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Characterization of bile acids and fatty acids from ox bile in oil paintings by gas chromatography–mass spectrometry☆

M.J. Casas-Catalán[a], M.T. Doménech-Carbó[a,*], R. Mateo-Castro[b], J.V. Gimeno-Adelantado[b], F. Bosch-Reig[b]

[a] *Department of Conservation and Restoration of Cultural Heritage, Polytechnic University of Valencia, Camino de Vera 14, Valencia 46022, Spain*
[b] *Department of Analytical Chemistry, University of Valencia. Avda. Dr. Moliner s/n, Burjassot 46100, Spain*

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Abstract

Characterization of ox bile, traditionally used in painting, is of interest in the fields of archaeometry and conservation and restoration of works of art. Bile acids, fatty acids (F), and cholesterol found in ox bile have been identified using a derivatization method that combines the formation of ethyl esters from the carboxylic groups and the trimethylsilyl ethers from hydroxyl groups. This method of analysis is consistent with these others proposed by the authors to analyze drying oils, proteins, and diterpenic resins usually used as binders and varnishes by the painters. Bile acids from binary samples such as animal glue/ox bile, casein/ox bile and Arabic gum/ox bile have been successfully analyzed using the proposed method. Finally, a method of analysis of mixtures of drying oil and ox bile has been also proposed attempting to quantitatively characterize samples in which ox bile was added to the drying oil for increasing the surfactant properties.

Keywords: Art analysis; Derivatization, GC; Paints; Ox bile; Oils; Bile acids; Fatty acids; Cholesterol; Ethyl chloroformate; Trimethylsilylimidazole

1. Introduction

Traditional recipes of painting techniques have reported the use of ox bile as additive of proteinaceous binders (animal glue, casein) used to prepare the gypsum grounds of the panel paintings [1] or glue-paste adhesive [2], as additive of plant gums and mucilages used as binders of water colors due to its surfactant properties [3] and as additive for combining oil and tempera techniques due to its emulsification properties [4]. Characterization of this product is of interest in the fields of the archaeometry and the conservation and restoration of works of art enabling the characterization of the artistic technique and, in this way, leading to establish the more adequate restoration treatment.

Ox bile used in fine arts is traditionally known as *ox-gall*. Basically this product is composed of bile extracted from ox bladders. Animal bile is composed of a variety of organic and ionic constituents. Major components of the animal bile are phospholipids, bile salts, and cholesterol which are secreted by the liver. The major phospholipid component of the bile is phosphatidylcholine which accounts to about 90% of the total phospholid content of the bile. Palmitic acid, linoleic acid, and oleic acid, among others fatty acids (F), have been found as main components of phospholipids from animal liver [5,6]. The main products of cholesterol metabolism are bile acids, which are products of cholic acid (C) and chenodeoxycholic acid (Cd), which are known as the primary bile acids because of their hepatic origin. Before secretion, the primary bile acids are conjugated at the carboxylic acid carbon with either glycine (G) or taurine, which increases their polarity and water solubility. This mechanism of conjugation leads to the formation of four bile acids, within cholesterol, glycocholic acid, taurocholic acid, glycochenodeoxycholic acid, and taurochenodeoxycholic acid. During the passage through the small intestines and colon the bile acids are changed by enzymes produced by bacterial floras, that give rise to the secondary bile acids, deoxycholic acid (D), and lithocholic acid (L) where the two are conjugated by glycine and taurine [7,8].

The present work has been devoted to the chemical characterization of traditional *ox-gall* and its identification from

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* Corresponding author. Tel.: +34-96-3877310; fax: +34-96-3877319.
E-mail address: tdomenec@crbc.upv.es (M.T. Doménech-Carbó).

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samples in which other organic compounds are found: in ground binders from panel paintings together with animal glue and casein; in glue-paste adhesives for lining of paintings together with animal glue, linseed oil, and Venice turpentine; in binders of water colors together with vegetal gums; in canvas painting together with animal glue or drying oils for combining techniques.

In this way, a derivatization method that combines the formation of ethyl ester from the carboxylic groups and the trimethylsilyl ether from hydroxyl groups of bile acids is proposed. This method of analysis is consistent with these others proposed by the authors for analyzing drying oils, proteins, and diterpenic resins usually used as binder and varnishes by the painters [9–11]. The proposed method combines the capability of the ethyl chloroformate (ECF) to form the ethyl esters and the capability of the trimethylsilylimidazole (TMSI) to form trimethylsilyl ethers, whose use has been reported separately in the literature [12–16]. The proposed method includes two steps in the experimental procedure consisting in: (1) formation of ethyl esters using ethyl chloroformate in ethanol–pyridine medium, (2) the esterified compounds are lead to react with trimethylsilylimidazole to form the corresponding trimethylsilyl ethers.

Cholic and deoxycholic bile acids and myristic, pentadecanoic (Pn), palmitoleic, palmitic, heptadecanoic (H), oleic, and stearic fatty acids and cholesterol found in ox bile used in painting have been successfully analyzed. A tentative method of analysis of mixtures of linseed oil and ox bile has been also proposed attempting to semiquantitatively characterize samples in which ox bile was added to the linseed oil for increasing the surfactant properties.

2. Experimental

2.1. Solvents and reagents

In order to check the applicability of the combined derivatization method using ethyl chloroformate and trimethylsilylimidazole to the analysis of artistic samples in which ox bile is found, the following experiments have been performed: optimization of derivatization method, study of the possible interference effect of the organic matrix (animal glue, polysaccharide, drying oil), testing of a method enabling to the determination of ox bile/linseed oil weight ratio.

The following reagents were used to treat the samples: ethyl chloroformate (purity > 98%) and absolute pyridine (Fluka, Buchs, Switzerland). Hydrochloric acid at 37% for analysis (Scharlau, Barcelona, Spain). Chloroform stabilized, spectrophotometric grade (purity > 99%) (Acros, NJ, USA). Absolute ethanol and hexane for analysis and sodium hydrogenocarbonate for analysis (Panreac, Barcelona, Spain), trimethylsilylimidazole (purity > 98%) (Aldrich, Steinheim, Germany).

A single historical sample of “ox-gall” used in fine art works was supplied by Dr. Luis Angel de La Fuente (Bilbao, Spain). Lithocholic acid, chenodeoxycholic acid, deoxycholic acid, cholic acid, pentadecanoic acid, docosanoic acid (behenic acid, B), eicosanoic acid (arachidic acid, Ar), hexadecanoic acid (palmitic acid, P), octadecanoic acid (stearic acid, S) tetradecanoic acid (myristic acid, M), and 9-octadecenoic acid (oleic acid, O) were purchased from Aldrich (Steinheim, Germany). The following compounds: cholesterol, heptadecanoic acid, pelargonic acid (Pg), capric acid (Cp), suberic acid (Sb), and azelaic acid (A), that were found in the binary samples studied, were identified by using the Willey Library of Mass Spectra.

A standard solution mixture was prepared at a concentration of 0.5 $\mu g\,\mu l^{-1}$ of each amino acid. The amino acids used to prepare the standard mixture were alanine (Al), glycine, valine (V), leucine (Le), isoleucine (I), proline (Pr), threonine (T), serine (Se), glutamic acid (E), aspartic acid (As), methionine (Me), hydroxyproline (OH-P), and phenylalanine (F). All the above standards were obtained from a Sigma LAA-21 kit (St. Louis, MO, USA). An amino acid standards Sigma LAA-21 kit was used.

Casein (from bovine milk, 95% purity) and animal glue type A (from porcine skin, 98% purity) were supplied by Sigma (Steinheim, Germany). Arabic gum, animal glue and casein were supplied by RCM Productos de Conservación, Barcelona, Spain. Linseed oil, (Kremer, Farbmühle, Aichsten/Allgäu, Germany) was supplied by AP Fitzpatrick, London, UK.

2.2. Test specimens

A series of test specimens were prepared in which ox bile was added to a previously prepared aqueous solutions of animal glue, ammonium caseinate and Arabic gum to form a 50% solution. Other series of test specimens were prepared in which ox bile was added in different proportion (0–100%, w/w) to fresh linseed oil and then the test specimens were dried at room temperature (for 15 days) and kept in the fridge until analysis.

2.3. Samples preparation

Analysis of different natural products usually present in artworks includes a first step of hydrolysis. The experimental conditions were: (a) 6 M HCl, 24 h at 110 °C in inert atmosphere for the ox bile-animal glue and ox bile-casein specimens; (b) 6 M HCl, 6 h at 110 °C in inert atmosphere for the ox bile-linseed oil specimens; (c) 37% HCl–EtOH–water (1:1:2, v/v/v), 30 min at 100 °C for the ox bile-Arabic gum specimens [17]. Samples, after hydrolysis, were lead to dryness and then, the derivatisation method was applied.

2.4. Experimental procedure of derivatization

This derivatisation procedure combines the formation of ethyl esters using ECF from the carboxylic groups and the formation of the corresponding trimethylsilyl ethers using

trimethylsilylimidazole from the hydroxyl groups of the previously esterified compounds.

Samples (1–4 mg) were dissolved with 50 μl of an ethanol–pyridine (4:1) solution mixture for their derivatisation with (2–8 μl) of ECF. After vigorous shaking for 10 s, the reaction mixture was extracted with 50 μl of 5% ECF solution in chloroform; then 50 μl of $NaHCO_3$ saturated solution was added. Two layers, aqueous and chloroformic were formed. After their separation, the organic layer, containing the ethyl ester derivatives, was evaporated to dryness. Then, (9–36 μl) TMSI was added under a N_2 atmosphere. The solution was left at 40 °C for 20 min. The derivatives were extracted with 50 μl of hexane and the excess of derivatising reagent was eliminated adding 100 μl of water. After vigorous but careful shaking an aliquot of 1 μl of the upper hexane layer was injected for analysis into the gas chromatograph.

2.5. Instrumentation

An Agilent 5973N mass spectrometer coupled to an Agilent 6890N gas chromatograph (Agilent Instruments, USA) was used. Agilent Chemstation software (MSD) was used for the integration of peaks and for the mass spectra evaluation.

GC separation was achieved in a chemically bonded fused-silica capillary column HP-5-MS (Agilent), (stationary phase 5% phenyl–95% methylpolysiloxane, 30 m × 0.25 mm i.d., 0.25 μm film thickness). Inlet, initial and final oven temperatures were established on the basis of the prior studies carried out on fatty acids [9]. The chromatographic conditions were: temperature initial of the gas chromatograph 100 °C. Oven temperature was programmed with a gradient of 40 °C min^{-1} up to 295 °C held for 17 min. The chromatographic separation was accomplished in 21.88 min. The chromatograms obtained exhibited a good resolution of the chromatographic peaks. The carrier gas was He with inlet pressure of 72.5 kPa and 1:20 split ratio. Ions were generated by electron ionization (70 eV) in the ionization chamber. Total ions measurements were obtained in the mass spectrometer that was scanned from *m/z* 20–800 at a scan rate of 1.97 s/scan.

3. Results and discussion

3.1. Identification of bile acids, fatty acids, and cholesterol of ox bile

A series of experiments were carried out in order to establish the optimal experimental conditions to analyze the main components of the traditional ox bile: bile acids, fatty acids, and cholesterol. Studies carried out were performed to check different conditions of reaction for the TMSI, in particular, temperature and time of reaction. Amount of reagent was established on the basis of stoichiometric calculations referred to 1 mg of sample. Experimental conditions corresponding to reaction with ECF (amount ECF, temperature and reaction time) were maintained, basically, the same that those proposed for the analysis of fatty acids [9].

Table 1 summarizes the ethyl esters and TMS derivatives obtained from the standard fatty acid, bile acid, and cholesterol firstly analysed to achieve the optimal experimental conditions to perform this study. Retention time and molecular weight for the derivative products formed for each standard have been also included. The experimental conditions used enable the formation of the ethyl esters of carboxylic groups in the fatty acids. Small amounts of TMS derivatives of fatty acid together with the main peaks corresponding to the ethyl ester derivatives have been observed in these preliminary tests. Bile acid derivative was mainly obtained in which ethyl ester of the carboxylic group together with the TMS derivatives of hydroxyl groups are formed. In this last case, small peaks were also obtained that correspond to the bile acid in which the TMS derivatives from hydroxyl and carboxylic groups were formed. Other products in which hydroxyl groups were partially derivatised by TMSI were also obtained, as secondary products. Fig. 1 illustrates these results in the case of cholic acid. In order to reduce the intensity of the TMS derivative peaks preliminary experiments were carried out in which the different parameters influencing the reaction process were varied. It was observed that the amount of TMSI reactive was the main factor influencing the peak corresponding to the TMS derivative of bile acids and fatty acids. An increase in the amount of TMSI reduces the formation of partially TMS derivatised products obtained from bile acids. Nevertheless, this increase has a negative effect on the formation of the completely trimethylsilylated bile acid, which slightly increases. No significant changes were observed in the intensity of the main product of reaction, the ethyl ester and TMS derivative of bile acid. The experiences carried out led to establish an amount of TMSI that optimizes those two opposite secondary effects, as indicated in the experimental section.

3.2. Characterization of ox bile in art work samples

Fig. 2 shows the chromatogram of ox bile obtained in the same experimental conditions that those used for standards of fatty acids and bile acids. Chromatographic peaks between 4.20 and 5.50 min correspond to the fatty acid derivatives. Peaks obtained between 10.50 and 12.80 min correspond to the ethyl ester and TMS derivatives of bile acids. It can be seen that the results obtained are in good agreement with those from the standards used, not only the retention time of the different components but also the type of derivatives formed from each bile acid and their relative peak areas. It is remarkable the high content in cholic and deoxycholic bile acids and palmitic, oleic, and stearic fatty acids. Myristic, pentadecanoic, palmitoleic, and heptadecanoic acid and cholesterol are found as minor components.

Table 1
Molecular mass, retention time, and main derivative products identified for cholesterol, fatty acids, and bile acids found in ox bile

Chemical compounds	M_r	Retention time (min)	Identification of main derivatives
Cholesterol	458	10.54	Trimethylsilyl ether
Bile acids			
Lithocholic acid	476	11.44	Ethyl ester and trimethylsilyl ether
	520	11.71	Bis-trimethylsilyl ether
Chenodeoxycholic acid	564	12.34	Ethyl ester and bis-trimethylsilyl ether
	536	12.58	Bis-trimethylsilyl ether
	492	18.03	Ethyl ester and trimethylsilyl ether
Deoxycholic acid	564	12.08	Ethyl ester and bis-trimethylsilyl ether
	608	12.36	Tri-trimethylsilyl ether
	492	12.71	Ethyl ester and trimethylsilyl ether
Cholic acid	652	12.40	Ethyl ester and tri-trimethylsilyl ether
	696	12.60	Tetra-trimethylsilyl ether
	580	17.58	Ethyl ester and bis-trimethylsilyl ether
Fatty acids			
Myristic acid	256	4.29	Ethyl ester
Pentadecanoic acid	270	4.55	Ethyl ester
	314	4.70	Trimethylsilyl ester
Palmitic acid	284	4.81	Ethyl ester
	328	4.93	Trimethylsilyl ester
Oleic acid	310	5.25	Ethyl ester
	354	5.37	Trimethylsilyl ester
Stearic acid	312	5.30	Ethyl ester
	356	5.42	Trimethylsilyl ester
Eicosanoic acid	340	5.86	Ethyl ester
	384	5.98	Trimethylsilyl ester
Docosanoic acid	368	6.54	Ethyl ester
	412	6.68	Trimethylsilyl ester

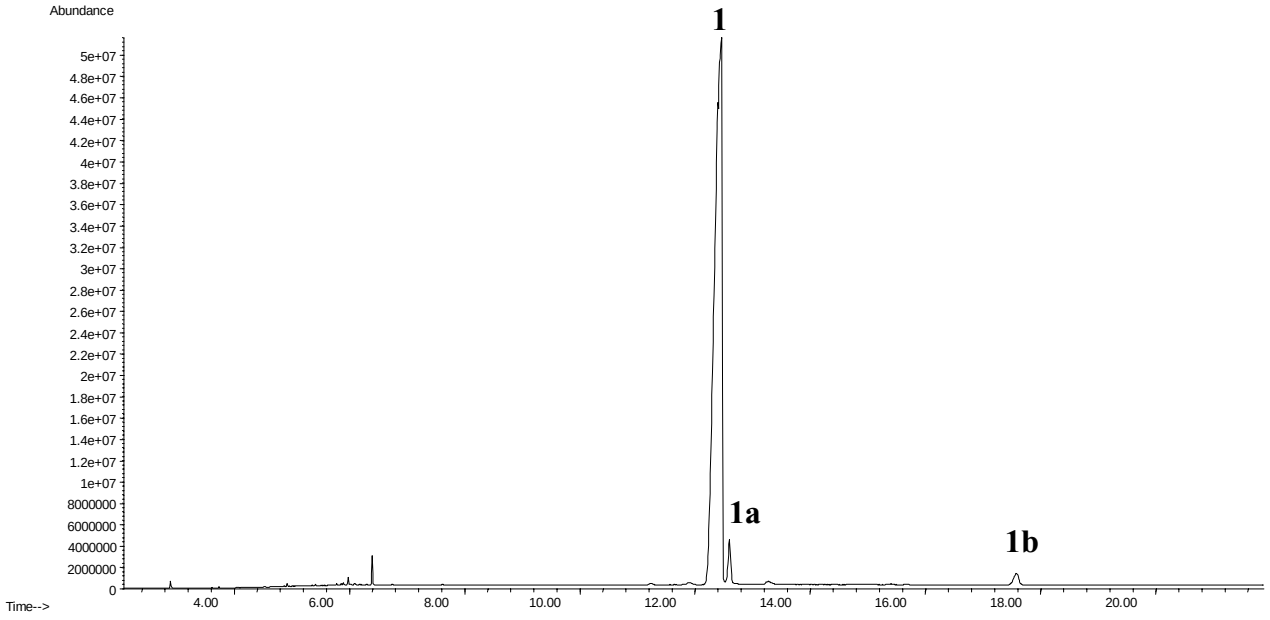

Fig. 1. Gas chromatogram of ethyl ester and TMS derivatives of cholic acid. Peaks: (1) ethyl ester and tris-trimethylsilyl ether; (1a) tetra-trimethylsilyl ether; (1b) ethyl ester and bis-trimethylsilyl ether. Time scale in minute.

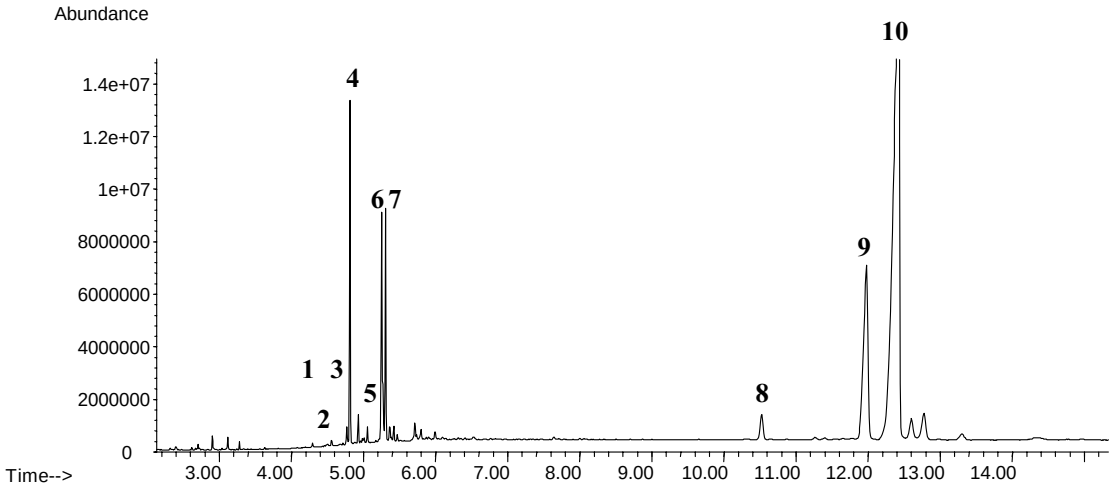

Fig. 2. Gas chromatogram of ethyl ester and TMS derivatives of ox bile. Peaks: (1) myristic acid ethyl ester; (2) pentadecanoic acid ethyl ester; (3) palmitoleic acid ethyl ester; (4) palmitic acid ethyl ester; (5) heptadecanoic acid ethyl ester; (6) oleic acid ethyl ester; (7) stearic acid ethyl ester; (8) cholesterol trimethylsilyl ether; (9) deoxycholic acid ethyl ester bis-trimethylsilyl ether; (10) cholic acid ethyl ester tri-trimethylsilyl ether. Time scale in minute.

Three independent replicates of ox bile from a single sample of "ox-gall" have been analysed in order to assess the reproducibility of the proposed method of analysis. Additionally, ratios of the total ions integrated peak areas for each fatty and bile acids have been calculated relatives to that from the cholic acid, as major component found in the ox bile (FA/C and BA/C, respectively). Values of these quotients, that are listed in Table 2, indicate that pattern of free bile acids found in the sample analysed reflect that of conjugated forms in ox bile, whereas predominant fatty acids found in the analysed sample of ox bile are oleic acid (0.094), palmitic acid (0.087), and stearic acid (0.064). Palmitoleic, heptadecanoic, pentadecanoic, and myristic acid have also been found in smaller amount. It should be noted the absence of linoleic acid whose content in liver phospholipids is significant. This result suggests that the sample of "ox-gall" analysed probably had undergone a process of ageing, in a similar way to the drying oils (oxidation due to the atmosphere oxygen and polymerization reactions). This process should be characterized by the drastic decreasing on the content of unsaturated acids and, in particular, linoleic acid. Values of standard deviation not higher than 0.03 have been found which are similar to those obtained in the analysis of other natural products used in works of art such as drying oils [18].

Similarly, a second series of experiments in which ox bile was successively diluted in ethanol–pyridine (4:1) were carried out in order to establish the sensitivity of the proposed method. The results obtained indicate that 1 μg of ox bile can be identified by using the proposed method.

To assess the capability of the proposed method, different test specimens were analyzed in which ox bile had been prepared together with animal glue, casein, Arabic gum, and linseed oil, at 50% (w/w), in the same way that traditionally has been used. For each pictorial binder a single sample of ox bile was used as indicated in the experimental section. The purpose of these experiments was to study the possible interference due to amino acids components of the animal glue and casein, fatty acids components of linseed oils and monosaccharides components of the Arabic gum.

A first series of analyses have been carried out in which a previous step of hydrolysis has been included as described in Section 2.3. Amino acids usually present in proteins were found at retention times ranging from 3.30 to 7.50 min. Major components of animal glue such as alanine, glycine, proline, glutamic acid, and hydroxyproline appeared at t_{Al}: 2.70 min; t_G: 2.73 min; t_V: 3.09 min; t_{Le}: 3.22 min; t_{Pro}: 3.48 min; t_{As}: 3.88 min; t_E: 4.22 min; $t_{OH\text{-}P}$: 4.06 min. Similarly, major monosaccharides from Arabic gum appeared at retention times of $t_{rhamnose}$: 3.84 min; $t_{arabinose}$: 3.91 min. In all cases, bile acids were not found in the chromatograms obtained. This result indicates that the strong acidic conditions applied in the process of hydrolysis act on the bile acids hindering their analysis and it suggests that identification of this compound in an old sample from a work of art requires no application of hydrolysis conditions.

A second series of analyses including (1:1) animal glue/ox bile, Arabic gum/ox bile, and linseed oil/ox bile binary systems (50%, w/w) were carried out in which hydrolysis step was suppressed.

Amino acids were not found in chromatograms corresponding to animal glue. Similar behavior was observed in the samples prepared with casein. Values of quotients of the different fatty acids, bile acids and cholesterol were, in general, similar to those from pure ox bile (see Table 2). Higher value of palmitic acid/cholic acid ratio obtained in casein was attributed to the fatty materials found in the commercial product.

Small quantities of arabinose, one of the main components of the Arabic gum, were found in the chromatogram obtained

Table 2
Peak area ratios of ethyl ester and TMS derivatives of bile acids (BA) and ethyl ester derivatives of fatty acids (FA) relative to ethyl ester and TMS derivative of cholic acid (C) from pure ox bile and animal glue/ox bile, casein/ox bile, Arabic gum/ox bile, and linseed oil/ox bile binary systems (1:1)

Fatty acids	FA/C	Bile acids and sterols	BA/C
Ox bile			
Myristic acid	0.0018 ± 0.0005	Cholesterol	0.026 ± 0.001
Pentadecanoic acid	0.004 ± 0.001	Deoxycholic acid	0.28 ± 0.02
Palmitoleic acid	0.007 ± 0.001		
Palmitic acid	0.087 ± 0.005		
Heptadecanoic acid	0.0063 ± 0.0005		
Oleic acid	0.094 ± 0.004		
Stearic acid	0.064 ± 0.002		
Animal glue/ox bile			
Myristic acid	0.003 ± 0.001	Cholesterol	0.020 ± 0.002
Pentadecanoic acid	0.008 ± 0.002	Deoxycholic acid	0.21 ± 0.01
Palmitoleic acid	0.006 ± 0.001		
Palmitic acid	0.089 ± 0.001		
Heptadecanoic acid	0.0073 ± 0.0005		
Oleic acid	0.086 ± 0.003		
Stearic acid	0.055 ± 0.004		
Casein/ox bile			
Myristic acid	0.005 ± 0.001	Cholesterol	0.022 ± 0.001
Pentadecanoic acid	0.005 ± 0.001	Deoxycholic acid	0.213 ± 0.01
Palmitoleic acid	0.0091 ± 0.0002		
Palmitic acid	0.104 ± 0.003		
Heptadecanoic acid	0.008 ± 0.002		
Oleic acid	0.073 ± 0.003		
Stearic acid	0.063 ± 0.002		
Arabic gum/ox bile			
Myristic acid	0.002 ± 0.001	Cholesterol	0.020 ± 0.001
Pentadecanoic acid	0.006 ± 0.001	Deoxycholic acid	0.220 ± 0.01
Palmitoleic acid	0.005 ± 0.002		
Palmitic acid	0.086 ± 0.001		
Heptadecanoic acid	0.005 ± 0.001		
Oleic acid	0.081 ± 0.002		
Stearic acid	0.053 ± 0.002		
Linseed oil/ox bile			
Pelargonic acid	0.004 ± 0.001	Cholesterol	0.020 ± 0.001
Capric acid	0.005 ± 0.001	Deoxycholic acid	0.25 ± 0.03
Suberic acid	0.0055 ± 0.0007		
Azelaic acid	0.021 ± 0.005		
Myristic acid	0.0072 ± 0.0007		
Pentadecanoic acid	0.0093 ± 0.0007		
Palmitoleic acid	0.011 ± 0.001		
Palmitic acid	0.092 ± 0.007		
Heptadecanoic acid	0.0090 ± 0.0007		
Oleic acid	0.081 ± 0.002		
Stearic acid	0.062 ± 0.002		

for the Arabic gum/ox bile sample. This compound, which appears at 3.91 min, does not interfere with the compounds from the ox bile as suggested by the agreement between values of fatty acid/cholic acid and bile acid/cholic acid ratio found in these samples and those others from the pure ox bile (see Table 2).

Finally, as it was expected, the chromatogram corresponding to the binary sample of linseed oil/ox bile showed an increase in the values of peak area of fatty acids. These higher values of fatty acid/cholic acid ratios are listed in Table 2.

3.3. Determination of the linseed oil/ox bile ratio

Study of binary systems linseed oil oil/ox bile is difficult owing to the analysis of fatty acids that simultaneously came from linseed oil and ox bile. In spite of the analysis of this type of samples is carried out without the previous step of hydrolysis, free fatty acids from the drying oil are obtained that result in the increase of the quotient of the peak area ratios of the different fatty acids referred to that from cholic acid. Nevertheless, this fact could tentatively be used for obtaining additional information on the quantitative

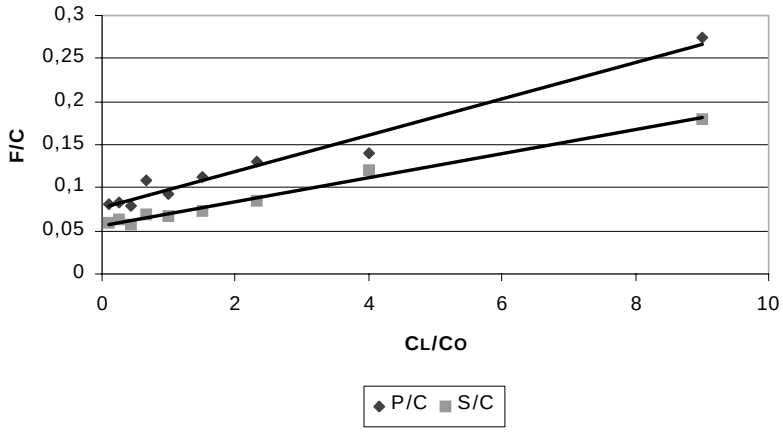

Fig. 3. Variation of the fatty acid/cholic acid peak area ratios (F/C) with linseed oil/ ox bile weight ratio (c_L/c_O). Values plotted correspond to the average of three independent replicates of binary samples prepared at different linseed oil/ox bile ratio. (◆) palmitic acid/cholic acid ratio (P/C); (■) stearic acid/ cholic acid ratio (S/P).

composition of this binary system. Determination of linseed oil/ox bile weight ratios could be obtained by the measurement of the peak area of a fatty acid that is found in both components of the binary system, and the cholic acid from the ox bile, and the further calculation of the peak area ratio (F/C). If a constant value for the fatty acid/cholic acid ratio (F_O/C_O) in the pure ox bile is assumed, a linear dependence between F/C and c_L/c_O should be expected for experimental values of the peak area ratio of each different fatty acid and cholic acid from series of linseed oil/ ox bile samples:

$$\frac{F}{C} = \frac{F_O}{C_O} + K\left(\frac{c_L}{c_O}\right)$$

where $K = k_L/k_O$, is the slope and F_O/C_O corresponds to the intercept of the linear function described.

Average values of palmitic acid/cholic acid ratio (P/C) and stearic acid/cholic acid ratio (S/C) of three independent replicates of different linseed oil/ox bile samples prepared at different c_L/c_O ratio, ranging from 9 (90% linseed oil: 10% ox bile) to 0.11 (10% linseed oil: 90% ox bile), are shown in Fig. 3. Palmitic and stearic acid have been selected for this quantitative study as the main saturated fatty acids found in linseed oil [9] and ox bile [18–21]. Experimental results plotted for each series corresponding to palmitic acid and stearic acid, satisfactorily fit to a straight line with slope values P/C: 0.02 and S/C: 0.013 and intercept values P/C: 0.077 and S/C: 0.055. Nevertheless, as can be seen in Fig. 3, values of P/C and S/C in the lower part of the calibration curve (c_L/c_O lower than 2:1) are close to the experimental values obtained in the pure ox bile (intercept). On the other hand, the differences between two consecutive values of P/C or S/C are higher than that from the standard deviation obtained in the reproducibility study for values of c_L/c_O higher than 0.67 (2:3). These results suggest that the proposed method could be tentatively used to assess mixtures of linseed oil and ox bile that exhibit values of c_L/c_O higher than 0.67 (2:3).

4. Conclusions

1. A derivatization method is proposed that combines the formation of the ethyl ester from the carboxylic groups and the trimethylsilyl ether from hydroxyl groups in the bile acids, that is consistent with these other proposed by the authors for analysing drying oils, proteins, polysaccharides, and terpenic resins usually used as binders and varnishes by the painters. The proposed method enables the identification of the main components of ox bile without interfering with the amino acids or monosaccharides from the animal glue, casein or Arabic gum.
2. The proposed method has been applied to the analysis of the traditional ox bile in which cholic acid and deoxycholic acid have been identified together with myristic, pentadecanoic, palmitoleic, palmitic, heptadecanoic, oleic, and stearic acid and cholesterol. Absence of linoleic acid suggests that the sample of “ox-gall” analysed, probably has undergone a ageing process, in a similar way to the drying oils.
3. A linear dependence of the fatty acid/cholic acid quotients on the linseed oil/ox bile weight ratio is observed that enables to quantitatively resolve this binary system if a constancy in the saturated fatty acid/cholic acid ratio from natural ox bile is assumed. Nevertheless, this statement has not been demonstrated owing to the difficulty to obtain different batches of “ox-gall”. On the other hand, the proposed method is applicable to mixtures of linseed oil and ox bile that exhibit c_L/c_O values higher than 0.67 (2:3). If it is taken into account that, as indicated in the literature [2], that values of c_L/c_O lower than 0.67 (2:3) should not be expected by artistic uses of ox bile together

with linseed oil, the proposed method could be suitable as a tentative quantitative approach to the chemical composition of old pictorial samples.

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