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Study of the GC–MS determination of the palmitic–stearic acid ratio for the characterisation of drying oil in painting: *La Encarnación* by Alonso Cano as a case study

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ABSTRACT

The correct identification of drying oils plays an essential role in providing an understanding of the conservation and deterioration of artistic materials in works of art. To this end, this work proposes the use of peak area ratios from fatty acids after ensuring that the linear responses of the detector are tested. A GC–MS method, previously reported in the literature, was revisited to its developed and validated in order to identify and quantify of eight fatty acids that are widely used as markers for drying oils in paintings, namely myristic acid ($C_{14:0}$), palmitic acid ($C_{16:0}$), stearic acid ($C_{18:0}$), oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$), suberic acid ($2C_8$), azelaic acid, ($2C_9$) and sebacic acid ($2C_{10}$). The quaternary ammonium reagent *m*-(trifluoromethyl)phenyltrimethylammonium hydroxide (TMTFAH) was used for derivatization prior to GC–MS analysis of the oils. MS spectra were obtained for each methyl ester derivative of the fatty acids and the characteristic fragments were identified. The method was validated in terms of calibration functions, detection and quantification limits and reproducibility using the signal recorded in SIR mode, since two of the methyl derivatives were not totally separated in the chromatographic run. The proposed method was successfully applied to identify and characterise the most widely used drying oils (linseed oil, poppy seed oil and walnut oil) in the painting *La Encarnación*. This 17th century easel painting is located in the main chapel of the cathedral in Granada (Spain) and was painted by the well-known artist of the Spanish Golden Age, Alonso Cano (1601–1667).

1. Introduction

The characterisation of drying oils used in paintings has always been one of the most important goals in conservation sciences. Drying oils such as poppy seed, linseed and walnut have been used alone or mixed as a binder medium throughout much of history. The number and kinds of drying oils used in a painting depend on the school of painting, the time period, and the painting technique employed by the artist. Therefore, their identification provides crucial information not only for art historians, but for effective and secure restoration and conservation purposes.

Drying oils are composed of triglycerides dried by both photo-oxidation and cleavage/decomposition of the unsaturated fatty acid through complex processes in the glycerides to create interchain bonds forming a cross-linked structure. Additionally, ageing involves the loss of unsaturated monocarboxilic acids and the concurrent formation of dicarboxilic acids, especially azelaic acid [1]. The resulting fatty acid composition has been used to discriminate between the different types of drying oils (linseed, walnut and poppy) from films of dried paint. Currently, one of the most widely used approaches for the identification of drying oils is based on the palmitic to stearic acid (C_{16}/C_{18}) ratio in the artistic sample estimated by means of gas chromatographic analysis [2]. This ratio can be used to identify each type of drying oil and is not affected by the ageing process. The azelaic to palmitic acid ($2C_9/C_{16}$) ratio and the total dicarboxylic acid content (azelaic, suberic and sebacic) also indicate the presence of drying oils in paintings [1] nevertheless they can be used to test for the presence of proteinaceous binder media such as egg or "tempera grassa" as stated Colombini et al. [3].

The analysis of drying oils in the field of art has been approached with several analytical techniques [4,5], however most of the proposed methods are based on gas chromatography (GC) or gas chromatography combined with mass spectrometry (GC–MS)

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[6–10]. In all cases, the treatment of drying oils – whether aged or not – prior to their analysis by GC or GC–MS requires the hydrolysis of the sample followed by the derivatization of the fatty acids to increase their volatility, in order to convert into the methyl ester derivatives (FAMEs). A variety of sample preparation procedures have been described in the literature with this aim, such as silylation [11], reaction with alkyl chloroformates [6,7,12] and the transesterification of the triglycerides [13–17]. Transesterification reactions are mainly used as one-step techniques. Different *N*-tetraalkylammonium hydroxide type reagents have been used for direct transesterification, namely *N*-tetramethylammonium hydroxide (TMAH) [15], trimethylsulfonium hydroxide (TMSH) [16] and *m*-(trifluoromethyl)phenyltrimethylammonium hydroxide (TFTMAH) [13,14,17,18]. The last of these reagents claims to have produced results superior to the more widely used TMAH, because it requires a lower reaction temperature when used in a direct thermochemolysis process and does not produce unwanted reactions [15].

The objective of this work is twofold: the development of a validated GC/MS method for the analysis of drying oils in paintings and its application to paintings by the fine Spanish Baroque artist, Alonso Cano.

This study revisits the direct procedure for transmethylation using methanolic *m*-(trifluoromethyl)phenyltrimethylammonium hydroxide (TFTMAH) previously reported in the literature [18], since, to the best of our knowledge, it was not entirely validated in terms of linearity, detection and quantification limits and reproducibility, as is currently required. Once the method was validated, it was applied to calculate the fatty acid concentration and, therefore, the above-cited ratios for the identification of drying oils.

It has recently been shown [19] that in the case of nonlinear GC instrument responses, C_{16}/C_{18} ratios depend on sample dilution. This fact can hinder proper drying oil identification and lead to erroneous interpretation. Hence, in the present paper the linear response of the detector was first confirmed at different concentration levels and the C_{16}/C_{18}, and $2C_9/C_{16}$ ratios expressed as area and concentration values, were obtained and evaluated.

The validated method for identifying drying oils was then successfully applied to analyse samples from a painting by Alonso Cano (Granada, Spain, 1601–1667). This painter is considered one of the most original and brilliant artists from the so-called Spanish Golden Age (17th century) and the founder of the school of Baroque painting in Granada. There, he began the series the Life of the Virgin, to which the painting studied in this work belongs. The enormous canvas (4.52 m × 2.52 m) that holds *La Encarnación* is part of the iconographic program about the life of the Virgin that Cano painted for the main chapel of the Granada cathedral, intended to be placed at a great height. The characterisation of the drying oil in this work is part of a larger project whose purpose is to identify and characterise the materials used in paintings and sculptures by Cano [20–22]. The study also intends to contribute to the understanding of his artistic technique, taking into account both the evolution of his painting style and the reconstruction of Cano's renowned palette.

2. Experimental

2.1. Reagents and solutions

The organic solvents toluene and methanol were supplied by Sigma (Sigma–Aldrich Química S.A., Madrid, Spain). The transesterification reagent of fatty acids was *m*-(trifluoromethyl) phenyltrimethylammonium hydroxide (TFTMAH) (known as Meth Prep II), supplied by Alltech (Alltech Associates, Inc., Belgium).

As fatty acid standards, all supplied by Alltech, mixtures of monosaturated acids (myristic ($C_{14:0}$), palmitic ($C_{16:0}$), stearic ($C_{18:0}$)), C18 insaturated fatty acids (oleic ($C_{18:1}$), linoleic ($C_{18:2}$)) and dicarboxilic acids (suberic ($2C_8$), azelaic ($2C_9$), sebacic ($2C_{10}$)) were used. Individual fatty acid standards (also Alltech) were examined in order to characterise their chromatographic retention data and to compile a mass spectra database for the fatty acids as methyl esters (FAMEs). The internal standard used was tridecanoic acid (Alltech). Stock solutions of each individual fatty acid and their mixtures in methanol (1000 μg g^{-1}) were kept at 4 °C.

The chemicals used were of analytical-reagent grade and all aqueous solutions were prepared using reverse-osmosis type quality water produced by a Milli-RO 12 plus Milli-Q purification system (Millipore, Bedford, MA).

2.2. Paint samples

2.2.1. Homemade paint samples

The drying oil used to prepare the homemade paint samples was linseed oil (boiled linseed oil) purchased from Talens (Apeldoorn, Holland). Rabbit skin glue (collagen) was purchased from Sigma. Chicken eggs were bought in a local supermarket. The egg glair and yolk were separated by the usual method of pouring the egg contents back and forth in the half shells. Then the yolk was rolled on a paper tissue to remove the layer of clinging egg white and most of the chalazae and then transferred to a jar. Then, the skin was punctured at the bottom by a pin and the liquid content poured into the jar [23].

Following the available literature [24], four kinds of model samples were prepared using the traditional recipes for the pictorial layer used by Cano: linseed oil alone (L), a 1:1 mixture of linseed oil and gypsum (L+G), 1.5:3 linseed oil blended with egg yolk (L+E), and finally, 1:1 linseed oil with rabbit glue (L+RG). These were uniformly spread on 45 mm × 20 mm glass slides to obtain thin films that were left to dry in daylight at room temperature in the laboratory for a period of fifteen weeks. Three replicates of each model sample were used as the reference standards for analysis.

2.2.2. Historical paint samples

For the memorial exhibition celebrating the 4th anniversary of Cano's birth held in Granada in 2001 [25], the seven paintings belonging to the Life of the Virgin cycle, including *La Encarnación* (Fig. 1), were brought down from the cathedral's main chapel and subjected to a restoration process. In cooperation with the restorer, we took microsamples from different places and in areas with different colours that were analysed to characterise the lipid binder using the proposed method.

2.3. Analytical procedures

2.3.1. Transesterification procedure

Small amounts of paint sample (0.05–0.10 mg) were introduced in a microvial with conical inserts. Each sample was treated with 15 μL of 0.2 M methanolic solution of TFTMAH and 200 μL of toluene, in order to prepare FAMEs by transesterification of the triglycerides present. The mixture was sonicated for 30–40 min followed by centrifugation at 2000 rpm for 3 min. The supernatant solution was transferred to a vial with conical inserts for injection into the gas chromatograph and to perform the gas chromatographic analysis.

2.3.2. Chromatographic and mass detection procedure

A Varian CP 3800 GC equipped with a CTC Analytics Combi-PAL autosampler and automatic injector and split/splitless injection portal was used. The chromatographic separations were done on a VF-5 MS Highly inert Varian capillary column (5% phenyl-methyl

Fig. 1. *La Encarnación* by Alonso Cano.

low bleed; 30 m × 0.25 mm i.d. × 0.25 μm d_f), interfaced to a Mass Detector Varian Saturn 2200 (Lake Forest, CA, USA). The GC inlet temperature was 240 °C and the MS interface 280 °C. The oven was programmed from 120 °C, with a 2 min hold, and then increased at 5 °C/min to 230 °C; the total run time was 24 min. The inlet was operated in splitless mode, with a split ratio of 50 with 2.10 purge-on time. Helium was the carrier gas (≥99.9995%) with a constant flow of 1.0 mL min^{-1}. The MS was run in selected ion mode (SIM), with the selected ions cited in Table 1. When the MS ran in scan mode, the *m*/*z* range was 45–400 *m*/*z*. The trap temperature was set at 200 °C. Electron ionisation energy was 70 eV, with a maximum ionisation time of 25,000 μs. Data were processed using Saturn GC/MS Workstation version 6.41. After the derivatization step, a GC/MS analysis was performed. For this purpose, 2 μL of solution (supernatant) was injected into the GC at an inlet temperature of 240 °C.

Table 1
Retention time (t_R), FAMEs (MW) and principal ions (*m*/*z*).

t_R (min)	FAMEs (MW)	*m*/*z*
8.8	$2C_8$ (202)	55,**69**,83,138,171
10.9	$2C_9$ (216)	**55**,83,152,168,185
13.1	$2C_{10}$ (230)	**55**,83,97,125,166,
14.8	$C_{14:0}$ (242)	55,**74**,115,129,143
18.8	$C_{16:0}$ (270)	55,**74**,143,227
22.0	$C_{18:2}$ (294)	**67**,81,95,135,149,164
22.2	$C_{18:1}$ (296)	**55**,37,166,264
22.6	$C_{18:0}$ (298)	55,**74**,143,255

3. Results and discussion

3.1. Derivatization process

Although the transesterification procedure chosen was previously reported by Robb and Westbrook [18], this study has taken it up again in order to optimise their performance. The methylation reagent *m*-(trifluoromethyl)phenyltrimethylammonium hydroxide (TFTMAH) was chosen because it gives direct methylation of glycerides with minimal sample manipulation. This is an important consideration for artwork samples, since they are necessarily very small and typically very complex. Fatty acids bound in the form of triglycerides esters from drying oils are converted into *m*-(trifluoromethyl)phenyltrimethylammonium salt, to yield fatty acid methyl esters (FAMEs) [16].

Despite the methanolic TFTMAH reagent was successfully used in earlier works [14,16–18,26], experimental investigations were made in order to establish the optimal amount of reagent to be used and also to obtain knowledge about the stability of the FAMEs derivatives.

The extension of the derivatization reaction mainly depends on the percentage of methanolic solution of TFTMAH mixed with benzene or toluene that is added to the reagent solution to facilitate the solubilisation of non-polar analytes [26]. Thus, the percentage of the methanolic solution of TFTMAH was tested from 2.5% to 15% in order to establish the best experimental conditions. With that aims, a mixture standard solution in methanol containing 30.0 μg g^{-1} of each fatty acid studied was prepared. Aliquots (100.0 μL) of this solution were introduced in 2 mL vial with conical inserts, increased volumes of TFTMAH from 5 to 30 μL (i.e. 5, 10, 15, 20, 25 and 30 μL) and the needed volume of toluene to have a final volume of the derivatization mixture of 200 μL were added. The mixture

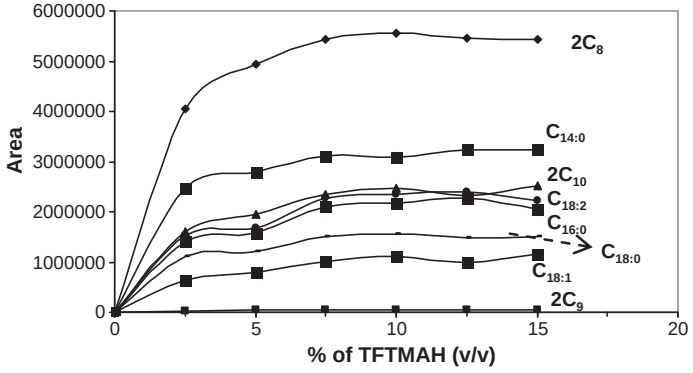

Fig. 2. Yield of the transesterification reaction of the fatty acids.

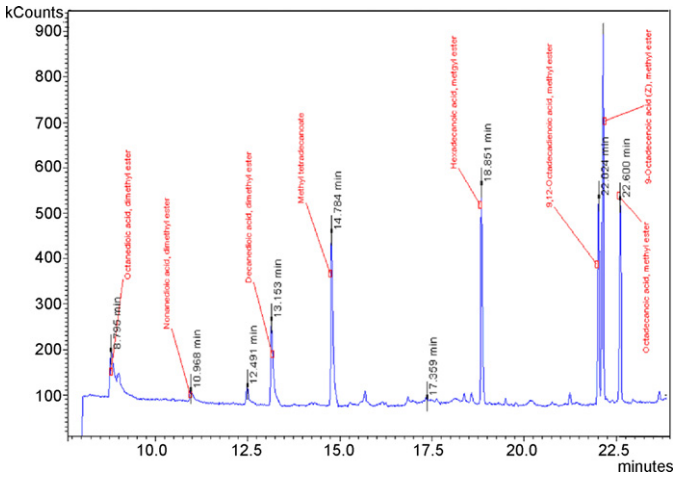

Fig. 3. Chromatogram of a standard mixture of the FAMEs.

was sonicated, centrifugated and injected into the chromatograph as indicated in the experimental section. The results of this experience are showed in Fig. 2. It can be seen that no improvements were obtained when percentages larger than 7.5% of TFTMAH were used. Thus, 15 μL of the methanolic solution of TFTMAH and 185 μL of toluene were selected and used in all the studies, since they provided the best results for all the studied FAMEs.

The stability of the FAMEs derivatives was tested by analysing them periodically from their preparation up to 280 min. For this, a set of methanolic solutions containing 10.0 $\mu g\,g^{-1}$ of each fatty acid was prepared and derivatized. After that, they were periodically GC–MS analysed as indicated in the experimental section. No significant variations in the corresponding concentration or significant tendencies were observed for any of the FAMEs studied. The variation in the concentration observed expressed as the variation coefficient (%) was between 1% (for C18) and 10% (for $2C_9$). Thus the stability of the derivatives was corroborated as being at least 280 min from their preparation. That meant that no precautions regarding the time after the derivatization step and before the GC–MS analysis had to be taken.

3.2. GC–MS method development

The starting point for the development of the GC–MS method was to obtain knowledge about the chromatographic behaviour and the mass spectrum of each of the FAMEs. Consequently, individual fatty acid standard solutions were prepared and analysed after applying the derivatization procedure, and the retention data and mass spectrum of each of the FAMEs were thus characterised.

The chromatographic separation was optimised using a standard mixture solution of fatty acids 1.0 $mg\,L^{-1}$ each. The FAMEs of the eight fatty acids were analysed within 24 min using a non-polar phenyl-methyl low bleed column under the optimised GC conditions. The best separation was achieved at an initial temperature of 120 °C, holding for 2 min, then ramping at 5 °C min^{-1} to a final temperature of 230 °C. The elution order of the FAMEs in a standard mixture sample is shown in the chromatogram in Fig. 3. As expected, the methyl derivatives of the dicarboxylic fatty acids were eluted first because of their higher polarity. Also as expected, the retention time increased with the number of carbons in the FAMEs, and the last compound to be eluted was the methyl derivative of the C18. As cited in the bibliography [27,28], the most difficult region of separation was C18:1 and C18:2. Nevertheless, it was not necessary to achieve a complete chromatogram peak resolution since the analytical signal was obtained by monitoring the base peak of the FAMEs (SIR mode).

The use of a mass detector to obtain the analytical signal provides a powerful combination of sensitivity and identification capability. Its advantages are particularly notable when analyzing complex samples, such as those from the field of cultural heritage. In this study, the mass spectrum of each of the FAMEs was obtained and several characteristic ions were identified (Table 1). These ions can be further used for purposes of identification when analyzing real paint samples. In all cases, the ion selected for further analytical analysis was the most sensitive one, that is, the base peaks from the mass spectra of the FAMEs. In this way, good sensitivity and prevention against interference were provided. The ion, **69** *m*/*z* (base peak) was chosen for $2C_8$; **55** *m*/*z* (base peak) for $2C_9$; **55** *m*/*z* (base peak) for $2C_{10}$; **74** *m*/*z* (base peak) for $C_{14:0}$; **74** *m*/*z* (base peak) for $C_{16:0}$; **67** *m*/*z* (base peak) for $C_{18:2}$; **55** *m*/*z* (base peak) for $C_{18:1}$; and **74** *m*/*z* (base peak) for $C_{18:0}$. The mass detector response differed significantly among the fatty acids analysed. The derivative from $C_{18:1}$ was the most abundant peak, followed by the C_{16} derivative, $C_{18:2}$ derivative, C_{18} derivative, C_{14} derivative, $2C_{10}$ derivative, $2C_8$ derivative, with the $2C_9$ derivative being the least abundant peak.

A typical chromatogram obtained in the conditions described above is shown in Fig. 4. This chromatogram corresponds to a real paint microsample extracted from a yellow part of the carpet area of the painting. By applying the proposed method, it was possible to satisfactorily separate the eight methyl derivatives from the main fatty acids usually present in glycerides from old oil paint samples in 24 min, providing useful retention times without any interference from the other peaks of the chromatogram. MS spectra were obtained for each compound and the characteristic fragments were subsequently identified.

3.3. Analytical method validation

Despite the fact that the GC–MS analysis of fatty acids in paint samples has been proposed before [18], to the best of our knowledge, the validation of the methods following the current recommendations of the Analytical Methods Committee [29] has not been provided to date. Thus, a validation in terms of linearity, detection limit and quantification, and reproducibility was carried out.

The mass spectra were measured in the single ion monitoring mode (SIM or SIR) for all quantitative purposes. Thus, the analytical parameters of the method were obtained from the registered chromatograms, monitoring the most abundant peak (base peak) of each of the FAMEs (Table 1). Ten concentration levels (from 0.5 $\mu g\,g^{-1}$ to 300.0 $\mu g\,g^{-1}$) with four replicates each were initially used to characterise the calibration functions. With this aims, methanolic solutions of the fatty acids were prepared and analysed as indicated in the experimental section. It was graphically corroborated the clear lose of the linearity for the higher concentration levels tested. Thus, the next step focused on a closer study of the smaller concentration interval i.e. 0.5–100.0 $\mu g\,g^{-1}$. The linearity of these functions was well defined by applying the lack-of-fit test. Finally, the calibration functions were satisfactorily established for each FAMEs. The results for this study, including the linear dynamic range, intercepts (*a*), slopes (*b*), correlation coefficients (*R*) and the probability levels of the lack-of-fit test (*P*, %) are summarised in Table 2.

The results were indicative of good linearity (higher than 97%) within the range of 2.0–50.0 $\mu g\,g^{-1}$ for $2C_8$, $2C_9$, $2C_{10}$, $C_{18:1}$ and $C_{18:2}$ and within the range of 1.0–50.0 $\mu g\,g^{-1}$ to $C_{14:0}$, $C_{16:0}$ and $C_{18:0}$ with a correlation coefficient higher than 99.4% (except for $2C_9$, which was 96.8%) and probability levels higher than 48.8%. Linearity was accepted if the *P*-value of the corresponding lack-of-fit test was greater than 5% according to the guidelines of the Analytical Methods Committee [29]. Thus, the results demonstrated the

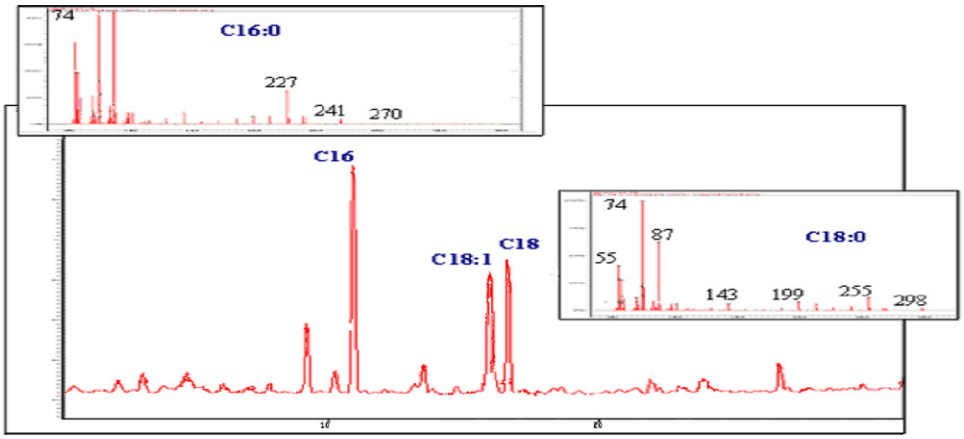

Fig. 4. Chromatogram from the yellow sample of the carpet area (*La Encarnación*).

Table 2
Analytical parameters of GC–MS proposed method for fatty acid derivatives.

Analytical parameters	Fatty acid derivative							
	$2C_8$	$2C_9$	$2C_{10}$	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$
Linear dynamic range ($\mu g\,g^{-1}$)	2–50	2–50	2–50	1–50	1–50	1–50	2–50	2–50
Intercept	−0.357	−0.100	−0.009	−0.021	−0.057	−0.028	−0.025	−0.204
Slope	0.352	0.042	0.034	0.087	0.088	0.075	0.046	0.205
R	0.9985	0.9688	0.9989	0.9986	0.9945	0.9967	0.9990	0.9988
[a] *P* (%)	65.5	48.8	68.9	84.2	65.3	52.6	60.9	78.7
Reproducibility (CV, %)	6.3	6.2	6.2	5.6	5.3	4.6	4.2	5.1
DL ($\mu g\,g^{-1}$)	1.9	2.0	1.8	0.9	0.9	1.0	1.4	1.9
QL ($\mu g\,g^{-1}$)	3.8	3.9	3.6	1.7	1.8	2.0	2.7	3.6
Linearity (%)	97.3	97.4	98.3	99.2	99.1	99.2	98.4	98.3
Sensitivity ($\mu g\,g^{-1}$)	1.1	1.1	1.0	0.5	0.5	0.5	0.8	1.0

[a] Lack of fit test (*P*, %).

good linearity of the response of the mass detector in the selected conditions for each of the FAMEs.

To study the reproducibility of the procedure, ten solutions containing an intermediate concentration of 25.0 $\mu g\,g^{-1}$ of each fatty acid were prepared and analysed as indicated in the experimental section. The reproducibility of the procedure, in terms of relative standard deviation value (RSD, %), was between 4.0 and 6.0%, and it could thus be concluded that the reproducibility was satisfactory for the analysis of fatty acids applying a previous transesterification step.

The detection limit (DL) and quantification limit (QL) were estimated from the calibration function [30]. DL and QL were calculated as 3.3 σ_{n-1}/S and 10 σ_{n-1}/S respectively, where σ_{n-1} is the standard deviation of the intercept and S is the slope of the calibration function. They were placed between 0.9 and 2.0 $\mu g\,g^{-1}$ (DL) and between 1.7 and 3.9 $\mu g\,g^{-1}$ (QL), indicating a satisfactory sensitivity of the proposed method for the intended purpose.

3.4. Analysis of samples from La Encarnación by Alonso Cano

3.4.1. Characterisation of drying oils

Drying oils have traditionally been characterised from the value of the ratio of areas from chromatographic peaks between the methyl ester of hexadecanoic acid and the methyl ester of octadecanoic acid or palmitate to stearate (C_{16}/C_{18} ratio). This approach was established for the first time by Mills in 1996 [1]. It is based on the hypothesis that the C_{16}/C_{18} ratio in seccative oils is typical and fairly constant with time. The main problem when using this ratio arises from the wide dispersion of the values published in the literature. As an example, Table 3 shows the proposed values for the C_{16}/C_{18} ratio depending on the type of drying oil used in the painting. The intervals of the ratio by the pioneer Mills [1] and others who have published more recently [8,10,31] are also shown in the same table.

The suitability of this C_{16}/C_{18} area ratio is also based on the constancy of the response factor (peak area/fatty acid concentration) for all the fatty acids, and hence both peak areas and fatty acid concentrations can be used to identify the kind of drying oils present in the picture, bearing in mind that the estimated values for the ratio C_{16}/C_{18} will be different when calculated from area or concentration. Recently Tsakalof et al. [19] demonstrated that the C_{16}/C_{18} ratio becomes dependent on sample dilution when there is a non-linear response from the GC–MS instrument. This could justify the great variability in the proposed intervals found in the literature (Table 3) for the value of the C_{16}/C_{18} ratio. Consequently, we propose to test the linearity of the response factors before applying the C_{16}/C_{18} ratio criterion. In this way, a correct interpretation of the results using area values can be assumed.

With that goal in mind, the validated GC–MS method was applied to standard samples and the response factor was calculated for $2C_9$, C_{16} and C_{18} in the concentration range between 0.5 and 50.0 $\mu g\,g^{-1}$. The results demonstrated that the response factor remains constant for $2C_9$ (0.21 ± 0.02), C_{16} (0.91 ± 0.13) and C_{18} (0.79 ± 0.10) acids in the studied interval of concentration. Fig. 5 shows the linear calibrations and slopes for C_{16} (palmitic acid), C_{18} (stearic acid) and $2C_9$ (azelaic acid). The smaller slope value for azelaic acid could lead to greater variability in the results for a given concentration value. This also could justify the great dispersion in the characteristic $2C_9/C_{16}$ ratio claimed by different authors [6,8].

Eleven microsamples extracted from the painting *La Encarnación* (described in the Paint Samples section) were selected to analyse the organic binding media using both the proposed validated method by GC–MS and the C_{16}/C_{18} ratio estimated after

Table 3
Mean values and standard deviation (SD) of area C_{16}/C_{18} ratio for lipid binders in the available literature.

C_{16}/C_{18}					Refs.
Linseed oil	Walnut oil	Poppy oil	Egg yolk	Egg/linseed oil	
1.9 ± 0.5	3.3 ± 1.1	5.5 ± 2.5	[a]	[a]	[1]
1.2 ± 0.2	2.2 ± 0.3	[a]	2.4 ± 0.3	[a]	[10]
1.5 ± 0.1	2.5 ± 0.4	3.0 ± 0.5	1.9 ± 0.2	[a]	[31]
1.6 ± 0.1	3.3 ± 0.1	3.9 ± 0.1	2.5 ± 0.1	1.8 ± 0.4	[8]

[a] Not available.

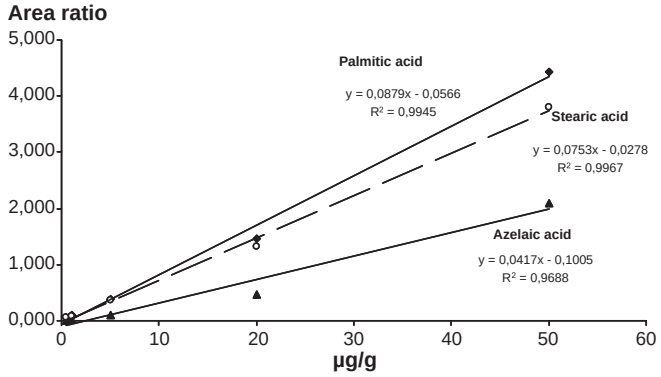

Fig. 5. Calibration graph for palmitic, stearic and azelaic acids.

testing that the response factor was constant. The results are listed in Table 4.

Comparing the results from Table 4 for the C_{16}/C_{18} ratio with the available values from the literature summarised in Table 3, it can be concluded that the drying oil used by Cano in *La Encarnación* was clearly linseed oil. All the analysed samples were characterised by an experimental value of the C_{16}/C_{18} ratio inside the 1.07–1.66 range, the characteristic ratio for linseed oil [2,8,10,31]. These results agreed with the data in the literature [20,24] on the pictorial techniques habitually used by Cano. Nevertheless, high values of C_{16}/C_{18} together with low values of $2C_9/C_{16}$ in the same real sample might suggest the presence of egg in some areas of the painting [2].

Once the presence of linseed oil was confirmed, the verification of the presence of egg in the painting was of a great importance, since it is related to the artistic techniques used by Cano. Therefore, in order to test and confirm the nature of the egg yolk, an important component because it contains fatty acids and can be mixed with linseed oil as a binder, a new experiment was carried out. Here, four model samples were prepared, as indicated in the experimental section, containing linseed oil alone (L) and mixtures of linseed oil with egg yolk (L+E), with gypsum (L+G) and with rabbit glue (L+RG). Those model samples tried to mimic the artistic materials that were habitually used for paintings during the Baroque period. These samples were left to dry and analysed by the proposed validated GC–MS method. They were used as reference samples, thus the azelate to palmitate ($2C_9/C_{16}$) and palmitate to stearate (C_{16}/C_{18}) ratios were calculated, always after testing that the response factor was a constant value (Table 5). Once these ratios were obtained, the results are consistent with the presence of egg in the sample.

Table 4
Mean values and standard deviation of C_{16}/C_{18} and $2C_9/C_{16}$ ratios in homemade paint samples.

Homemade paint samples	C_{16}/C_{18}	$2C_9/C_{16}$
L	1.2 ± 0.1	0.7 ± 0.1
L+G	1.0 ± 0.1	1.7 ± 0.1
L+E	1.3 ± 0.2	0.6 ± 0.1
L+RG	1.0 ± 0.1	1.2 ± 0.2

L, linseed oil; L+G, linseed oil+gypsum; L+E, linseed oil+egg yolk; L+RG, linseed oil+rabbit glue; $n = 3$ samples.

From the results of the analysis of the real samples (Table 5), it could be concluded that in the majority of the paint samples, the values of the $2C_9/C_{16}$ ratio inside the 0.17 and 0.68 range. A comparison of these data with the results from the model samples (Table 4) could are agree with the presence of linseed oil alone (L model sample = 0.71 ± 0.08), or even the use of traces of egg yolk added to linseed oil (L+E model sample = 0.57 ± 0.11). The lower amounts of dicarboxylic acids (especially azelaic acid) in aged egg yolk than in aged drying oils are due to lower content of more reactive polyunsaturated acids (linoleic and linolenic acids) when the egg was fresh [1]. However, the $2C_9/C_{16}$ ratio should be carefully examined for a proper interpretation, because many factors can affect the formation of the dimethyl ester of azelate ($2C_9$), not only the ageing process, but also the presence of colour pigments, as confirmed by the literature [28]. Table 5 also includes the dyes and pigments present in the painting that were previously identified by our Research Group using Scanning Electron Microscopy and X-ray microanalysis (SEM–EDX) [20,24]. The low values of the $2C_9/C_{16}$ ratio together with the high values for the C_{16}/C_{18} ratio in the same paint sample could suggest that both components (linseed oil and egg yolk) are present in several locations in the painting.

In brief, the results of the analyses confirm the hypothesis about the pictorial technique reported in the literature by Alonso Cano during his artistic stage in Granada [20]. The reported experimental data shows evidence of the use of linseed oil as the main component of the binding medium concluding that the pictorial technique used in *La Encarnación* is an oil painting. Nevertheless, traces of egg yolk have also been detected in some areas of the paint. The addition of a little amount of egg in oil painting agrees with the consulted literature [23]; it was used to improve the flow of the drying oil and to extend it easily on a support, especially in large size paintings. Furthermore, the use of egg is also described to prevent a colour change when blue colours were used in oil paintings where linseed oil was used as binder [23,24]. Blue colours turn green due to the yellowing of aged linseed oil. In the Cano painting studied here, traces of egg were found in blue areas such as the curtain and the Virgin's mantle.

3.4.2. Others remarks

The surprising presence of traces of the unsaturated acid $C_{18:1}$ was revealed by the analysis of some samples. Usually oleic acid disappears in aged samples after several months; the presence of oleic acid in a 400-year-old painting is difficult to explain. The most obvious explanation attributes its presence to residues from a repainting during a restoration carried out in 1967 [25], when commercial painting oils with high oleic acid content were used.

The analysis of the real paint samples by the proposed method showed the presence of other compounds that were also methylated, apart from those already discussed above.

The chromatogram of several samples showed the presence of the characteristic chromatographic peaks of dehidroabietic and 7-oxo-dehidroabietic acids. These acids are formed when the abietic acid of the varnishes compound by diterpenic resins from the pinaceae family degrades over time [1]. These results are consistent

Table 5
Values of C_{16}/C_{18}, $2C_9/C_{16}$ and $C_{18:1}/C_{16}$ in analysed samples from Cano's *La Encarnación*. Pigments and dyes present in samples [20–22].

Area samples	Pigments and dyes	C_{16}/C_{18}	$2C_9/C_{16}$	$C_{18:1}/C_{16}$	Binders
Red tunic (Virgin)	Red lacquer	1.4	0.3	0.1	Linseed oil traces egg
Blue mantle (Virgin)	Lapis lazuli, traces carbon black	1.5	0.3	–	Linseed oil traces egg
Carnation foot (Angel)	Vermilion, red lacquer, smalt, lapis lazuli, iron oxides, lead-tin yellow, traces black bone	1.3	0.2	0.1	Linseed oil traces egg
Tunic (Angel)	Smalt, vermilion, traces carbon black	1.1	0.2	–	Linseed oil traces egg
Red carpet	Vermilion, iron oxides, red lacquer	1.2	0.6	–	Linseed oil
Yellow carpet	Lead-tin yellow	1.3	0.7	–	Linseed oil
Red curtain	Red lacquer, lapis lazuli	1.3	0.7	–	Linseed oil
Blue carpet draw	Smalt	1.2	0.2	–	Linseed oil traces egg
Carnation (Virgin)	Vermilion, white lead, red lacquer, smalt, lapis lazuli, iron oxides	1.4	0.2	–	Linseed oil traces egg
Prayer stool (Virgin)	Copper resinate, smalt	1.7	0.2	0.1	Linseed oil traces egg
Hair (Angel)	Lead-tin yellow, iron oxides	1.3	0.4	–	Linseed oil

with the remaining of original varnish in some areas of the picture. In addition, samples located at the blue carpet draw and hair of Angel Announcer (at the bottom of the painting), suggest the presence of traces of beeswax due to the presence of esters of the even, saturated, straight chain (C20, C22, C24) [1]. It has been suggested that traces of beeswax was possibly added to the original varnish composition in order to achieve an aesthetic matte effect on the painting's surface. No traces of either conifer resins or waxes can be found in the rest of the analysed samples. This could be attributed to a non-homogeneous cleaning of the original varnish during the restoration process.

4. Conclusions

A GC–MS method has been developed and validated for the analysis of drying oils used in painting samples from works of art. Prior to the analysis, a direct off-line transmethylation of the oils with TFTMAH as the methylation reagent was optimised. The chromatographic system consists of a non-polar capillary column running under an optimised temperature program combined with single-ion monitoring MS detection. It yielded satisfactory separation efficiency and resolved of all the studied compounds. The proposed methodology is of considerable interest, since it makes it possible to identify the lipid nature of micro samples with minimum preparation time. This work also proposes the use of the traditional ratios (C_{16}/C_{18} and $2C_9/C_{16}$) expressed from the areas under the corresponding chromatographic peak, but after the confirmation of the linear response of each fatty acid. This ensures a correct interpretation of the results.

The method was further applied to characterise the type of drying oil in the painting *La Encarnación* by Alonso Cano. The binding media in this famous painting was mainly composed of linseed oil. Several areas of the painting were characterised by the presence of traces of egg. The remaining original varnish was still observed since diterpenic resins were detected along with traces of beeswax. All the results found agree with the pictorial technique described in treatises on seventeenth-century painting. Taking all of this into consideration, this work represents an important step in the technical, material and aesthetic knowledge about Cano's paintings, which will support the confirmation or rejection of his authorship in some cases of controversial attribution.

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