Paper C15

Gas Chromatography as a Tool for Quality Control in Production of Bovine Tallow Olein

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Abstract

This present work aims to present the results for the test and adaptation of an analytical methodology used in the characterization and quantification of major fatty acids of bovine tallow formers. This byproduct, which has its origin in the waste material processing meat in slaughterhouses, or waste from processing hides into leather, has high commercial value when fractionated in the form of olein (fraction composed of unsaturated fatty acids), being used as input for the production of fatliquoring emulsions for leather or grease to produce biodiesel. Analysis of fatty acid profile of tallow and products of fractionation was performed on the fractions of methyl esters formers of the samples through the use of gas chromatography. The results for the fractions of major fatty acids in tallow and products division, allows a quality assessment by the industry using these materials acids as raw material and features a reliable quantitative tool for the evaluation of efficiencies and optimization of the fractionation processes.
Keywords: olein, tallow, gas chromatography

1. Introduction

1.1. Bovine Tallow and its Fractionation

Tallow is the animal fat and its use is declined due to changing feeding habits of people and the soap industry cannot take up all the excess animal fat produced. It can be used for the production of biodiesel and other oleochemicals (Bhatti *et al.*, 2008).

This material is considered a byproduct of the slaughterhouse industry and tanneries. According to Aquim (2004), the quantities of solid waste generated in the pre-fleshing are from 6 kg per 26 kg of salted skin, approximately 23% (by weight) of raw material.

Apart the source of generation, the decomposition process of the hide and subcutaneous tissue is initiated by the bacterial action and enzymes from the time of slaughter of animals, leading to changes in the characteristics of tallow quality. The degradation of the material can be observed both in its color as the free fatty acids content. Thus, differences are observed in sensitive analytical parameters of quality, according to the difference in time from the slaughter of animals to the beginning of the tallow extraction.

The extraction of animal tallows is performed by cooking at low pressure steam for a short period of time, in which occurs the melting and partial emulsion of the constituents in water and separation from the solid phase by gravity or centrifugation. The fatty matter present in the subcutaneous tissue is composed of triglyceride (glyceride esters of fatty acids), whose presence or absence of unsaturated carbon chains determines many of its physical characteristics. According to Zheng and Hanna (1996), the major constituents found in bovine tallow are palmitic acid (16:0), stearic acid (18:0) and oleic acid (18:1).

Aiming at the production of high quality oils, such as low cloud point fraction in olein with high concentration of oleic acid and iodine number, is necessary to remove waxes and saturated triglycerides (solid at room temperature). Fractionation is a processing form in which the triglycerides formers of a crude oil are separated into fractions with different melting points and compositions. The fractionation process via "Winterization" aims to separate those higher melting point triglycerides, based on its precipitation in the form of crystals, in specific temperature and time. The process is a true fractional crystallization, in which three factors are fundamentally important in the nature and crystals formation, temperature, time and agitation (Cunha *et al*., 2002).

In the present work were evaluated the bovine tallow and the resulting current from their fractionation namely olein. The olein consists of triglycerides whose fatty acids are mostly unsaturated and have the oleic acid as the major component.

1.2. Gas Chromatography

The chromatography as analytical technique applied to triglycerides, was developed primarily for adulteration characterization of oils used in food industry. For example, the intentional adulteration of olive oil by the addition of soybean oil (which has a smaller value). There are several methods to determine physical and chemical properties of oils, but the complexity of the samples complicates the recognition and measurement of possible tampering (Pereira and Aquino, 1999).

According to Sleeter (1985), the chromatographic analysis in order to determine the oil composition by their triglyceride formers can be used in the fractionation monitoring esterification and blending of oils. The most common procedures adopt capillary columns for complex mixtures separation, the column construction is commonly made by fused silica, using a thin liquid film of dimethyl polysiloxane as the stationary phase. The detection and quantification of species are obtained by flame ionization (FID), whose cell is arranged transversely to the gas flow.

The analytical methodology development capable of determining the composition, in terms of fatty acids mass fractions of bovine tallow and the obtained currents after fractionation, can be regarded as a refinement of the work's analytical part. Concerns about the composition determination of the olein produced raised from the tanning industry are regarding the presence of natural fatty materials with high saturated triglyceride levels, causing the phenomenon known as grease efflorescence.

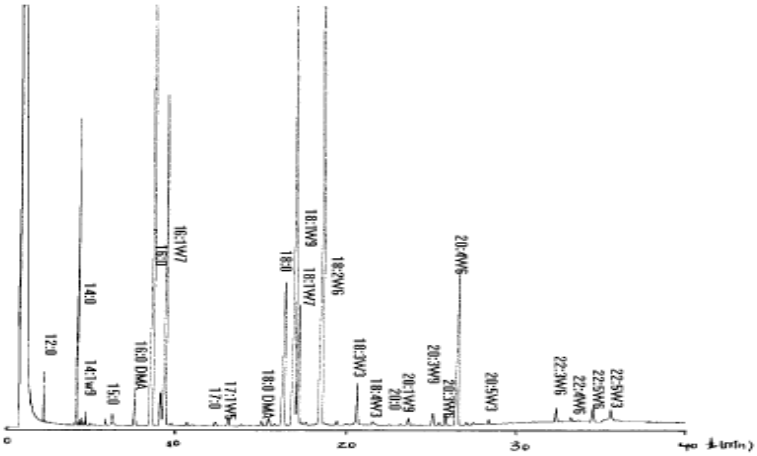
Gas chromatography is a technique for separation and analysis of mixtures composed of volatile substances. The sample is introduced and vaporized into an appropriate gas flow called mobile phase or carrier gas. The gas stream containing the vaporized sample passes through a tube containing the stationary phase (chromatographic column), where the separation of the mixture takes place. The rapid popularization of the technique is undoubtedly due to the simplicity of use, applicability for a wide range of situations and substrates combined with a remarkable separation capacity, particularly useful in complex mixtures analysis (Pereira and Aquino, 1999).

The stationary phase can be a solid adsorbent or, more commonly, a thin liquid film (liquid non-volatile) supported in an inert solid in granulated form or on the internal wall of a fused silica tube. In gas chromatography the two predominant factors in separating the constituents of a sample are: the interaction between the components of the sample (the greater the interaction of constituents with the stationary phase, the longer retention within the column) and the volatility (the more volatile substance, the faster it is vaporized and the greater its speed of passage through the system). Moreover, according to Wuilloud *et al.* (2004), the step of sample preparation is crucial for the specific compounds analysis in order to obtain accurate and reproducible results.

The mixture is eluted and separated in the carrier gas inside the column are continuously evaluated at

the end of the system by a detector (a device that generates an electrical signal proportional to the amount of material eluted). The record of the electrical signal as a function of time is called the chromatogram, a chart in which the substances are observed as peak potential with proportional areas to the mass fractions of mixture components. Figure 1 shows a typical chromatogram of a mixture of methyl esters of fatty acids present in tallow.

According to Moretto and Fett (1998), the gas chromatographic method can be applied to synthetic and natural mixtures of triglycerides and partial glycerides, fatty acid esters and lipid components that are less volatile, can be converted to volatile derivatives or pyrolyzed in reproducible manner, without the occurrence of decomposition.



Flame ionization detector, 280ºC; injector, 250 ºC, split ratio, 100:1; fused silica capillary column, DB-WAX (30m, 0,30 mm and 0.25 cm), column temperature: 150ºC for 11min, up to 210ºC at 3ºC/min.

Figure 1: Characteristic chromatogram of pig fat fatty acids

Source: Bragagnolo and Rodriguez-Amaya, 2002

2. Materials and methods

2.1. Tallow Samples and Olein

The beef tallow samples were collected at the company the Anabe Com. de Couros e Rep. Ltda.. These samples were used in the test and for adaptation of the analytical methodologies used to characterize the oils.

The Anabe oil refinery plant operates with two types of beef tallow, and the differentiation between them is made according to their origin, tallow from tanneries or from slaughterhouses. Currently, in view of the market price of tallow has increased significantly, some slaughterhouses have installed tallow extraction plants on its facilities. This tallow has superior characteristics to that obtained in tannery.

Measurements of acidity index were based on ABNT NBR 11115 - Determination of Acidity (1998). This measure is defined as the number of potassium hydroxide milligrams needed to neutralize the free fatty acids present in one gram of fatty material.

Analyses of saponification index were performed according to NBR 10448 - Determination of Saponification Index (2000). The saponification number is defined as the number of potassium hydroxide milligrams needed to saponify one gram of fatty matter.

The methodology used in the analysis of iodine number where based on the NBR 9231 -

Determination of Iodine Number (2002). The definition of iodine number is given as the number of halogen grams fixed per 100g of fatty matter, in the test conditions, expressed as iodine.

The first step in fatty acids quantification was the study of a methodology to crack the triglycerides and make a subsequent esterification of the resulting fatty acids with methyl alcohol. This methodology has a fundamental importance, since the determination of triglycerides in their original form would require very specific methodologies for their quantification. Gas chromatographic analyses linked to a mass spectrometer were performed to identify the major fatty acids present in beef tallow that were later quantified by gas chromatography with flame ionization detector.

The investigations of a chromatographic methodology able to quantify the relative mass fractions of the compounds present in these oils aims to enable the evaluation of the tallow fractionation efficiency of the processes. The obtaining refined olein (unsaturated fraction) with low percentage of saturated fatty acids is a condition imposed by the tanning industry that seeks to minimize the problems related to the fat efflorescence occurrence in leathers treated with natural oils by fatliquorig in wet finishing process.

2.2. Esterification Methodology

The methyl esters preparation was based on ASTM D 3457-91 - Standard Test Method for Preparation of Methyl Esters from Fatty Acids for Determination of Fatty Acid Composition by Gas-Liquid Chromatography, apart from works published by Sant'anna *et al.* (2001) and Baylin *et al.* (2006).

Esterification proceeded as follows: weighed at approximately 0.25 g of oil sample in flat-bottomed flask of 125 mL; added 6 mL of sodium hydroxide 0.5 N methanol solution and kept the solution refluxing for 20 minutes; added by the top of the condenser 7 mL of 20% boron trifluoride (BF_3) at reflux for 2 more minutes; added 5 mL of heptane by the top of condenser and reflux system for 1 minute; after cooling at room temperature, was dripped a small amount of saturated sodium chloride solution until the observation of two well-defined phases; withdrew the upper phase using a Pasteur pipette and wrap it in a test tube, discarding the lower phase; dried up the sample with a small amount of anhydrous sodium sulfate, and after, stored the samples in sealed vials under refrigeration for a maximum period of two days.

2.3. Fatty Acids Identification by GC-MS

The fatty acid composition analysis was performed using a gas chromatograph Shimadzu model GCMS - QP5050 A, coupled to a mass spectrometer (GC-MS). This preliminary analysis was necessary due to unfamiliarity of the compounds present and the difficulty in identification of the major compounds present in tallow samples.

The chromatographic column used was a capillary column DB-5, 30m length, 0.25 mm internal diameter and stationary phase of dimethyl-phenyl-polysiloxane with 0.25 mm thickness. The conditions were: injection split ratio 60:1; column temperature set to 80°C for 0.2 minutes, heating to 140°C at 8°C/min, isotherm at 140°C for 2 minutes, heating to 215°C at 8°C/min and remained at that temperature until the end of analysis; carrier gas helium at 5 mL/min; injector temperature at 210°C, detector temperature at 270°C. The samples were injected into the chromatograph at volumes of 2µL. The qualitative composition of compounds and identification of the majority species was performed by comparing retention times of peaks with standards of high purity fatty acid esters and confirmed through the internal library of the software Class 5000. The volume of the standards injected into the equipment was restricted to only the amount retained in the needle of the microsyringe filled with 1000 to 1200 ppm solutions.

From the results obtained was prepared a standard solution of fatty acid esters containing 200 ppm of

each species identified. The choice of such compounds was based on the peak areas of higher concentrations. This solution was used in chromatographic analysis in order to determine the compositions of the oils obtained in dry fractionation experiments.

2.4. Mass fractions of Fatty Acids

The relative composition of fatty acids present in oil samples was performed by area normalization and was expressed as mass percentages. This procedure was based in the work performed by Chiu *et al*. (2002), and ASTM D 5974-96 - Fatty and Rosin Acids in Tall Oil Fractionation Products by Capillary Gas Chromatography.

The mass percentage of tallow components and the fractions obtained after fractionation was performed using gas chromatography with flame ionization detector (GC-FID). Analyses were done by injecting previously prepared methyl esters in a chromatograph AutoSystem XL / GC, equipped with a capillary column PE-WAX (0.53 mm internal diameter, stationary phase 0.25, 30m long), Perkin -Elmer on the following operating conditions: Column temperature: 80°C for 4 minutes, heating to 170°C at 10°C/min, isotherm for 5 min, heating to 190°C at 2°C/min, isotherm for 5 min, and heating to 215°C at 2°C/min until the end of analysis. Detector temperature: 270°C. Injector temperature: 210°C. Carrier gas flow: Helium, 4mL/min. Split ratio: 40:1. Sample volume injected: 2µL.

The response factors (RF) calculation for each fatty acid present in the standard solution was obtained from equation 2.1 in order to calibrate the equipment. Although the concentrations of various fatty acids present in the standard solution being equal, the response factors show a variation due to chemical conformations and molecular mass of the compounds. Thus the correction of nonlinearity of response was required.

$$RF_i = \frac{m_i}{A_i} \tag{2.1}$$

in which:

RF_i = response factor relative to each fatty acid;
m_i = mass of each fatty acid present in the solution;
A_i = peak area of the fatty acid considered.

The mass fractions determination compensated by the response factors for each methyl ester peak was performed by calculating the percentage of the total area as shown in equation 2.2. Individual values of chromatographic peak areas were obtained through the use of software *Turbochrom 4.0*.

$$\%FattyAcid_i = \frac{A_i \times RF_i}{\sum A_j \times RF_j} \times 100 \tag{2.2}$$

in which:

$\%Fatty\ Acid_i$ = mass fraction of each methyl ester;
A_i = chromatographic peak area related to the interest methyl ester;
RF_i = response factor for the interest methyl ester;
A_j = peak area for each compound observed in the chromatogram;
RF_j = response factor for each methyl ester observed in the chromatogram.

It is worth to emphasize that the use of a standard solution for mass fraction determination is valid only in cases where the compounds concentration in the sample are similar to the standard solution and is not valid for individual quantification. If the interest was focused on the individual quantification would require the construction of calibration curves (internal or external standard technique) for each compound.

3. Results and discussion

3.1. Tallow and Olein Characterization

Table 3.1 presents the characterization results of the tallow used in this work. The tallow samples were characterized by the acidity index, saponification index and iodine number for comparison with the olein fraction obtained at the end of the fractionation process.

The acidity index results obtained are higher than expected for tallow from a slaughterhouse where very low values near 1 mg/g according to Cunha et al. (2002) would be expected. The other results are within or very close to the intervals mentioned by Bailey (1979) which are 40.2 to 49.5 for the iodine number and 196 to 199 for the saponification number values referred to North American bovine tallow. As the acidity index is a measure related to the degradation of tallow triglycerides two different situations can infer this characteristic: problems occurred during the hide storage (pré-fleshing waste with a high degree of degradation) and / or the extraction process employed makes uses very drastic conditions in its operation.

According to Keenan (1979) acidity indexes exceeding 6 makes unviable the utilization of tallow as a feedstock for fractionation plants due to the losses associated with the free fatty acids neutralization. Therefore it is necessary a more detailed assessment regarding the operating conditions of the extraction process, profits in extraction efficiency (more drastic conditions of the process) may result in loss of quality of tallow by its degradation.

Table 3.1: Bovine tallow characterization

Parameter	Results
Iodine Number (g /100g)	50,6
Saponification Index (mg/g)	188,06
Acidity Index (mg/g)	11,75

The characterization results of the unsaturated fat fraction generated from the dry fractionation process are presented in Tables 3.2. From these results a number of considerations can be made regarding its physical and chemical characteristics. One of the important control parameters in the evaluation of process variability which quantify the losses is the acidity index. Oils that present high values for the acidity index require large volumes of alkali neutralization which results in loss of product by the formation of fatty soaps that are eliminated by dissolution in water. There was also an increase in iodine number and saponification index which suggests an increase in unsaturated fatty acids the content.

Table 3.2: Olein characterization

Parameter	Results
Iodine Number (g /100g)	58,81
Saponification Index (mg/g)	199,39
Acidity Index (mg/g)	14,37

3.2. Fatty Acids Identification by GC-MS

The major fatty acids identification by GC-MS started by testing the more appropriate chromatographic conditions to the compounds separation, obtaining of a satisfactory tallow chromatogram in order to determine the compounds retention times and the compounds identification using the equipment library. From this, it was proceeded the preparation a standard solution with known fatty acids concentrations used in the quantification via GC-FID.

Figure 3.1 shows the tallow chromatogram obtained which were performed the major compounds

identification. Retention times referred to the compounds is presented in Table 3.3.

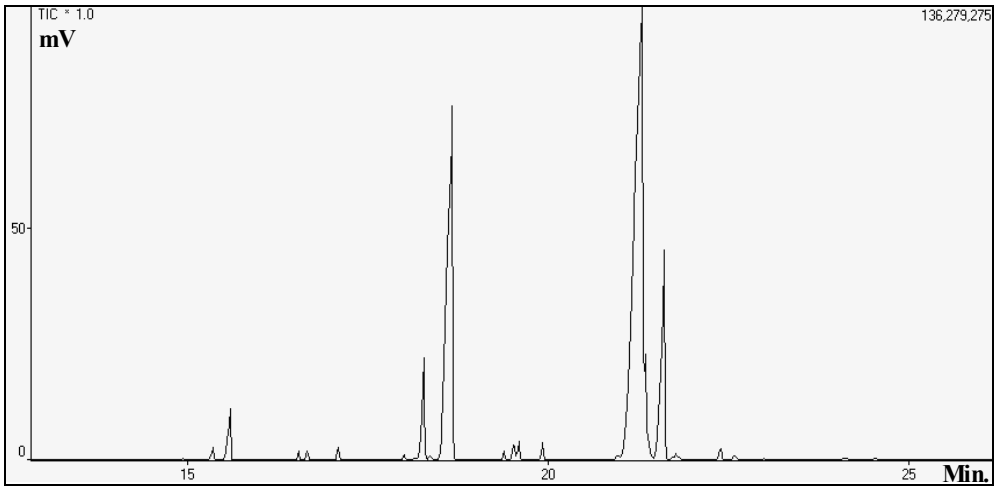


Figure 3.1: Mass chromatogram of bovine tallow

Table 3.3: Fatty acid methyl esters from bovine tallow and their retention times

Fatty Acid Methyl Ester	Retention Time (min)
C14:0 Methyl-Miristate	13,72
C16:1 Methyl-Palmitoleate	17,05
C16:0 Methyl-Palmitate	17,7
C18:2 Methyl-Linoleate	22,6
C18:1 Methyl-Oleate	23,13
C18:0 Methyl-Stearate	24,55

3.3. Results of Fatty Profile Analysis

Initially were calculated the response factors for each methyl esters present in the 200 ppm standard solution prepared previously in order to calibrate the equipment. It was observed that despite the equal concentrations of several esters the response factors show a variation due to compounds chemical conformations and its molecular weights. Table 3.4 presents the response factors obtained for the various methyl esters calculated from the average of three eluting procedures with individual area values of the chromatographic peaks obtained by employing the software Turbochrom 4.0. Figure 3.2 shows the chromatogram obtained for standard methyl esters solution containing 200ppm of each compound.

It can be observed from Figure 3.2 that the peak areas related to the compounds methyl palmitate, methyl oleate and methyl stearate are much higher than other peak areas. This behavior justifies the need of using a standard solution to determine the response factors for each compound of interest.

The results obtained by analyzing tallow samples as its fatty acid composition are presented in Table 3.5. Fatty acids with larger mass fractions are oleic acid, followed by palmitic and linoleic acids.

Table 3.6 presents the results obtained for the unsaturated fraction (olein) of bovine tallow after its dry fractionation. This process seems quite efficient in the removal of saturated triglycerides that have in their chain stearic acid as the main trainer. This behavior is not observed for the triglycerides that have in its constitution the palmitic acid which removal are much more difficult.

Table 3.4: Response factors of major fatty acid methyl esters considered

Fatty Acid Methyl Ester	Response Factor
C14:0 Methyl-Miristate	338,85
C16:1 Methyl-Palmitoleate	287,61
C16:0 Methyl-Palmitate	3357,63
C18:2 Methyl-Linoleate	838,72
C18:1 Methyl-Oleate	1339,40
C18:0 Methyl-Stearate	1290,55

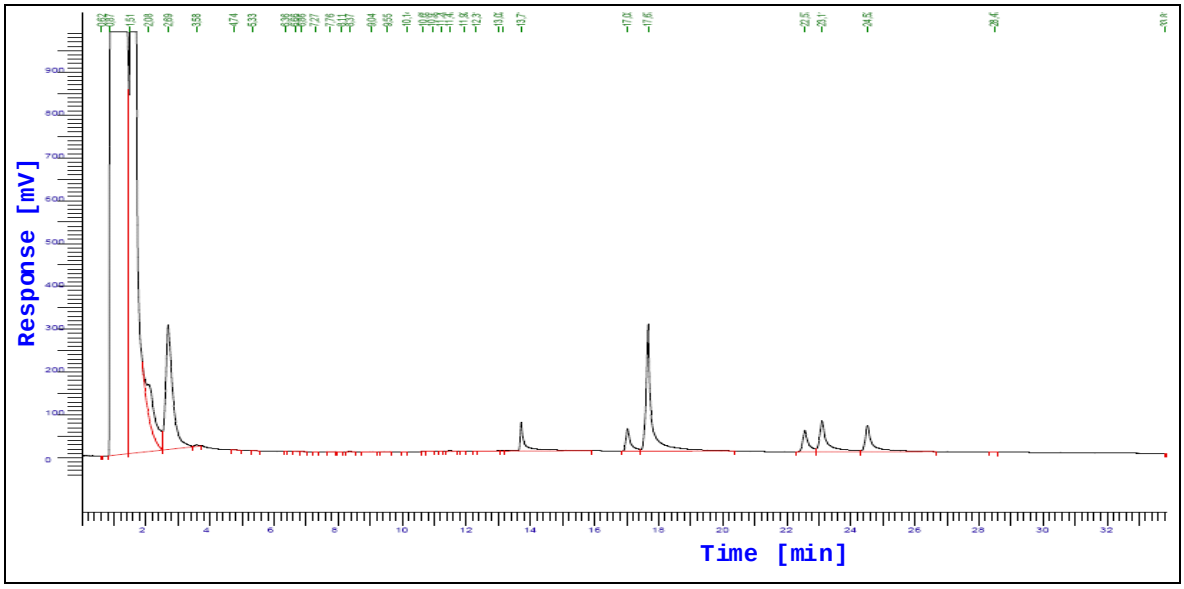


Figure 3.2: Chromatogram of 200ppm methyl esters standard solution

Table 3.5: Fatty acid composition of bovine tallow

Fatty Acid Methyl Ester	Mass Fraction (%)		Confidence Interval (95%)
C14:0 Methyl-Miristate	1,20	±	0,03
C16:1 Methyl-Palmitoleate	7,74	±	0,47
C16:0 Methyl-Palmitate	18,04	±	0,85
C18:2 Methyl-Linoleate	11,59	±	0,65
C18:1 Methyl-Oleate	55,89	±	0,68
C18:0 Methyl-Stearate	5,53	±	0,60
Σ Saturated	24,78	±	0,56
Σ Unsaturated	75,22	±	0,56

Table 3.6: Fatty acid composition of olein

Fatty Acid Methyl Ester	Mass Fraction (%)		Confidence Interval (95%)
C14:0 Methyl-Miristate	0,61	±	0,10
C16:1 Methyl-Palmitoleate	5,25	±	0,20
C16:0 Methyl-Palmitate	15,90	±	0,54
C18:2 Methyl-Linoleate	5,65	±	0,13
C18:1 Methyl-Oleate	71,45	±	0,96
C18:0 Methyl-Stearate	1,14	±	0,02
Σ Saturated	17,65	±	0,64
Σ Unsaturated	82,35	±	0,64

4. Conclusions

The chromatographic method developed for evaluating the quality characteristics of tallow and olein related to the presence of impurities (saturated triglycerides) was a very useful tool. This methodology when compared to other analytical methods has a much higher reliability and provides more specific data on the type and fractions of detected impurities. In other studies from Priebe and Gutterres (2011), the extraction/digestion of tallow assays were carried out to quantify the effect of factors such as the presence of agitation, temperature and extraction time in terms of the efficiency of the extraction operation and acidity index of the tallows obtained. The oils were characterized according to the acidity index, iodine index, saponification index, water content, volatile matter content and fractions of saturated and unsaturated fatty acids. The oil composition was quantified using gas chromatography.

5. References

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