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

by

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Abstract

This work presents the results of a Gas Chromatography methodology used to characterize and quantify the major fatty acids of bovine tallow. Tallow is a byproduct from processing waste meat in slaughterhouses and from tannery waste. Tallow has a high commercial value, especially when fractionated to olein that is predominantly composed of unsaturated fatty acids. Olein is used as a raw material for the production of leather fatliquoring emulsions or biodiesel production. The fatty acids of tallow and its products of fractionation were first converted to methyl esters, and these esters were then analyzed by Gas Chromatography. The results showed the major fatty acids and their weight distribution. This analytical methodology will allow a quality assessment of tallow by the end user industry. It is also a reliable quantitative tool to evaluate the efficiency and optimization of the tallow fractionation processes.

Resumen

Este trabajo presenta los resultados de una metodología de cromatografía de gases utilizados para caracterizar y cuantificar los principales ácidos grasos del sebo bovino. El sebo es un subproducto de la industria cárnica en los mataderos y de los residuos de curtiembres. El sebo tiene un alto valor comercial, especialmente cuando se fracciona en oleína, que está compuesto principalmente por ácidos grasos insaturados. Oleína se utiliza como materia prima para la producción de emulsiones de engrase de cuero o de producción de biodiesel. Los ácidos grasos de sebo y sus productos de fraccionamiento se convirtieron primero en ésteres metílicos y estos ésteres fueron analizados por cromatografía de gases. Los resultados mostraron cuales son los ácidos grasos principales y su distribución por peso. Esta metodología de análisis permitirá evaluar la calidad del sebo por la industria de usuaria final. También es una herramienta cuantitativa fiable para evaluar la eficiencia y la optimización de los procesos de fraccionamiento de sebo.

Introduction

Bovine Tallow and its Fractionation

Tallow is the animal fat. Its use is declined due to changing feeding habits of people and the inability of the soap industry to use the excess tallow produced. It can be used for the production of biodiesel and other oleochemicals.[1,2] This material is considered a byproduct of the slaughterhouse industry and tanneries. In tanneries, the quantities of solid waste generated in the pre-fleshing are about 6 kg per 26 kg of salted skin, approximately 23wt% of raw material.[3]

The decomposition of the hide and subcutaneous tissue that the fat is extracted is initiated by bacterial action and by enzymes from the time of slaughter of animals thus leading to changes in the characteristics of the tallow quality. The degradation of tallow can be observed both in its color change and its free fatty acids content. Thus, these changes can/are observed by sensitive analytical parameters of quality, as a function of the time from the slaughter of animals to the beginning of tallow extraction.[4] The extraction of animal tallow is performed by cooking the waste material with low-pressure steam for a short period of time so as to melt and partial emulsify the constituents in water. Tallow is then separated from the solid phase by gravity or centrifugation. The fatty matter present in the subcutaneous tissue is composed of fatty acid triglycerides, whereby the presence or absence therein of unsaturation determines many of its physical characteristics. The major constituents found in bovine tallow are palmitic acid (16:0), stearic acid (18:0) and oleic acid (18:1).[5]

The production of high quality oils from tallow requires that its olein fraction has a low cloud point, a high concentration of oleic acid, and lastly a high iodine value. This requires the prior removal of waxes and saturated triglycerides that are solid at room temperature by fractionation. Fractionation via "winterization" is a process whereby triglycerides in tallow are separated into fractions with different melting points and

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compositions. Winterization separates the high melting point triglycerides in the form of crystals at specific temperatures and time. Winterization is a true fractional crystallization in which three factors are fundamentally important over the nature and crystal formation: temperature, time and agitation of the oil.[6] The investigations of a chromatographic methodology were able to quantify the relative mass fractions of the compounds present in these oils and will enable the evaluation of the fractionation efficiency of the tallow processes. The refined olein unsaturated fraction containing a low percentage of saturated fatty acids is a condition imposed by the leather industry to minimize the alleged problems of fat efflorescence in leathers treated with fatliquors of natural oils.

Gas Chromatography

Chromatography as an applied analytical technique for triglycerides was primarily developed to characterize adulterated oils in the food industry. An example is the adulteration of olive oil with soybean oil of lesser commercial value. There are several methods to determine physical and chemical properties of adulterated oils, but the sample complexity difficult the recognition and measurement of possible tampering.[7] The chromatographic analysis used to determine the oil composition by their main triglycerides can be used in the fractionation, the monitoring of esterification and blending of oils. The most common procedures adopt capillary columns for the separation of complex mixtures. The column construction is commonly made of 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), with its detecting cell arranged transversely to the gas flow. [8] The analytical methodology developed and presented in this paper can be regarded as a very convenient tool when a fast and efficient evaluation of fractionation processes or oil quality needs to be held. This testing method will help the leather industry manage its concern about the composition of olein. The presence of highly saturated triglyceride levels in olein is believed tanners to cause the phenomenon known as grease efflorescence. Recently, Wang *et al*.[9] have shown that the predominant components of fatty spew were methyl esters of fatty acids and saturated species were the predominant culprit of efflorescence or fatty spew on leather.

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 the 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 popularity of this technique is undoubtedly due to the simplicity of use, and its applicability to a wide range of situations and substrates combined with a remarkable utility for complex mixtures analysis.[7] The stationary phase can be a solid adsorbent or, more commonly, a non-volatile thin liquid film supported on 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*.,[10] the prior step of sample preparation is crucial for the specific compounds analysis in order to obtain accurate and reproducible results.[10] The mixture is eluted and separated in the carrier gas inside the column are then continuously evaluated at its end by a detector which generates an electrical signal proportional to the amount of material eluted. The plot 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.

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, or converted to volatile derivatives or pyrolyzed in a reproducible manner, without the occurrence of decomposition.[11]

Material and Methods

Tallow Samples and Olein

The beef tallow samples were collected at the Anabe Com. de Couros e Rep. Ltda. Company. These samples were used in the tests and for adaptation of the analytical methodologies used to characterize the oils. The Anabe oil refinery plant uses beef tallow from tanneries and from slaughterhouses. Nowadays, slaughterhouses are installing tallow extraction on location because the current market price of tallow has significantly increased and became profitable. Tallow from slaughterhouses has superior commercial characteristics than tallow obtained from tanneries. Measurements of acidity index were based on ABNT NBR 11115.[12] This measure is defined as the weight of potassium hydroxide in milligrams needed to neutralize the free fatty acids present in one gram of fatty material. Analyses of saponification index were performed according to ABNT NBR 10448.[13] The saponification Index is defined as the weight of potassium hydroxide in milligrams needed to completely hydrolyze one gram of fatty matter. The methodology used in the analysis of iodine value were based on the ABNT NBR 9231.[14] The definition of iodine value is given as the weight of this halogen in grams fixed per 100g of fatty matter, in the test conditions.

Samples Preparation

The first step used to quantify the fatty acids of tallow was to hydrolyze all glycerides and in a subsequent step esterify the resulting fatty acids with methyl alcohol. This aforementioned methodology is fundamentally important, because the determination of triglycerides in their original form requires very specific and complex methodologies. Analyses by gas chromatography-mass spectrometry (GC-MS) were performed to identify the major methyl esters obtained, representing the major fatty acids from beef tallow. Afterward the methyl esters were also quantified by gas chromatography equipped with a flame ionization detector (FID).

Esterification Methodology

The preparation of the methyl esters of fatty acids was based on ASTM D 3457-91[15], apart from works published by Sant'anna *et al.*[16] and Baylin *et al.*[17] Esterification proceeded as follows: weighed approximately 0.25 g of oil sample in a flat-bottomed flask of 125 mL; added 6 mL of 0.5 N sodium hydroxide methanolic solution and kept the solution refluxing for 20 minutes; added at the top of the condenser 7 mL of 20% boron trifluoride (BF_3) at reflux for 2 more minutes; added 5 mL of heptane at the top of condenser and reflux system for 1 minute; after cooling at room temperature, a small amount of saturated sodium chloride solution was dripped until two well-defined phases were observed; withdrew the upper phase using a Pasteur pipette and wrapped it in a test tube, discarding the lower phase; dried up the sample with a small amount of anhydrous sodium sulfate, and after, storage the sample was sealed in a vial under refrigeration for a maximum period of two days.

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 micro syringe filled with 1000 to 1200 ppm solutions (in heptane). 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 (winterization).

Mass fractions of Fatty Acids

The relative composition of fatty acids, identified previously 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.*[18], and ASTM D 5974-96.[19]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 the equation in order to calibrate the equipment. Although the concentrations of various fatty acids present in the standard solution were equal, the response factors showed a variation due to chemical conformations and molecular mass of the compounds. Thus the correction of nonlinearity of response was required.

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

Where: RF_i = response factor relative to each fatty acid; m_i = mass of each fatty acid present in the solution and; 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. Individual values of chromatographic peak areas were obtained through the use of software *Turbochrom 4.0*.

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

Where: $\%Fatty\ Acid_i$ = 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 this would require the construction of calibration curves (internal or external standard technique) for each compound.

RESULTS AND DISCUSSION

Tallow and Olein Characterization

Table I presents the characterization results of the tallow used in this work. The tallow samples were characterized by the acidity index, saponification index and iodine value for comparison with the olein fraction obtained at the end of the fractionation process. The acidity index results obtained were higher than expected for tallow from a slaughterhouse where very low values near 1 mg/g, would be expected.[5] The other results are within or very close to the ranges mentioned by Bailey which are 40.2 to 49.5 for the iodine value and 196 to 199 for the saponification index, values referred to North American bovine tallow.[20] As the acidity index measures the degradation of tallow triglycerides two different situations can cause this characteristic problem. It occurred during the hide storage (pre-fleshing waste with a high degree of degradation) and/or the extraction process employed used very drastic conditions in its operation. Acidity indexes exceeding 6 make unviable the utilization of tallow as a feedstock for fractionation plants due to the losses associated with the free fatty acids neutralization.[21] Therefore it is necessary to have 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 I
Bovine tallow characterization

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

The characterization results of the olein unsaturated fraction generated from the dry fractionation or "winterization" process are presented in Table II. 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/emulsion in water. There was also an increase in iodine value and saponification index; both of which suggested an increase in unsaturated fatty acids content.

TABLE II
Olein characterization

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

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, obtained from satisfactory tallow chromatogram in order to determine the compounds retention times and the compounds identification using the equipment library. Following this, we proceeded to prepare a standard solution with known fatty acids concentrations used in the quantification via GC-FID. Figure 1 shows the tallow chromatogram used for the major compounds identification. Retention times referred to the compounds is presented in Table III.

Results of Fatty Profile Analysis

Initially the response factors for each methyl esters present were calculated 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 showed a variation due to the compounds chemical conformations and their molecular weights. Table IV 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 2 shows the chromatogram obtained for standard methyl esters solution containing 200ppm of each compound. It can be observed from Figure 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 V. Fatty acids with larger mass fractions are oleic acid, followed by palmitic and linoleic acids. Table VI presents the results obtained for the olein unsaturated fraction of bovine tallow after its dry fractionation. This process seems quite efficient in the removal

TABLE III
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

TABLE IV
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

TABLE V
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
_Saturated	24,78	±	0,56
_Unsaturated	75,22	±	0,56

TABLE VI
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
_Saturated	17,65	±	0,64
_Unsaturated	82,35	±	0,64

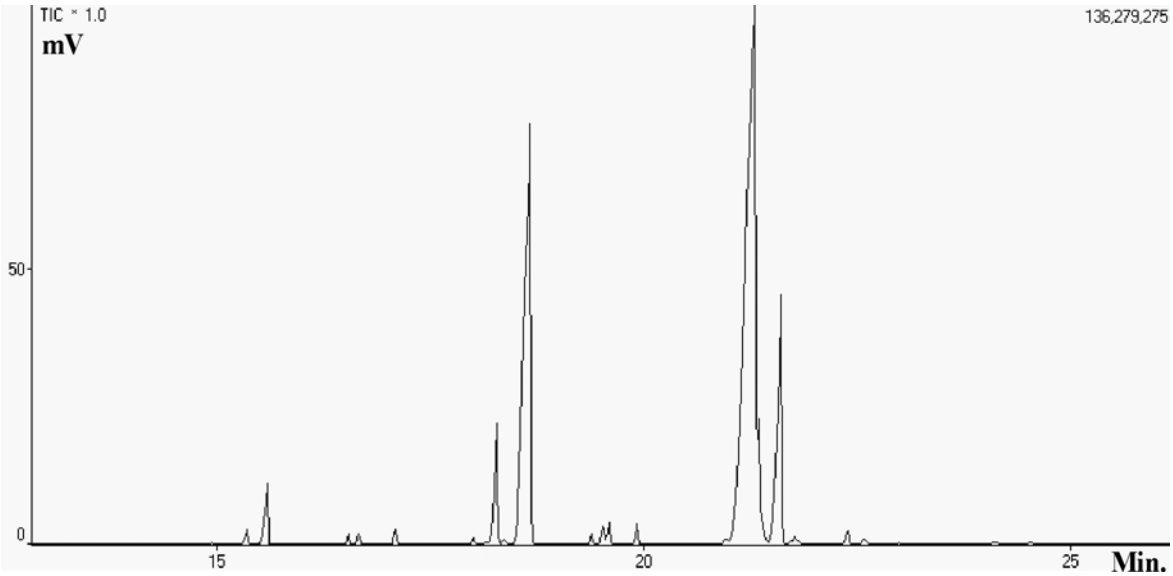

Figure 1. Mass chromatogram of bovine tallow

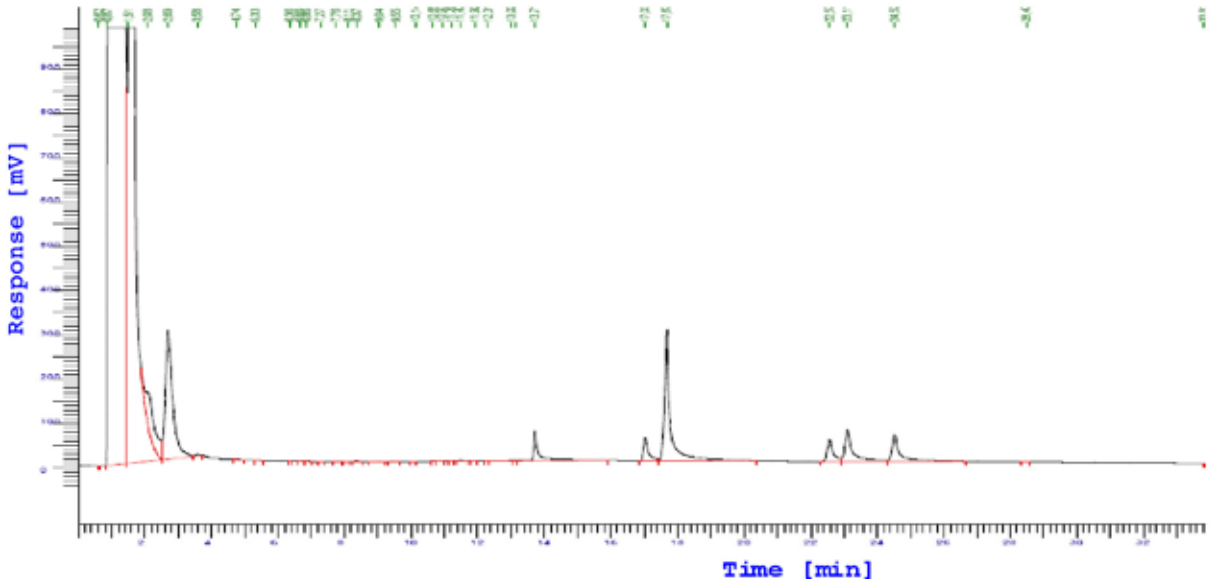

Figure 2. Chromatogram of 200ppm methyl esters standard solution

of saturated triglycerides that have in their chain stearic acid as the main component. This behavior was not observed for the triglycerides that have in its composition palmitic acid, the removal of which are much more difficult.

CONCLUSIONS

A Gas Chromatography method and methodology were developed to evaluate the quality characteristics of tallow and olein. It was found to be a very useful tool in identifying and quantifying the saturated triglyceride impurities of concern to the tanning industry regarding spew. When compared with other analytical methods, this methodology had a much higher reliability and provided more specific data on the type and fractions of detected impurities.

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