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Identification of lipid binders in old oil paintings by separation of 4-bromomethyl-7-methoxycoumarin derivatives of fatty acids by liquid chromatography with fluorescence detection☆

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Abstract

A HPLC-fluorescence method for identification of drying oils from binding media or protective film used in pictorial works of art prior to conservation or restoration is proposed. Fluorescence derivatization of fatty acids released by hydrolysis of structural drying oils is studied. The derivatization reagent was 4-(bromomethyl)-7-methoxycoumarin with 18-crown-6 as catalyst. Mobile phase was programmed from methanol–water (90:10 v/v) to methanol–water (100:0 v/v) in 25 min. The excitation and emission wavelengths were 325 and 395 nm, respectively. Under these chromatographic conditions, coumarin derivatives of myristic, palmitic, oleic and stearic acids were satisfactorily resolved. The method shows good sensitivity, with a detection limit of 6.0×10^{-8} mmol, and good linearity between 1.0×10^{-7} and 1.8×10^{-4} mmol of each analyte. Peak area ratios among fatty acids derivatives, especially the stearic acid/palmitic acid peak area ratio, are useful to identify the drying oils. The proposed method has been successfully applied to artistic samples from items of the cultural heritage of Valencia (Spain).

Keywords: HPLC; Drying oils; Fatty acids; Derivatization; Art analysis; Coumarin; Fluorescence

1. Introduction

Information about the materials that have been used originally by the artist is highly important in the filed of restoration and preservation of works of art. The materials that are present in old pictorial works of art are the result of the degradation undergone by the original material. This degradation process is usually complex, unknown and difficult to model, because the environment of the work of art is, in general, unknown. Then, it is difficult to know which materials have been originally present in the work of art, beginning from the materials present in the aged work of art. The difficulty is additionally increased by the small size usually available as well as the complexity of the sample [1].

Drying oils are among the most employed materials in pictorial works of art, especially in oil paints, and have been used habitually as binding consolidants and protective films. Depending on the period and pictorial technique, linseed oil is the most usual drying oil, although poppy seed, nut and sunflower seed oils have also been used [2].

Drying oils are complex mixtures of triglycerides, which are triple glycerol esters of some medium size fatty acids, such as myristic, palmitic, oleic and stearic acids, mainly in the aged oils. The relative amount of each fatty acid

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in the triglyceride mixture is a characteristic parameter of the drying oil. When the oil is exposed to environmental action or due to passage of time, it deteriorates and the fatty acid ratio also changes [3]. Highly unsaturated fatty acids, specially linoleic and linolenic acids are only present in the fresh drying oils. These components justify the siccative properties of the drying oils by means of its polymerization, and therefore these disappear in the reactions [2].

Drying oils have been identified by fatty acid analysis. Spectrophotometric techniques such as FTIR [4] or Raman [5] and electrochemical methods [6] found lipidic substances in ancient pictorial works of art, but these techniques were usually unable to identify each drying oil. To make this identification possible separation techniques such as GC–FID [7] or GC–MS [8] has been used. Previous pyrolysis of the sample has been carried out by some authors [9]. Fatty acids are released from drying oils by means of acidic hydrolysis in order to determinate their relative amount. HPLC–UV–vis has been used to analyze this kind of samples, prior derivatization of fatty acid with an UV–vis chromofore [10]. Although the fatty acid derivatives were clearly resolved, sensitivity was quite low. As UV–vis spectroscopy provides usually less sensitivity and selectivity than fluorescence, this last technique is preferred as detection procedure.

Fatty acids have not fluorescent groups, so a derivatization reaction with a fluorescence reagent is needed. Some derivatization reagents, such as 9-(2-hydroxyethyl)-carbazole [11], 9-(hydroxymethyl)anthracene [12], 2-(4-hydrazinocarbonylphenyl)-4,5-diphenylimidazole [13], 9-anthyldiazometane [14], *N*-(1-naphthyl)ethylenediamine [15], 5-(4-pyridyl)-2-tiophenmethanol [16] and 2-(2,3-naphthalimino)ethyl trifluoromethane-sulfonate [17] have been used to make fluorescent derivatives of fatty acids. These methods, however, have not been always satisfactory with regard to separation and analysis time, while some reagents are difficult to find and need a previous synthesis in laboratory. The derivatization reagent here tested is 4-bromomethyl-7-methoxycoumarin, employed in fatty acids analysis, which provides good sensitivity [18].

These fatty acid derivatives are usually resolved by HPLC on reversed-phase columns with isocratic or gradient elution systems using methanol or acetonitrile and water in various proportions. When the proportion of organic modifier increases, the derivatives are eluted early [19]. The elution time of each fatty acid derivatives principally depends on the number of carbon atoms and the number of unsaturated bonds in the aliphatic acid chain [20].

This aim of this paper was to do an analytical study of drying oils by analysis of the fatty acids obtained after acidic hydrolysis of the oils, using HPLC with fluorescence detection, with the purpose of obtaining best resolution of peaks and detector selectivity [7] than with GC–FID methods, and better sensitivity than that achieved with HPLC–UV–vis detection [10].

2. Experimental

2.1. Reagent solutions

A 0.11 g/100 ml of 4-bromomethyl-7-methoxycoumarin (Br-Mmc) (97% purity, Aldrich, Alcobendas, Spain) was prepared by dissolving the reagent in acetone. A 0.07 g/100 ml solution of 18-crown-6 (99.5% purity, Aldrich), was prepared by dissolving the reagent in acetonitrile. These reagent solutions were kept below 5 °C and protected from light.

Standard of oleic acid (C18:1, ole) (95% purity) was from Fluka (Switzerland). Standards of myristic (C14:0, myr), palmitic (C16:0, pal), stearic (C18:0, ste) and arachidic (C20:0, ara) acids (all 99% purity) were obtained from Sigma (Alcobendas, Spain).

$KHCO_3$ was 98% pure (Aldrich). HCl and acetone were analysis grade and acetonitrile and methanol used in chromatographic separation were HPLC grade (Carlo Erba, Val de Reuil, France). Hexane was analysis grade (Fisher Chemicals, Loughborough, UK). Ethanol used was HPLC grade (Scharlau, Barcelona, Spain). All used water (Nanopure II grade) was generated in laboratory by a Nanopure water production device (Sybron-Barnstead, Dubuque, IA, USA).

2.2. Standards and solutions

Solutions of myristic, palmitic, oleic and stearic acids (250 μmol/l) were made by dissolution of the appropriate amount of each fatty acid in ethanol. Arachidic acid was dissolved in ethanol in order to make a 1 mmol/l solution.

Standards of the most usual drying oils (linseed, poppy seed and nut oil from Kremer Pigments, Germany) have been analyzed. In order to know how the relative amount of each fatty acid can be affected depending on ageing, the standards were aged by two kinds of accelerated artificial ageing processes (thermal treatment and UV irradiation treatment), and by naturally ageing:

- Thermal ageing was accomplished by putting the drying oils on slides into a Dycometal DI-100 climatic chamber at 50 °C with 40% relative humidity for 14 days [21].
- For UV irradiation ageing, the slides with the drying oils were irradiated with OSRAM L36/37 fluorescent lamp at 350–400 nm and 36 W, for 4 weeks at a distance of 12 cm [22].
- Natural ageing was made keeping the drying oils on slides for 3 years without any treatment.

All used glassware were washed with sulfochromic mixture, distilled water, rinsed with acetone and dried at 110 °C to avoid possible contamination.

2.3. Artistic samples

The real samples were taken from the surface of pictorial works of art with a scalpel. The following pieces

from artistic heritage of Valencia (Spain) were analyzed:

- Sample 1: Crucifixion, 17th Century, anonymous, but assigned to Tintoretto.
- Sample 2: Saint Therese, 18th Century, anonymous.
- Sample 3: Saint Martha image, 16th Century, anonymous.
- Sample 4: Unclassified and strongly damaged work, Yañez de la Almedina, 16th Century (Valencia Cathedral).
- Sample 5: Unclassified and strongly damaged work, Yañez de la Almedina, 16th Century (Valencia Cathedral).
- Sample 6: The Virgin intercedes in the presence of the Trinity, Palomino, Marian Museum (Valencia Basilica).
- Sample 7: Sketch of the linen of the niche of the Virgin, Stolz, Marian Museum (Valencia Basilica).
- Sample 8: The *Virgen de los Desamparados* between angels which carry badalquin and innocent children with candles, anonymous, Marian Museum (Valencia Basilica)
- Sample 9: The *Virgen de los Desamparados*, anonymous, Marian Museum (Valencia Basilica).

2.4. Apparatus

Chromatographic analysis was carried out using a Model 1100 liquid chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with quaternary pump, on-line vacuum degassing system, injector (20 μl loop volume), column oven and fluorescence detector. Signals were processed by the Agilent Chemstation for LC, Rev. A 09.03-847.

Separation of fatty acids was achieved in a Zorbax XDB-C18 C_{18} main column (15 cm × 4.6 cm I.D., particle size 5 μm) thermostated at 30 °C. A pre-column filled with the same stationary phase was used. The composition of the mobile phase changed following a gradient programme, from MeOH–H_2O (90:10) to MeOH–H_2O (100:0) in 25 min at 1.5 ml/min. The selected excitation and emission wavelengths were 325 and 395 nm, respectively [23].

2.5. Hydrolysis of drying oils

Drying oils standards (0.5–1 mg) were placed in a 0.3-ml microvessel (Supelco, Bellefonte, USA) and treated with 100 μl of 12 mol/l HCl for 24 h at 110 °C, avoiding HCl vaporization [7]. After cooling, 250 μl of hexane was added and the mixture was shaken in order to favour extraction. Hexane phase was separated and two other extractions were done again by adding 250 μl of hexane to aqueous phase and shaking. Finally, the three hexane phases were mixed in a Pyrex test tube (Bibbi Sterilin, Staff, UK), and dried at 70 °C. The residue was dissolved in 0.1 ml of ethanol and 20–30 mg of $KHCO_3$ powder was added. After cooling the mixture was ready to be derivatized.

For real sample analysis, solid parts scalpered from the pictorial work of art (0.5–1 mg) were derivatized in the same way as drying oil standards.

2.6. Derivatization procedure

Ethanol was removed by heating at 90 °C. After cooling, 250 μl of Br-Mmc solution and 100 μl of 18-crown-6 solution were added [18,24]. The mixture was protected from light and heated in a water bath, at 55 °C for 25 min [18]. Finally, the solution was diluted with 250 μl of acetone, centrifuged at 5000 rpm for 2 min in order to separate the suspension formed by $KHCO_3$ excess and cooled in running tap water [25,26]. An aliquot of 20 μl of the resulting derivative mixture was injected directly into the chromatograph.

After each injection, the column was cleaned by passing pure methanol during a minimum of 10 min after the last peak of interest.

3. Results and discussion

3.1. Derivatization conditions

Coumarin is a well-known derivatization reagent in fluorescence analysis. The conditions used in this work are similar that presented by Lam and Grushka [18], however, the use of 250 μl of Br-Mmc solution instead of 0.5 ml, permitted the use less quantity of reagent being used while maintaining the sensitivity.

3.2. Chromatographic separation

Retention times for analyte derivatives increase as methanol level in the mobile phase decreases [20]. Different trials using methanol–water mixtures showed that a high proportion of methanol (90% or more) is needed to obtain useful (low) retention times. The gradient is useful to improve the separation of the derivatives of the fatty acids, especially those from palmitic and stearic acids, which show closely retention times. A gradient of methanol–water from 90:10 (v/v) to 100:0 (v/v) in 25 min is enough to obtain good separation and satisfactory retention times. The excess of reagent is not a problem, because it appears at the beginning of the chromatogram and does not interfere with any peak. The cleanage after each injection is suitable because in absence of cleanage, excess of derivatization reagent causes absorption problems after roughly a hundred injections.

The temperature is an important factor in the separation, affecting mainly the retention time. It is important to keep the temperature constant to avoid possible influence of environmental temperature fluctuation in the chromatograms [26]. Low temperatures increase retention times and the pressure of the quaternary pump, and separation between palmitic and oleic acids decrease. High temperatures reduce retention times, but no separation improvement is observed, and they are aggressive to the column; thus, a temperature of 30 °C was chosen for the analysis.

The choice of the excitation and the emission wavelengths is essential to obtain good sensitivity and repeatability in

fluorescence methods. The coumarin derivatives have their maximum excitation wavelength in methanol medium at 325 nm [20]. The maximum emission wavelength was found to be 395 nm from the emission spectrum obtained by exciting the analytes at 325 nm.

3.3. Assay with fatty acids

The calibration parameters for the main fatty acids found in drying oils used in pictorial works of art (myristic, palmitic, oleic and stearic acids), were determined using 2.5×10^{-5} mmol of arachidic acid (ara) as internal standard. The method was tested for myristic, palmitic, oleic and stearic acids and calibration lines were linear between 1.0×10^{-7} and 1.8×10^{-4} mmol. The following equations were obtained by linear regression:

$$\frac{A(\text{myr})}{A(\text{ara})} = (32900 \pm 300)[\text{myr}] + (0.07 \pm 0.02) \text{ with } R^2 = 0.9990,$$

$$\frac{A(\text{pal})}{A(\text{ara})} = (31800 \pm 700)[\text{pal}] + (0.05 \pm 0.06) \text{ with } R^2 = 0.9990,$$

$$\frac{A(\text{ole})}{A(\text{ara})} = (29100 \pm 500)[\text{ole}] + (0.06 \pm 0.02) \text{ with } R^2 = 0.9992,$$

$$\frac{A(\text{ste})}{A(\text{ara})} = (22900 \pm 300)[\text{ste}] + (0.09 \pm 0.02) \text{ with } R^2 = 0.9997,$$

for $n = 6$ calibration points. A means peak area and amounts are given in mmol. The detection limit was 6.0×10^{-8} mmol (measured as quotient between three times the standard deviation of the blank and sensitivity) in all cases. The sensitivity found with this method was 25 times higher than was found with the UV method [10].

In order to determine the ratio between the signals given by the fatty acid standards and verify their separation, 0.1 ml of 0.25 mmol/l solution of each fatty acid standard in ethanol, spiked with 0.025 ml of 1 mM solution of arachidic acid in ethanol was analyzed. The five fatty acids are in the same quantity (2.5×10^{-5} mmol). As it can be seen in Fig. 1, the peaks of the fatty acid derivatives are clearly resolved. Peaks seem approximately Gaussian but areas are different for each fatty acid, which may be due to different derivatization yield or different fluorescence yield of each derivative. However, the myr/pal, ole/pal and ste/pal peak area ratios remained roughly constant.

3.4. Assay with standard drying oils

Fresh and aged standard oils were analyzed by HPLC using the proposed method. Characteristic chromatograms were found for each kind of drying oil and ageing procedure. The chromatograms for linseed oil can be seen in Fig. 2. For each combination of drying oil and ageing procedure, the ratio between the peak area of each fatty acid derivative and the area of the palmitic acid derivative was calculated. The results for five different replicates of each kind of drying oil and ageing procedures are shown in Table 1.

Recovery was calculated by spiking 100 μl of hydrolysed poppy seed oil standard, aged under UV treatment (before extraction with hexane) with 0.025 ml of 1 mM ethanolic solution of fatty acids. Myristic and oleic acids already present in this poppy seed oil sample is neglected (see

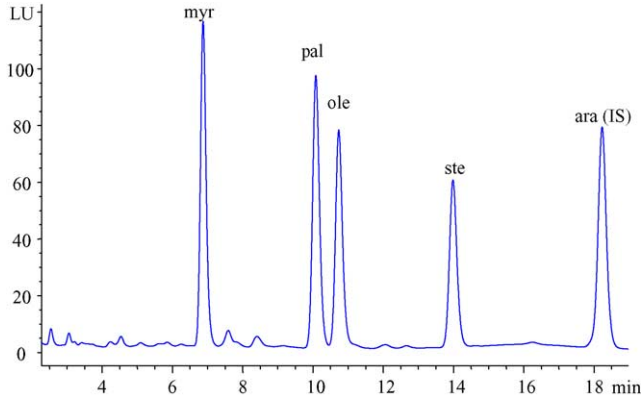

Fig. 1. Liquid chromatogram obtained by optimized derivatization with 4-bromomethyl-7-methoxycoumarin of 2.5×10^{-5} mmol of each myristic (myr), palmitic (pal), oleic (ole), stearic (ste), and arachidic (ara) acids. Conditions: mobile phase: gradient from MeOH–H_2O (90:10) to (100:0) in 25 min at 30 °C. Flow-rate: 1.5 ml/min. Excitation wavelength 325 nm, emission wavelength 395 mn, injected volume: 20 μl.

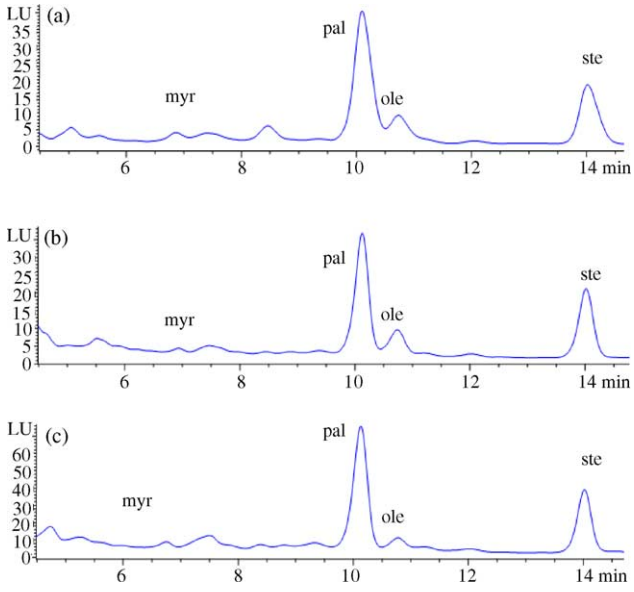

Fig. 2. Liquid chromatograms obtained by optimized derivatization with 4-bromomethyl-7-methoxycoumarin of linseed oil standard: (a) aged for 3 years without treatment, (b) aged by thermic treatment, and (c) aged by UV-irradiation treatment. Conditions as in Fig. 1.

Table 1
Values for myristic/palmitic (myr/pal), oleic acid/palmitic (ole/pal) and stearic acid/palmitic (ste/pal) peak area ratios in fresh, 3 years aged (without treatment) and artificially aged drying oil standards

Drying oil	Type of ageing	myr/pal	Linolenic/pal	Linoleic/pal	ole/pal	ste/pal
Linseed	Fresh	0.03 ± 0.01	7 ± 1	3 ± 1	3.5 ± 0.5	0.48 ± 0.05
	3 years	0.04 ± 0.01	–	–	0.20 ± 0.01	0.49 ± 0.01
	Thermic	0.014 ± 0.004	–	–	0.21 ± 0.05	0.59 ± 0.03
	UV	0.05 ± 0.01	–	–	0.07 ± 0.01	0.49 ± 0.03
Poppy seed	Fresh	0.04 ± 0.03	0.04 ± 0.04	3 ± 1	1.7 ± 0.2	0.16 ± 0.03
	3 years	0.021 ± 0.009	–	–	0.04 ± 0.02	0.21 ± 0.04
	Thermic	0.04 ± 0.01	–	–	0.088 ± 0.007	0.24 ± 0.03
	UV	0.011 ± 0.003	–	–	0.029 ± 0.002	0.19 ± 0.01
Nut	Fresh	0.03 ± 0.02	2 ± 1	8 ± 2	2.0 ± 0.3	0.28 ± 0.03
	3 years	0.06 ± 0.03	–	–	0.18 ± 0.04	0.31 ± 0.03
	Thermic	0.015 ± 0.005	–	–	0.26 ± 0.07	0.31 ± 0.06
	UV	0.02 ± 0.01	–	–	0.03 ± 0.01	0.29 ± 0.05

Values are average ± SD for $n = 5$ (different hydrolysed drying oil standards).

Table 1). Recovery was found to be (70 ± 10)% for myristic acid and (80 ± 5)% for oleic acid.

These chromatograms were more complex than those obtained with fatty acid standard mixtures, because drying oils have more complex matrixes, and some substances can appear in the chromatograms. Nevertheless, there was no peak interfering with the studied fatty acids and the area is easily calculable. Some of the peaks that appear at low retention times may be are fatty acids at low levels and without interest. The chromatograms permit the peak area ratio of each fatty acid with respect to palmitic acid to be determined.

As shown in Table 1, the ste/pal ratio is the parameter that allows the used drying oil to be identified [7]. The myr/pal ratio provides no information, because the amount of myristic is very small, whereas the ole/pal ratio provides information with regard to the ageing process, because oleic acid disappears with UV irradiation ageing. The drying oils can be then easily recognized.

3.5. Analysis of drying oils from painted works of art

Samples of real oil paintings were taken in order to identify which drying oil was used by artists using the developed method. Samples were analyzed in the same way as the standards (see Sections 2.5 and 2.6). The chromatograms were more complex than in the cases of drying oil standards and other peaks appeared. However, the resolution of the fatty acids is not impaired, and their peak area ratios can be calculated without difficulty, as can be seen in Fig. 3. As the case of the standard drying oils, the myr/pal, ole/pal and ste/pal peak area ratios and their standard deviation were calculated for three replicates (three different samples taken from the pictorial work of art). The results obtained for these ratios are shown in Table 2.

The standard deviations for samples were found higher than in the case of aged standard oils. The considerable heterogeneity and complexity of the pictorial works of art may be the cause of this behaviour [2]. The ratios obtained for the painting samples sometimes differ from those found for the aged drying oils. Ideal ratios were calculated by means of fresh standard drying oils artificially aged. But real ageing cannot be produced only by UV irradiation, because pictures are kept inside buildings (churches, museums, castles and houses) where they are relatively protected from sun radiation. Real ageing is not produced only by thermic attack, because pictures are not usually exposed to high temperatures (except in an occasional fire). Real ageing process occurs by addition of some causes, such as slow attack by environmental substances (dioxygen, moisture, microorganisms, pollution agents) and irradiation from light, and is usually unknown [2,28]. Then it is not feasible to model. As a result, artificial ageing processes are not equivalent to real ageing processes and provide only little information about changes in fatty acid relative amounts. Drying oils aged without treatment are neither representative because this ageing process was for 3 years and our real pictorial works of art are aged for 3 centuries at least. The presence of pigments and other substances in the paintings can also modify the ageing process because they can inhibit or retard the oxidation of some fatty acids. Although stearic and palmitic acids peak areas are affected, the ste/pal ratio can be considered reliable [27].

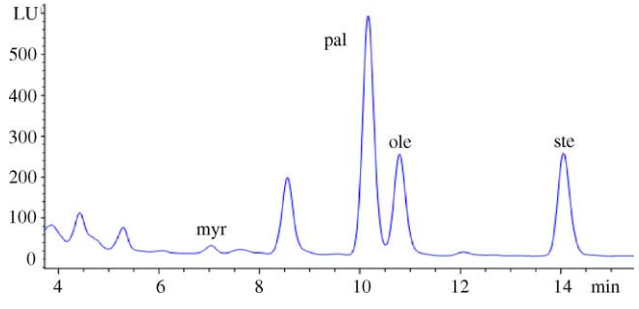

Fig. 3. Liquid chromatogram obtained by optimized derivatization with 4-bromomethyl-7-methoxycoumarin of a sample from the painting 'Crucifixion, 17th C, anonymous, but assigned to Tintoretto'. Conditions as in Fig. 1.

Table 2
Values for myristic/palmitic (myr/pal), oleic/palmitic (ole/pal) and stearic/palmitic (ste/pal) peak area ratios in sample of pictorial works of art and identification of used drying oil

Painting sample	myr/pal	ole/pal	ste/pal	Suggested drying oil
1	0.11 ± 0.07	0.5 ± 0.1	0.43 ± 0.06	Linseed oil
2	0.22 ± 0.03	0.5 ± 0.2	0.35 ± 0.05	Nut oil
3	0.18 ± 0.04	0.6 ± 0.3	0.45 ± 0.04	Linseed oil
4	0.20 ± 0.08	0.28 ± 0.07	0.7 ± 0.1	Linseed oil
5	0.28 ± 0.05	0.35 ± 0.09	0.43 ± 0.08	Linseed oil
6	0.33 ± 0.09	0.38 ± 0.09	0.7 ± 0.2	Linseed oil
7	0.6 ± 0.1	0.6 ± 0.1	0.51 ± 0.09	Linseed oil
8	0.4 ± 0.2	0.5 ± 0.2	0.55 ± 0.06	Linseed oil
9	0.18 ± 0.07	0.2 ± 0.1	0.6 ± 0.2	Linseed oil

Values are average ± SD for $n = 3$ (different samples taken from the pictorial work of art).

All painting samples present myr/pal ratios much higher than the ideal ratios (Table 2). It may be caused by a process in real ageing that does not take place in thermic or UV irradiation ageing processes.

Samples also have high ole/pal peak area ratios. It probably indicates that the mechanisms of ageing do not strongly affect the oleic acid, maybe due to special protection of oleic acid by the chemical environment (pigments) [27]. It is also possible that the ageing process had strongly affected the palmitic acid.

Except for sample 2, all samples exhibit ste/pal peak area ratios going from 0.43 to 0.7, which point to the probably use of linseed oil as lipidic binder in the pictorial works of art. Sample 2 shows a ste/pal area ratio that indicates that nut oil has probably been used.

The ole/pal area ratio in sample 3 is very high, which suggests that the ageing has not strongly affected the value of oleic acid, probably due to the chemical environment [27].

In sample 4 the ageing process has strongly affected the amount of palmitic acid, probably due to pigment effects [27].

Linseed oil has been found in many samples which is reasonable because it is the most used drying oil in painting works of art.

4. Conclusions

Identification of drying oils in pictorial works of art is possible by HPLC-fluorescence detection. Separation of the fatty acids obtained from the drying oils by HCl hydrolysis and robust and fast extraction by hexane is needed in order to carry out the proposed methodology for oil protective film and binding media in oil paintings. Fatty acid derivatization with 4-bromomethyl-7-methoxycoumarin provided sensitivity enough. Derivatives of stearic and oleic acids were clearly resolved, despite their similar formulas, and derivatives of palmitic and oleic acids were clearly resolved, regardless their similar retention times. The chromatograms for different standard drying oils (linseed, nut, poppy seed) provide sufficient data to differentiate among them by means of fatty acid peak area ratios. This HPLC method is useful to study the drying oils after undergoing thermic or UV irradiation treatment. The suggested HPLC method permits the easy identification of drying oils used in pictorial works of art, which is essential to conservation or restoration treatments. Even if artificial ageing treatment of drying oils is very different to real ageing of these materials in pictorial works of art, the ste/pal parameter is useful to identify the used drying oil.

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