yeasts even in SGM without the supplementation of linoleic acid (Figure 6). Moreover, it was observed that all yeasts were capable of taking up linoleic acid from the exogenous environment. However, lipid uptake varied between strains, as the highest amount of linoleic acid was observed in *M. pulcherrima,* whereas *S. cerevisiae* incorporated this fatty acid into its membranes the least highlighting differences in the metabolism and transport of lipids in yeasts. In a previous study, a high amount of squalene in the cell pellet was previously attributed to high ergosterol biosynthetic activity [59]. Thus, given the divergent fermentative performances of the yeasts in this study, it was assumed that these yeasts were at different physiological stages when samples were harvested for lipid analyses with *T. debrueckii* and *S. cerevisiae* being at a later stage of fermentation. From this perspective, further studies are necessary to evaluate the impact of lipids at defined physiological growth stages.

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