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Abstract: Traditionally, the determination of lipid binders in paintings has relied on evaluating characteristic profiles and ratios of fatty acids amounts by gas chromatography/mass spectrometry (GC/MS). The presence of mixtures in contemporary and modern oil paints makes the GC/MS determination of fatty acids not sufficient to fully characterize lipid binding media. In this study, we prove that a precious contribution to this issue can be achieved by triacylglycerols (TAGs) profiling by high-performance liquid chromatography with high-resolution tandem mass spectrometry, using ESI in positive and negative ionization modes. We exploited this analytical approach to study the curing and degradation processes undergone by six plant oils used in the formulation of media in modern paints, applying both natural and artificial ageing experiments. This is the first time that negative ionization mode has been applied to this purpose and that a survey with HPLC-ESI-Q-ToF is carried out to study the ageing kinetics of plant oils. While the discrimination of the lipid source cannot be achieved by GC/MS analysis, TAGs profiling allowed us to study the evolution over time of the constituents of modern oils, with respect to curing and ageing. The data achieved in this study demonstrate that the proposed analytical approach is efficient to study the oxidation of TAGs during ageing, and improve the current knowledge on the properties of vegetable oils possibly leading to the development of new paint materials and conservation treatments for modern and contemporary artworks.

Opposed Reviewers:

Cover Letter

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Dear Editor,

Please find in the attached files the manuscript "Model study of modern oil-based paint media by triacylglycerols profiling in positive and negative ionization modes" by Ilaria Degano, Jacopo La Nasa, Elisa Ghelardi, Francesca Modugno and Maria Perla Colombini.

This paper summarize the results of a complete ageing study on six oils used by paint manufacturers in modern oil paints: linseed, safflower, soybean, sunflower, tung and castor oils.
The aim of the paper is to provide an analytical tool and data interpretation database for the identification of plant oils in modern oil paint samples on the basis of the recognition of the main triglyceride components and their oxidation products. We evaluated this analytical approach as a tool for the study of the chemical transformations induced by ageing in oil paint layers, e.g. oxidation reaction of acyl chains and formation of diacids.

Both natural and artificial ageing experiments were carried out to study the curing and degradation processes. The paint films, prepared with TiO_2, were subjected to curing in indoor conditions and then to photo-induced artificial ageing. The set of samples was analysed both by GC/MS and HPLC-ESI-Q-ToF.
In this paper we present the first systematic and comprehensive survey on the artificial ageing of modern oils, followed by HPLC-ESI-Q-ToF. TAGs profiling, performed both in positive and negative ionization modes, allowed us to obtain a deeper knowledge on the TAGs constituting the oils used in modern paints and of their evolution over the time with respect to the curing and ageing. We also identified, for the first time, specific oxidised TAGs, which can act as specific molecular markers.

The manuscript is unpublished and has not been submitted for publication elsewhere.

We sincerely hope that the paper will be considered for publication in Talanta.

Best regards

Novelty statement

This paper summarize the results of a complete ageing study on six oils used by paint manufacturers in modern oil paints: linseed, safflower, soybean, sunflower, tung and castor oils.

The aim of the paper is to provide an analytical tool and data interpretation database for the identification of plant oils in modern oil paint samples on the basis of the recognition of the main triglyceride components and their oxidation products. With respect to the literature, we proved that the discrimination of the lipid source cannot be achieved on the basis of the P/S ratio, being the values displayed by the investigated oils in the same range as that of linseed oil.
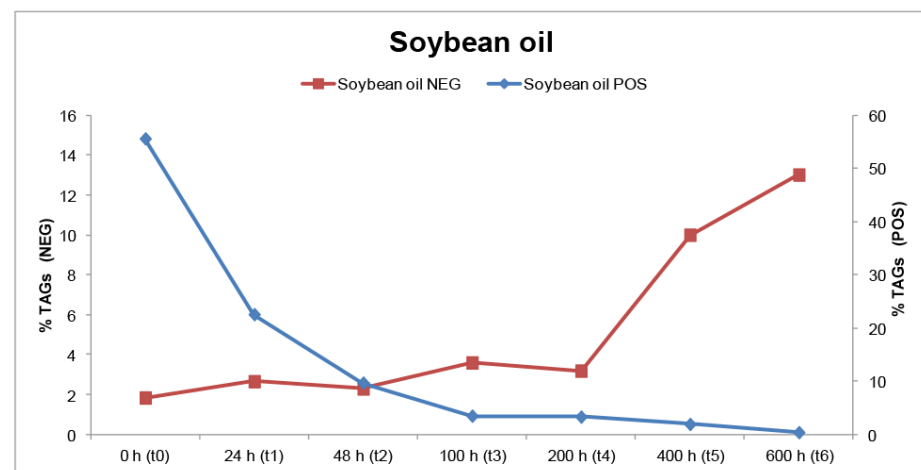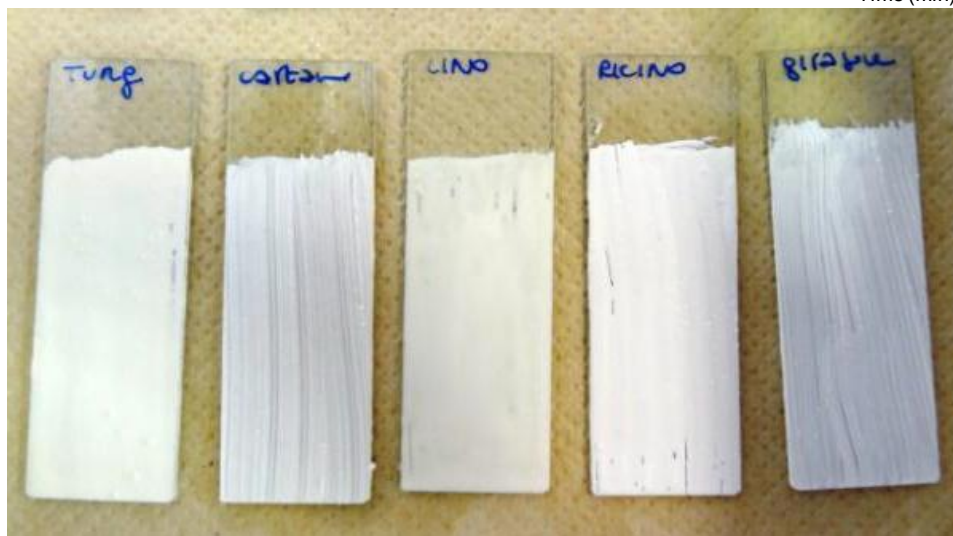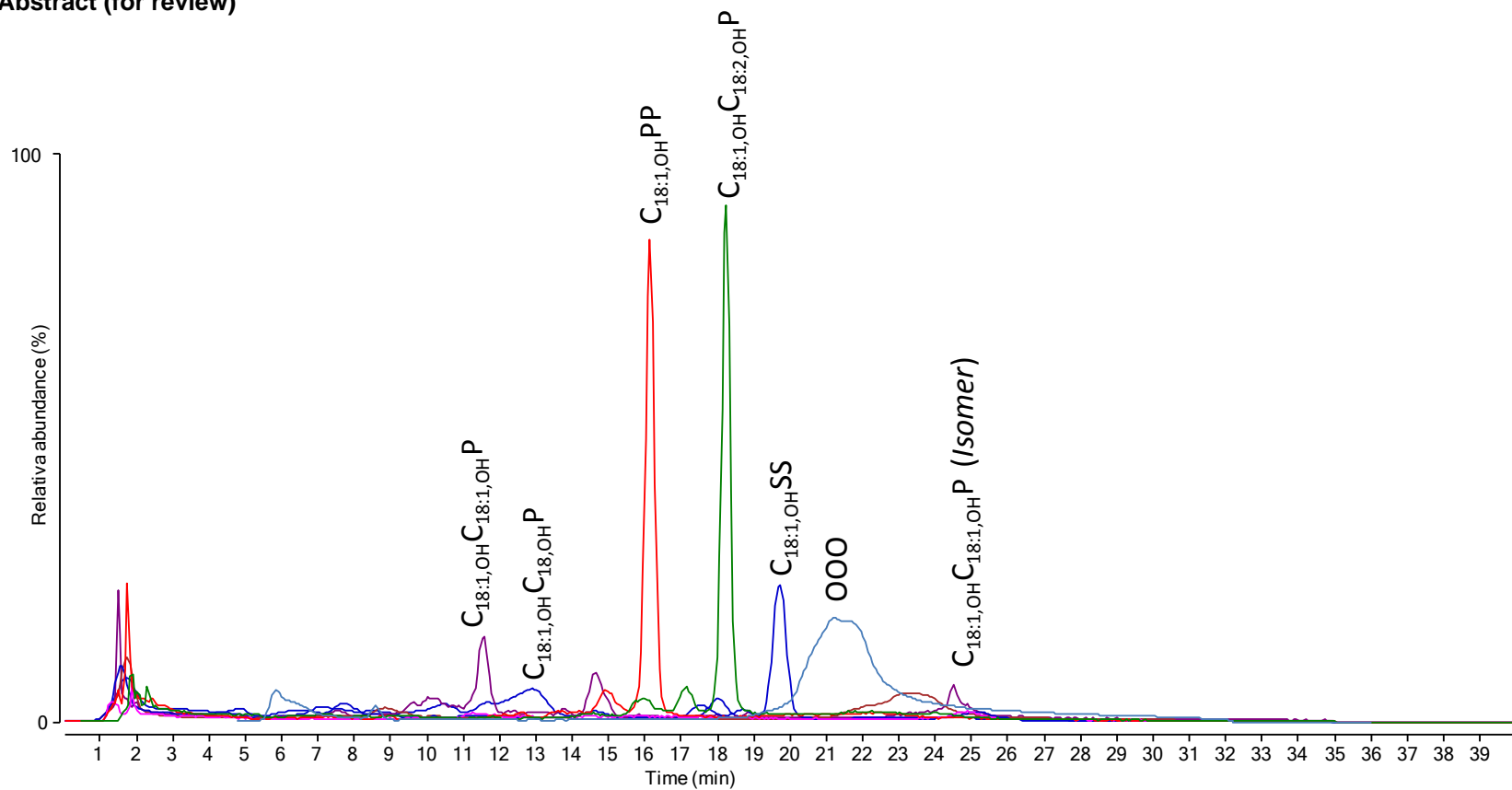
Highlights

We applied a multi-analytical approach based on mass spectrometry

We complemented the GC/MS study by performing TAGs profiling

We applied HPLC-ESI-Q-ToF for the TAGs profiling of aged oil paint layers

We validated a method for the identification of TAGs in modern oils paint samples

Model study of modern oil-based paint media by triacylglycerols profiling in positive and negative ionization modes

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Abstract

Traditionally, the determination of lipid binders in paintings has relied on evaluating characteristic profiles and ratios of fatty acids amounts by gas chromatography/mass spectrometry (GC/MS). The presence of mixtures in contemporary and modern oil paints makes the GC/MS determination of fatty acids not sufficient to fully characterize lipid binding media. In this study, we prove that a precious contribution to this issue can be achieved by triacylglycerols (TAGs) profiling by high-performance liquid chromatography with high-resolution tandem mass spectrometry, using ESI in positive and negative ionization modes. We exploited this analytical approach to study the curing and degradation processes undergone by six plant oils used in the formulation of media in modern paints, applying both natural and artificial ageing experiments. This is the first time that negative ionization mode has been applied to this purpose and that a survey with HPLC-ESI-Q-ToF is carried out to study the ageing kinetics of plant oils. While the discrimination of the lipid source cannot be achieved by GC/MS analysis, TAGs profiling allowed us to study the evolution over time of the constituents of modern oils, with respect to curing and ageing. The data achieved in this study demonstrate that the proposed analytical approach is efficient to study the oxidation of TAGs during ageing, and improve the current knowledge on the properties of vegetable oils possibly leading to the development of new paint materials and conservation treatments for modern and contemporary artworks.

1. Introduction

TAG (triacylglycerols) profiling by liquid chromatography – tandem mass spectrometry is a fundamental technique for the characterization of vegetable oils and lipids in foodstuffs, cosmetics, pharmaceuticals, and other matrices. Vegetable oils mainly consist of mixtures of triacylglycerols (TAGs), glycerol tri-esters of fatty acids with a variable content of double and triple unsaturated fatty acids. Secondary components include phytosterols, vitamins, etc. TAGs profiling by liquid chromatography based techniques often permits the unambiguous identification of the raw source of the oil, allowing the analyst to assess the occurrence of adulteration or, in general, of modification in the expected composition of the commercial material under study [1-3].

This powerful technique has only rarely been applied to the characterization of lipid materials in the field of cultural heritage, mostly due to the occurrence of reticulation, hydrolysis and oxidation reactions of the original TAGs in aged objects, related to the exposure to heating sources, light, or simply atmospheric oxygen [4-6]. In particular, vegetable oils historically used as paint materials (mainly linseed, walnut and poppyseed oils in Europe) are characterized by a high number of double or triple unsaturated acyl chains, whose presence give rise to polymerization phenomena collectively known as "siccative properties". The curing process alters the TAGs profile, entailing cross-linking and oxidation reactions, and decreases the solubility of the lipid fraction. The identification of lipid materials thus usually relies on the hydrolysis, derivatization, and quantitation of derivatized fatty acids by gas chromatography/mass spectrometry (GC/MS) [7-13]. This approach does not identify TAG molecular species, but only determines the type and amount of individual fatty acids present in the total TAG fraction. The siccative oil is identified, even in aged paint samples, on the basis of characteristic ratios between palmitic acid and stearic acid amounts (P/S). The P/S approach proves successful in discriminating between the traditional paint media linseed, walnut and poppyseed oils, whether no other source of lipids (e.g. wax or egg) is present in the sample, and if no mixture of oils is used [3]. Moreover, GC/MS allows the discrimination between egg lipids and vegetable oils, both historically used as paint binders, on the basis of the fact that aged egg tempera paints contain a significantly lower amount of dicarboxylic acids than aged drying oil films. Dicarboxylic acids, mainly nonanedioic acid (azelaic), accompanied by octanedioic (suberic) and decanedioic (sebacic) acids are indeed formed in relevant amount during curing and ageing of drying oils, rich in polyunsaturated fatty acids, while they are formed only in low amount during the ageing of egg lipids.

When modern art is concerned, the GC/MS determination of fatty acids and dicarboxylic fatty acids is not adequate for the identification the lipid paint media. This is due to the exploitation, by 20th century paint manufacturers, of semi-siccative or even non siccative vegetable oils in their artists' materials, such as sunflower, safflower, soy, castor, coconut, cotton, oiticica, peanut, rapeseed, tall and tung, sometimes mixed together, to partially replace the more expensive traditional drying oils[13]. The introduction of non traditional oils in the industrial formulation of paint media was mainly driven by production sustainability reasons. Moreover, the use of a less siccative binder allowed to produce paints that were more stable during storage, to be sold in tubes. The presence of mixtures and the occurrence of semi-siccative oils, whose fatty acid profiles do not significantly differ from each other and from that of linseed oil, make the identification of these materials, addressed to as "modern oils" by conservation scientists, impossible by GC/MS. In particular, the characteristic P/S ratio of fatty acids amounts for castor, sunflower and tung oil unaged paint layers lie in the range usually assigned to linseed oil (palmitic to stearic acid ratio <1.4), as shown in [13].

In addition, aluminum and zinc stearates acting as surfactants have been frequently included in the formulations of industrial oil paints since the beginning of last century, introducing modifications of the P/S value respect to that of the oil [14].

This is why we explored the triacylglycerol determination approach for the unambiguous characterization of modern oil paints. TAGs profiling of castor, sunflower and tung oil by applying a procedure based on high-resolution tandem mass spectrometry (HPLC-ESI-Q-ToF) with a Poroshell solid-core column proved reliable for their unambiguous identification, even in mixtures or after modifications, and in samples smaller than 0.1 mg [5, 15]. In particular, the interpretation of spectra obtained by tandem mass spectrometry allows the identification of the exact mass of each acylic substituent, from which their chain lengths and degrees of unsaturation can be inferred. The discrimination within isobaric TAGs, e.g. to determine the positions of fatty acid substituents on the glycerol backbone and to localize double bonds within fatty acid chains, represents a more critical analytical task. The application of negative mode ionization for the characterization of the aged oils allowed us to detect the triacylglycerols containing azelaic acid as acyl substituents: the use of high resolution tandem mass spectrometry allowed us to characterize for the first time their fragmentation patterns and to identify the acyl substituents in the acyl chains.

With regard to aged samples, TAGs profiling by HPLC-APCI-MS has been used to study the effects of different oil processing methods in model samples of linseed oil, but no

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complete studies have been published on the modeling of a complete set of oils used in paint manufacturing after ageing [16].

Consequently, although oil paint is still widely used by contemporary and modern artists and conservation of modern oil paintings represents a critical issue in the preservation of contemporary art, there is a general lack of knowledge on the characterization of modern oils as binding media and on their chemical behavior upon photo-ageing [17].

In the present work we took into consideration six oils used by paint manufacturers in modern oil paints: linseed, safflower, soybean, sunflower, tung and castor oils. The fresh oils were characterized by GC/MS to obtain their fatty acids profile and compare the results with literature data. Then, artificial ageing experiments were carried out to study the curing and degradation processes undergone by these oils. The six oils, mixed with titanium white and casted as paint films, were first subjected to curing, achieved by exposing the oils to natural light (indoor) for 3 months. The same paint films were then subjected to photo-induced artificial ageing. The set of samples was analyzed both by GC/MS and HPLC-ESI-Q-ToF. The aims of the study were twofold: obtaining reliable data on the kinetics of the curing and ageing processes to devise photo-ageing pathways, and validating the TAGs profiling procedure for the unambiguous identification of modern oils in paintings by the detection of residual and/or oxidized glycerides. Moreover we implemented the analytical information by the application of a new ESI-Q-ToF method operating in negative ionization mode allowed us to characterize for the first time the presence of the triacylglycerols containing azelaic acid as acyl substituent deriving from the oxidation of the oils and to evaluate their abundances variation during the ageing.

2. Materials and methods

2.1 Chemicals

The derivatization agent used for the GC/MS analyses was N,O-bis(trimethyl-silyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (Sigma-Aldrich, USA). The fatty acids stock solution in acetone contained lauric (5.41 mg g^{-1} of Lau), suberic (5.68 mg g^{-1} of Su), azelaic (4.04 mg g^{-1} of A), myristic (3.99 mg g^{-1} of My), sebacic (4.28 mg g^{-1} of Se), palmitic (4.70 mg g^{-1} of P), oleic (6.51 mg g^{-1} of O) and stearic acids (6.43 mg g^{-1} of S). Tridecanoic acid solution in iso-octane, 140.6 µg g^{-1}, was used as acidic and neutral fraction derivatization internal standard. Hexadecane solution in *iso*-octane, 212.9 µg g^{-1}, was used as injection internal standard. All acids and hexadecane, purity 99%, were purchased from Sigma-Aldrich (USA). Standard solutions were used to derive

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calibration curves. Solvents: bi-distilled water (pesticides-grade, Carlo Erba); *iso*-octane, diethyl ether and acetone (HPLC-MS grade, Sigma Aldrich); *n*-hexane, *iso*-propanol and methanol (HPLC-MS grade, Fluka).

2.2. Reference materials

2.2.1. Reference oils

Linseed and safflower oils were purchased from Maimeri (Italy), tung oil from Kremer Pigmente (Germany), castor oil from Zeta Farmaceutici S.p.A (Italy), while sunflower and soybean oils were bought on the local market. Titanium white (TiO_2) pigment was purchased from Zecchi (Florence). The fresh oils were analyzed without any further treatment. For the ageing study, the six oils were individually mixed with titanium white (TiO_2) and cast on glass slides as thin layers. TiO_2 was chosen for two main reasons. It is white, thus allowing the qualitative observation of changes in colour on the paint replicates. Most important, TiO_2 is known to photo-catalyze oxidation processes in organic materials[18]: thus we expect it to accelerate the curing and ageing of the paint references.

2.2.2. Curing and artificial ageing

A first step of curing was achieved by exposing the oils to natural light (indoor) for 3 months. Subsequently, the artificial photo-ageing was performed in a Solar Box 3000e (CO.FO.ME.GRA., Italy), equipped with a Xenon lamp and a UV-IR filter that absorbs wavelengths lower than 295 nm and higher than 780 nm, corresponding to outdoor exposure. Irradiation was set at 700 W/m2 and the maximum temperature on the samples was controlled with a BST, set at 30 °C. The ageing kinetics entailed sampling and analyzing the paint films at different times, after 0, 24, 48, 100, 200, 400 and 600 hours.

2.3. Sample preparation

The GC/MS analytical procedure was derived from the combined procedure previously published [5, 9], modified and adapted for the analysis of the lipid-resinous fraction only. About 1 mg of each sample was subjected to the following procedure: the fresh oils were sampled in liquid form, whereas a portion of the aged paint films was scraped off and subjected to analysis. The analytical procedure entails a saponification assisted by microwaves with 300 µL of KOH in ethanol (10% wt) at 80 °C for 60 min. After saponification, the solution was diluted in bi-distilled water, and the unsaponifiable fraction extracted in n-hexane (400 µL, three times). Subsequently, the residue of the n-hexane

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extraction was acidified with HCl (6 M) and then extracted with diethyl ether (400 µL, three times). The extracts were admixed and an aliquot of the obtained solution, containing both the organic acids and the neutral fraction, was subjected to derivatization with 20 µL of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), 50 µL of iso-octane (solvent), and 5 µL of tridecanoic acid solution (internal standard 1) at 60 °C for 30 min. Finally, 100 µL of iso-octane and 5 µL of hexadecane solution (internal standard 2) were added and a total of 2 µL of the iso-octane solution of derivatized neutral and acid compounds were analysed by GC/MS

The analytical procedure for the HPLC-ESI-Q-TOF analyses was the following: about 1 mg of each sample was subjected to extraction assisted by microwaves with 300 µL of a chloroform-hexane (3:2) mixture at 80 °C for 25 min. The extracts were dried under a nitrogen stream, diluted with 600 µL of elution mixture (90:10 methanol-iso-propanol) and filtered on a 0.45 µm PTFE filter (Grace Davison Discovery Sciences, USA) just before injection.

2.4. Apparatus

2.4.1. Microwave oven

The saponification of lipids for the GC/MS analysis and the extraction for HPLC-ESI-Q-ToF analysis were performed using a microwave oven model ETHOS One (High Performance Microwave Digestion System), power 600 W, Milestone (Italy).

2.4.2. GC/MS instrument and working conditions

The GC/MS analyses were carried out with a gas-chromatograph Trace GC 2000 equipped with a PTV injection port and coupled to a ITQ 900 ion trap mass spectrophotometer (ThermoQuest, USA).

Samples were injected in splitless mode at 280 °C. GC separation was performed on a fused silica capillary column HP-5MS, stationary phase 5% diphenyl- 95% dimethyl-polysiloxane, 30 m length, 0.25 mm i.d., 0.25 µm film thickness (J&W Scientific, Agilent Technologies, USA). The chromatographic conditions were: initial temperature 80 °C, 2 min isothermal, 10 °C/min up to 200 °C, 4 min isothermal, 6 °C/min up to 280 °C, 40 min isothermal. The helium (purity 99.9995%) gas flow was set in constant flow mode at 1.2 mL/min. MS parameters: electron impact ionization (EI, 70 eV) in positive mode; ion source temperature 230 °C; scan range 50-700 m/z; interface temperature 280 °C. The injection volume was 2 µL. The chromatograms were acquired in TIC (total ion current)

mode. The quantitative analysis was performed by normalizing amount of each acid with respect to the internal standard (tridecanoic acid, IS2) and then calculating the relative abundance (%) of each compound for each chromatogram.

Mass spectral assignment was based on the direct match with the spectra of NIST 1.7 and Wiley 275 libraries, and with a library created by the authors for trimethylsilyl derivatives of fatty acids. Comparisons with mass spectra of reference standards, when available, were also made. In the absence of reference spectra, the peak assignment was based on mass spectra interpretation.

2.4.3. HPLC-ESI-Q-ToF instrument and working conditions

The HPLC-ESI-Q-TOF analyses were carried out using a 1200 Infinity HPLC (Agilent Technologies, USA), coupled by a Jet Stream ESI interface (Agilent) with a quadrupole-time of flight tandem mass spectrometer 6530 Infinity Q-ToF detector (Agilent Technologies, USA). An Agilent Poroshell 120 EC-18 column (3.0 mm x 50 mm, 2.7 µm) with a Zorbax Eclipse Plus C-18 guard column (4.6 mm x 12.5 mm, 5 µm) was used for the chromatographic separation. The injection volume was 3 µL. Working conditions for the positive mode ionization were published in [15] and reported in detail in the Supplementary Information.

The separation was achieved using a gradient of methanol (eluent A) and iso-propanol (eluent B). The elution was programmed as follows: 90% A for 5 min, followed by a linear gradient to 90% B in 25 min, then held for 5 min. The re-equilibration time for each analysis was 10 min. The chromatographic runs were performed at a flow rate of 0.3 mL/min.

The ESI operating conditions for the positive mode ionization were: drying gas (N2, purity >98%): 350 °C and 10 L/min; capillary voltage 4.5 kV; nebulizer gas 35 psig; sheath gas (N2, purity >98%): 375 °C and 11 L/min. High resolution MS and MS/MS spectra were acquired both in positive and negative modes in the range 100-1700 m/z. The fragmentor was kept at 200 V, nozzle voltage 1000 V, skimmer 65 V, octapole RF 750 V and collision energy for the MS/MS experiments was set at 50 V. The collision gas was nitrogen (purity 99.999%). The data were collected by auto MS/MS acquisition with a MS scan rate of 1.03 spectra/s and MS/MS scan rate of 1.05 spectra/s; only one precursor was acquired per cycle (relative threshold 0.010%), active exclusion after 3 spectra and 0.50 min (selection by abundance only). The mass axis was calibrated using the Agilent tuning mix HP0321 (Agilent Technologies) in acetonitrile and water. MassHunter workstation Software

(B.04.00) was used to carry out mass spectrometer control, data acquisition, and data analysis. The relative abundances of the triglycerides were calculated on the integrated areas and normalized to 100% as reported in the literature. For the ageing kinetics, instead, the integrated areas were normalized to the weight (μg) of the sample.

The ESI operating conditions for the negative mode ionization were: drying gas (N_2, purity >98%): 350 °C and 10 L min^{-1}; capillary voltage 4.5 KV; nebulizer gas 35 psig; sheath gas (N_2, purity >98%): 375 °C and 11 L min^{-1}. The tandem mass spectra were acquired in negative mode ionization in the range 100-1700 m/z, with nozzle voltage 1000 V, skimmer 65 V, octapole RF 750 V. For the MS/MS experiment different fragmentor energy were tested in the range 100-200 V. Several voltages in the range 30-100 V in the collision cell were tested for Collision Induced Dissociation, to obtain information on fragmentation pathways of selected analytes. The best results were obtained setting the fragmentor energy at 125 V and the voltage in the collision energy at 30 V. The data were collected by auto MS/MS acquisition with an MS scan rate of 1.03 spectra/sec and an MS/MS scan rate of 1.05 spectra/sec; only one precursor was acquired per cycle (relative threshold 0.010%). The mass axis was calibrated using the Agilent tuning mix HP0321 (Agilent Technologies) in acetonitrile and water.

3. Results and discussion

3.1. GC/MS results

To highlight the different fatty acids profiles of the six oils analyzed, the chromatograms of the extracts of fresh and aged oils after saponification were compared both by a qualitative and a quantitative point of view. The compounds identified are listed in Table S-1. The characteristic palmitic/stearic acids (P/S), azelaic/palmitic acids (A/P) ratios and the sum of the dicarboxylic acids (Σdic) values are reported in Table 1. The relative standard deviation of the amounts of fatty acids (RSD%) calculated performing the analysis in triplicates was lower than 2%. For the fresh oils the results are consistent with literature data both on traditional siccative oils [11] and for modern oils [13]. Thus, no further experiment was performed on raw materials from different crops/manufacturer/suppliers.

As discussed in the introduction, it appears that even in the case of fresh oils the discrimination of the vegetal material employed as binding medium cannot rely on the P/S ratio, being the values of all the investigated oils in the range 0.9-2.0, a range which includes also the P/S ratio of linseed oil (1.3). Within this range, safflower oil featured the highest P/S value (2.0), tung oil and castor oil present the lowest P/S values (around 1),

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while linseed, soybean and sunflower oil have intermediate P/S ratio values, between 1 and 2.

Notwithstanding, fresh linseed, tung and castor oil can be distinguished on the basis of the presence of specific fatty acids acting as molecular markers: linolenic acid, eleostearic acid and ricinoleic acid, respectively.

Walnut oil and egg lipids feature higher values of the P/S ratio: 2-3 for walnut and 2.5-3..5 respectively according to literature data.

When dealing with the cured and artificially aged specimens, identification becomes even more critical. A general trend can be observed, in agreement with the literature, entailing the disappearance and/or strong decrease of unsaturated fatty acids in the aged samples, together with the appearance of dicarboxylic acids (the most abundant being azelaic acid) and hydroxy acids. In particular, polyunsaturated fatty acids (e.g. linolenic and linoleic acids) completely disappear with ageing, while mono-unsaturated fatty acids, such as oleic acid, strongly decrease. The specific molecular markers of linseed and tung oils (linolenic and eleostearic acid, respectively) are absent after ageing. On the other side in aged castor oil, ricinoleic acid, the characteristic marker, is still the most abundant peak in the chromatogram.

The predominance of azelaic acid, formed in all the sample during ageing, together with the presence of 9,10-dihydroxylated acids, is due to the occurrence of unsaturation mainly at position 9, consistent with the presence of large amounts of oleic and linoleic acids before ageing. The azelaic/palmitic (A/P) ratio of all the investigated oils significantly increases after artificial ageing, thus proving that the oxidation of unsaturated fatty acids has occurred in the reference films. Another index of oxidation is the sum of the relative content of dicarboxylic acids (Σdic), which is usually very high in aged siccative oils in respect of raw oils. Amongst the investigated oils, aged linseed Tung, safflower and sunflower all show a Σdic>40% after ageing. Soybean and castor oils have the lowest values, being non-siccative

With regard to the P/S ratio in the aged specimens, we observed that this value does not show a relevant change in the curing process. The changes in the P/S ratios during ageing are within the 15% of the original value. The values of this ratio for all the cured and artificially aged oils still lies in same the range of linseed oil, with slightly lower values for tung oil and castor oil.

We can conclude that the assignment of the type of siccative oil in a modern or contemporary paint, where the use of a modern oils cannot be excluded, shall not be based on the evaluation of the P/S ratio.

3.2. HPLC-ESI-Q-ToF results

We performed an ageing kinetics by analyzing linseed, sunflower, safflower, soybean, tung and castor oil paint films at different ageing times, after 0, 24, 48, 100, 200, 400 and 600 hours of artificial ageing in a Solar Box. To our knowledge, this is the first time a survey with HPLC-ESI-Q-ToF on the ageing kinetics of these oils is performed. We aimed both at studying the ageing pathways of triacylglycerols by determining intermediates, and at identifying reliable molecular markers for the unambiguous identification of the raw oils. The results obtained with the application of this analytical approach allowed us to build a new database of acyl glycerides species that can be used to identify the analyzed oils used as binding medium alone or in mixture with other oleochemicals, e.g. surviving TAGs, or oxidized species.

The oils were compared at the seven different ageing times, and the results are summarized in Figure 1 as histograms, which highlight the variation of the normalized abundances of the different TAGs. The identified specific molecular markers detected in positive mode ionization present after accelerated curing and ageing of the paint layers are summarized in Table 2.

For all the oils analyzed the amount of TAGs detected in positive mode in the extracts decreased with the ageing time. This category includes the original saturated and unsaturated TAGs plus those containing hydroxylated acyl chains, formed during photo-ageing. This decrease is mainly due to the cross-linking reactions taking place during the curing of the oils, which prevent the solubilization of the TAGs and their consequent detection, but it is also related to the oxidation of the hydroxylated acyl chains, leading to the formation of shorter, acidic acyl chains. The TAGs containing those chains are detected in negative mode only, and even if not very abundant, might be used in the kinetic study.

After curing (t0), the TAGs detected in positive mode from castor are the most abundant (in Figure 1 for the positive experiment we arbitrarily assigned 100% to total TAGs in castor t0, taken as reference to compare the samples), followed by soybean (60%), while for all the other films, extractable represent less than 10%. At the same time, the soluble fraction detected by the negative mode ionization increases during the ageing. After the

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curing step (t0), the amount of soluble TAGs detected in negative mode is in the range 1.1-8.7 %: castor oil, characterized by the lowest amount of triacylglycerols with unsaturated acyl chains, is characterized by the lowest amount of extractable triacylglycerols detectable in negative mode, while linseed and tung, characterized by an high amount of triacylglycerols containing double bonds, by the highest amount (in Figure 1 for the negative experiment we arbitrarily assigned 100% to total TAGs in linseed t6, taken as reference to compare the samples).

The chromatograms acquired in positive ionization mode of the extracts after 600 hours of artificial ageing are reported in Figure 2. The chromatograms were obtained by overlapping the extract ion chromatograms of all the identified TAG species. It must be noted that in the extract ion chromatograms more than a peak corresponding to a raw formula can be present, corresponding to positional isomers of the same TAG specie. The identification of the un-oxidized species was performed by comparison with the literature, and the acyl chains in the oxidized species were identified by interpretation of the tandem mass spectra. Although the sn-1 and sn-3 losses are energetically more favored than the loss in the sn-2 position and thus the corresponding diacylglicerol ions are more abundant in the MS/MS spectrum, it is not possible to assign the position of the acyl chains in absence of a proper standard, and also because the co-elution of positional isomers cannot be ruled out. It is also important to note that the interpretation of the tandem mass spectra of sodiate adducts does not allow the identification of epoxy groups or the position of double bonds [19]. Nonetheless, we considered the hydration of the double bonds as the main ageing processes undergone by TAGs, and proposed tentative formulas for oxidized species are reported in Table S-2.

Figure 3 shows the chromatogram acquired in negative mode ionization obtained for sunflower oil, and the mass spectra obtained for AzSO and AzAzP. The chromatograms obtained for the six reference oil are characterized by the presence of the same species with different relative abundances. Triacylglycerols with one or two α,ω -nonandioaic acid (azelaic acid) substituents bound to the glycerol are the most abundant species detected in negative mode in all the samples after ageing. The fragmentation patterns of the triacylglycerols containing one and two azelaic acid substituents are described by Table S-3 and S-4 respectively.

A general trend can be observed comparing the chromatograms of the oils at different ageing times: the content of the TAGs containing unsaturated fatty acids decreases in the aged ones, with the increase in oxidized TAGs (detected both in positive and in negative

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modes), consistently with GC/MS data. Most interestingly, even after the oxidation and reticulation processes have occurred (curing and ageing), some oils can still be unambiguously identified thanks to the presence of specific molecular markers or to the distribution of oxidized triacylglycerols detectable in positive mode, as discussed in the following paragraphs.

3.2.1 Linseed oil

In linseed oil POP, OOP, OOO, OSP, OOS, SOS, $C_{18:2,OH}$OP, $SC_{18:1,OH}$O strongly decrease from the less aged to the more aged oils. The abundance of the highly oxidized TAGs detected in negative mode increases with ageing time, as expected, and consistently with GC/MS data (Figure 1). The amount of the intermediate oxidation product $SC_{18:1,OH}$P (or positional isomer), increases and then decreases until it disappears completely in the most aged samples (t6). $C_{18:1,OH}C_{18:1,OH}$P, produced by oxidation of LLP, is present only in the most aged films (Figure 2a). Interestingly, in linseed oil films none of the original TAGs containing three polyunsaturated fatty acids (linolenic and linoleic acids) was detected, not even in the less aged film (t0). It must be noted, however, that the hours of ageing are referred exclusively to the hours of artificial ageing, thus the oils labeled as t0 or "0 hours" were not fresh, but they were left to cure for three months before the insertion in the Solar Box. Therefore, after three months of natural drying, the polyunsaturated fatty acids of the TAGs in linseed oil were already oxidized. Numerous oxidized triacylglycerols were characterized by two double bonds and two hydroxyl groups, whose presence can be related to the oxidation of triacylglycerols characterized by three double bonds in the acyl chain. Nonetheless, linseed oil can be unambiguously identified by the presence of PLnP even after a strong oxidation and reticulation has occurred. The Ln acyl substituent is still present in this TAG after ageing, most probably due to steric hindrance that prevented the oxidation in sn-2 position.

3.2.2 Sunflower oil

Sunflower oil (Figure 2b) shows a very similar ageing behavior to that of linseed oil, featuring the decrease of the TAGs containing unsaturated fatty acids (POP, OOP, OOO, OSP, OOS, SOS, $C_{18:1,OH}$OO) during ageing. Like in linseed oil, in sunflower oil paint none of the TAGs containing three polyunsaturated acyl chains was detected, even if the most abundant fatty acid in the extract of fresh sunflower is linoleic acid. The presence of $C_{18:1,OH}C_{18:2,OH}$P derives from the oxidation of linolenyl acyl substituents contained in the

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un-oxidized triacylglycerol LLnP. No specific biomarker of this oil can be detected in the chromatogram of the aged specimen; as expected, the distribution of oxidized TAGs is typical of an oil with an high linoleic acid content and the abundance of the highly oxidized TAGs detected in negative mode increases with ageing time (Figure 1).

3.2.3 Safflower oil

The analysis of safflower oil (Figure 2c) shows the decrease of the TAGs containing unsaturated fatty acids, with the presence of two early oxidation products: $C_{18:2,OH}SP$ and $C_{18:1,OH}SS$ (or their positional isomers). Unlike linseed and sunflower oils, the extracts of the less aged safflower oil paint layer contains un-oxidized TAGs and is characterized by a low amount of highly oxidized TAGs detected in negative mode (Figure 1). This may be due to the semi-siccative nature of safflower oil, which also accounts for the slow decrease in the total amount of extractable TAGs (see the histogram in Figure 1). Consistently, even after 600 hours of artificial ageing, un-oxidized OOO and OOS can be detected along with $C_{18:1,OH}C_{18:2,OH}P$ deriving from LLnP. The origin of other oxidized triacylglycerols is unclear, unless we hypothesize the occurrence of transesterification reactions.

3.2.4 Soybean oil

The chromatogram of soybean oil (Figure 2d), like that of safflower oil, shows TAGs containing linolenic and linoleic acids (LnLnP, LLnP) in the less aged paint layer, pointing out the semi-siccative nature of the oil. As expected, the TAGs containing unsaturated fatty acids, such as LnLnP, LLnP, $C_{18:2,OH}OP$, $C_{18:1,OH}OP$, POP, OOP, OOO, OSP, OOS, SOS, strongly decrease during ageing and, at the same time, an increasing of the amount of triacylglycerols with a carboxylic groups can be observed (Figure 1). The aged soybean oil is characterized by the presence of an high amount of $PC_{18:1,OH}P$ (deriving from PLP), $C_{18:1,OH}SS$ and $C_{18:1,OH}C_{18:2,OH}P$ (probably deriving from LSS and LLnP, present in traces in the fresh oil), which are characteristic of an oil with an high linoleic acid content.

3.2.5 Tung oil

For tung oil, the TAGs detected and identified were fewer, compared to the other five oils analyzed (Figure 2e). Moreover, the decreasing trend of the TAGs containing unsaturated fatty acids is less evident, characterized by a drastic variation in abundance only after 100 hours of ageing; besides, the rate of formation of triacylglycerols containing azelaic acid detected in negative mode appears lower.

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The most aged tung oil film oil was characterized by the presence of $C_{18:1,OH}OP$ and $C_{18:2,OH}SP$, whose origin is unidentified, $C_{18:1,OH}OO$, deriving from OOL, $C_{18:1,OH}C_{18:2,OH}P$, $C_{18:2,OH}C_{18:2,OH}P$, deriving from LLnP and LnLnP respectively, and $C_{18:1,OH}C_{18:1,OH}P$, oxidation product of LLP. We identified an oxidized TAG whose mass and fragmentation point to a $C_{18:1,OH}C_{18:2,OH}P$ structure, whose un-oxidized form is not clearly assignable. Interestingly, $C_{18:1,OH}C_{18:2,OH}P$ identified in tung oil has a different retention time respect to the isobaric TAG (positional isomer) identified in the other aged oils. Thus, it is a molecular marker specific for tung oil, most probably deriving from the oxidation of EEE, the molecular marker of this oil as reported in [15].

3.2.6 Castor oil

In castor oil (Figure 2f), the TAGs containing ricinoleic acid (Rn) decrease from the less aged to the more aged samples. Even if the amount of TAGs extracted in the most aged paint layer is very low, the oil is still well identifiable thanks to the detection of RnRnRn, and RnRnS. Moreover, $C_{18:1,2OH}C_{18:1,2OH}C_{18:1,OH}$ from RnLL and $C_{18:1,2OH}C_{18:1,OH}C_{18:1,OH}$ from RnRnL were identified as characteristic oxidized triacylglycerols of this lipid material (Figure 2f). As for the other oils, the abundance of the highly oxidized TAGs and TAGs containing carboxylic functionalities detected in negative mode increases with ageing time (Figure 1).

3. Conclusions

The application of analytical GC/MS and HPLC-ESI-Q-ToF methodologies enabled the characterization of the triacylglycerol fraction of the oils selected for the study. For each oil, the fatty acids profile after saponification was determined with GC/MS, for both the fresh oils, the naturally cured (3 months) and the artificially aged paint replicas. Our data are consistent with those reported in the literature for the fresh oils [13]: most of them did not show a characteristic fatty acids profile, except for tung and castor oil. The naturally aged paint layers were characterized by very similar profiles, featuring the same dicarboxylic fatty acids and hydroxy fatty acids, with azelaic acid being the most abundant for tung, sunflower, soybean and linseed oils. We proved that the discrimination of the lipid source cannot be achieved on the basis of the P/S ratio, being the values displayed by the investigated oils in the same range as that of linseed oil.

We complemented the GC/MS study by performing TAGs profiling on unaged and aged paint films. An innovative HPLC method to perform analyses on lipids, previously

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developed and optimized, was applied to this purpose. The HPLC-MS-MS analyses in positive and negative mode led to the characterization of the majority of the triacylglycerols constituting the oils before ageing, and of several oxidized triacylglycerols containing hydroxylic and carboxylic fatty acids in the aged specimens, some of them being intermediate oxidation products. The results of this survey have permitted to describe the changes in the triacylglycerol composition of oils during ageing highlighting:

- a decrease of TAGs containing unsaturated fatty acids;

- an increase in oxidized TAGs;

- a decrease of the total amount of TAGs detected in positive mode ionization;

- a increase of the total amount of TAGs detected in negative mode ionization.

Most important, our HPLC-MS-MS study allowed us to obtain a deeper knowledge of the TAGs constituting the oils used in modern paints and of their evolution over the time with respect to the curing process. We validated a reliable method for the unambiguous identification of these oils not only in paint samples, but also in historical materials collected from manufacturers' archives or ateliers, as long as surviving oxidized TAGs are extractable from the paint matrix. Moreover, the application of ESI in negative mode ionization allowed us to identify for the first time triacylglycerols containing azelaic acid as acyl substituent and to described their fragmentation patterns.

4. Acknowledgements

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Figure captions

Figure 1 - Total extracted TAGs for each oil (negative and positive mode), normalized towards sample weight and extract weight and presented as normalized to the highest amount amongst all the extracts (i.e. TAGs in castor oil, t0, for positive mode, and TAGs in linseed oil, t0, for negative mode). From top left to right: linseed oil, sunflower oil, safflower oil, soybean oil, tung oil, castor oil.

Figure 2 - Extracted ion chromatograms of the extracts of the oils after artificial ageing obtained in positive mode: a) linseed; b) sunflower; c) safflower; d) soybean; e) tung; f) castor.

Figure 3 - Extracted ion chromatograms acquired in negative mode of the most abundant triglycerides for Sunflower oil (a) and tandem mass spectra and fragmentation pattern of AzAzP (b) and AzSO (c).

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Table 1 - Characteristic fatty acids values observed in fresh, cured, and artificially aged (aa) oils. * in brackets, the literature range of values reported for the identification of lipid binders [10]; P: palmitic acid, S: stearic acid, A: azelaic acid; dic: dicarboxylic acids.

	Safflower oil	Soybean oil	Tung oil	Sun-flower oil	Castor oil	Linseed oil*	Walnut oil*	Egg*
P/S fresh oil	2.0	1.3	0.9	1.5	1.0	1.3 (1-2)		
P/S cured oil	1.8	1.5	0.7	1.1	0.9	1.1 (1-2)	(2-3)	(2.5-3.5)
P/S aa oil	2.0	1.5	0.9	1.3	1.0	1.3 (1-2)		
A/P cured oil	1.6	0.5	1.8	0.6	0.0	1.0 (>1)	(>1)	(<0.3)
A/P aa oil	2.1	0.6	3.2	1.4	0.2	1.4 (>1)		
Σdic cured oil	49.7	23.9	40.4	24.1	1.6	35.1 (39.3-46.3)	(34.1-42.3)	(10.1-13.7)
Σdic aa oil	59.0	26.6	57.7	43.4	8.6	44.9 (39.3-46.3)		
Molecular markers			Eleostearic acid (fresh oil)		Ricinoleic acid	Linolenic acid (fresh oil)		Cholesterol, proteins

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Table 2 - Specific molecular markers for the aged oils, detected after 600h of artificial ageing using positive mode ionization.

Oil	Original TAGs still detected after ageing	Oxidised species (positive mode)
Safflower oil	OOO OOS	$C_{18:1,OH}PP$ $C_{18:1,OH}C_{18:2,OH}P$ $C_{18:1,OH}SS$ $C_{18:2,OH}C_{18:2,OH}P$ $C_{18:1,OH}C_{18:1,OH}P$
Tung oil	-	$C_{18:1,OH}OP$ $C_{18:2,OH}SP$ $C_{18:1,OH}OO$ $C_{18:1,OH}C_{18:2,OH}P$ $C_{18:2,OH}C_{18:2,OH}P$ $C_{18:1,OH}C_{18:1,OH}P$
Soybean oil	OOO	$C_{18:1,OH}C_{18:1,OH}P$ $C_{18:1,OH}C_{18,OH}P$ $PC_{18:1,OH}P$ $C_{18:1,OH}C_{18:2,OH}P$ $C_{18:1,OH}SS$
Sunflower oil	-	$C_{18:1,OH}C_{18:1,OH}P$ $C_{18:1,OH}C_{18,OH}P$ $C_{18:1,OH}PP$ $C_{18:1,OH}C_{18:2,OH}P$ $C_{18:1,OH}SS$ $C_{18:2,OH}C_{18:2,OH}P$
Castor oil	RnRnRn RnRnS	$C_{18:1,2OH}C_{18:1,2OH}C_{18:1,2OH}$ $C_{18:1,2OH}C_{18:1,OH}C_{18:1,OH}$
Linseed oil	PLnP POP OOP OSP	$C_{18:1,OH}C_{18:1,OH}P$ $C_{18:1,OH}C_{18:1,OH}P$ $C_{18:1,OH}C_{18:1,OH}S$ $C_{18:2,OH}C_{18:2,OH}P$

Figure1
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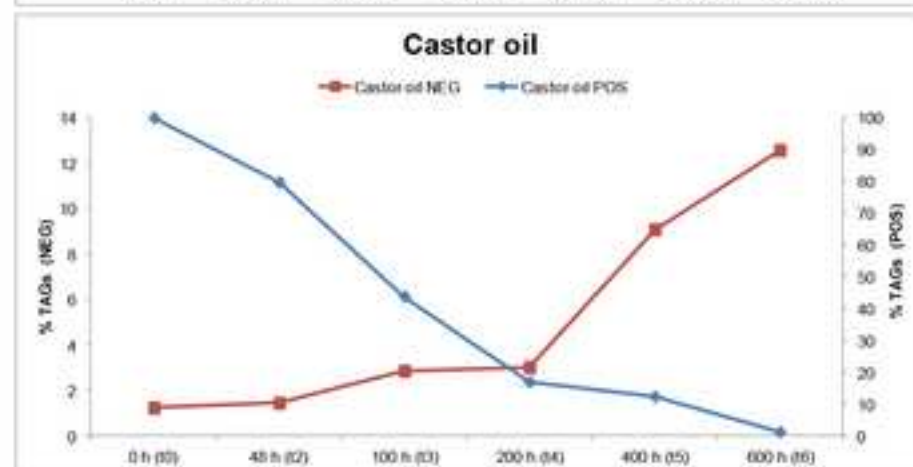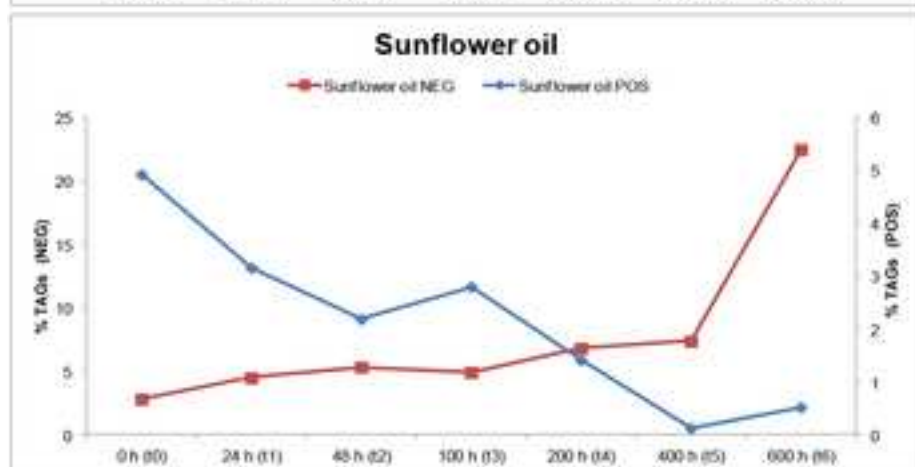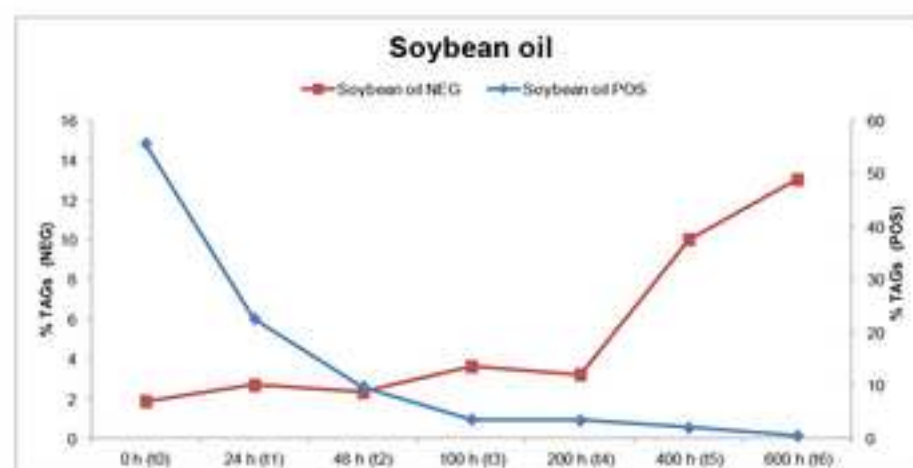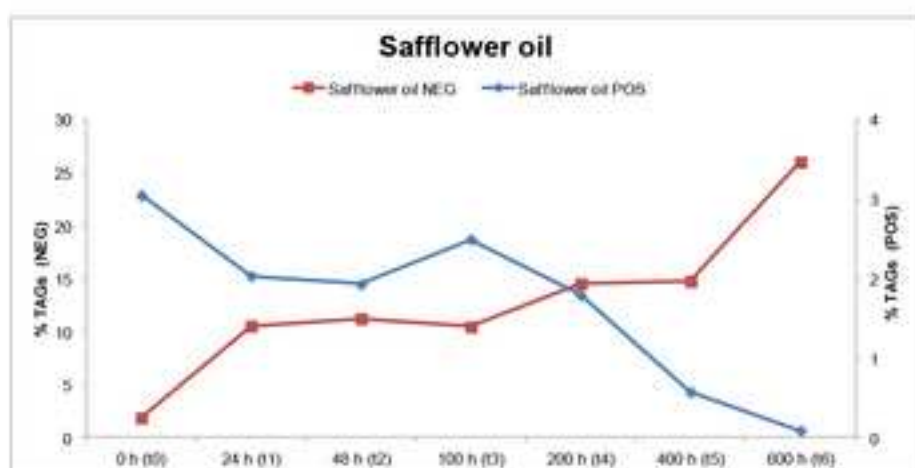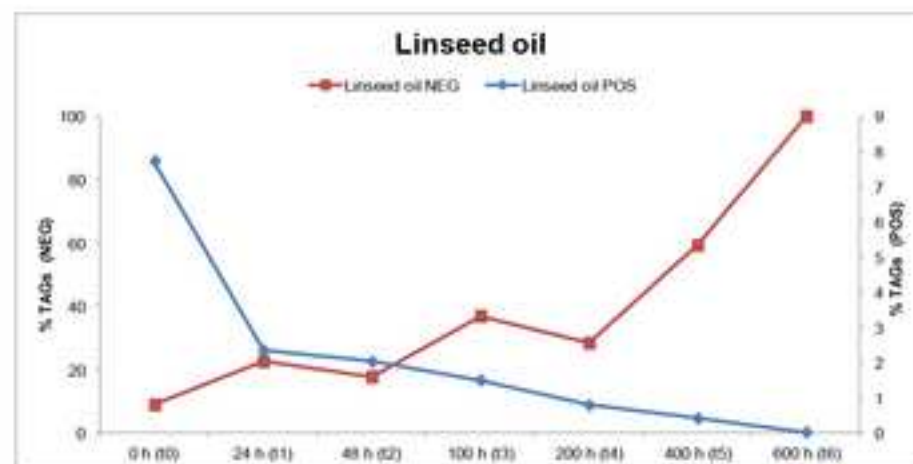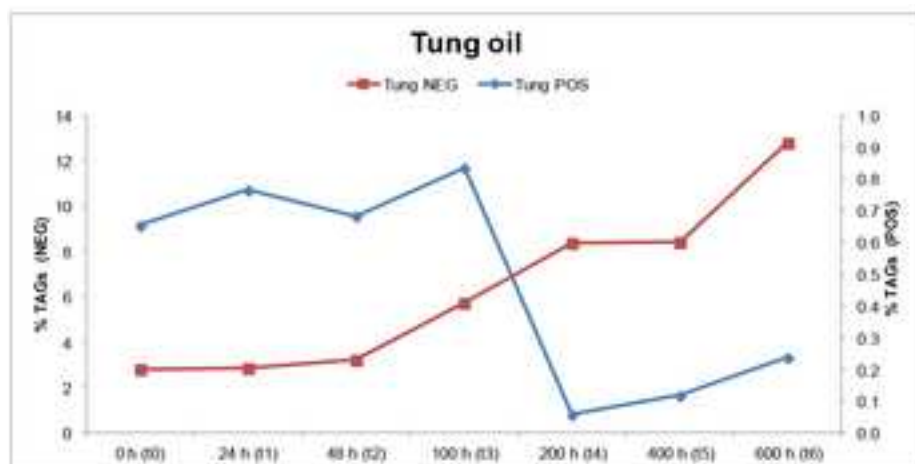
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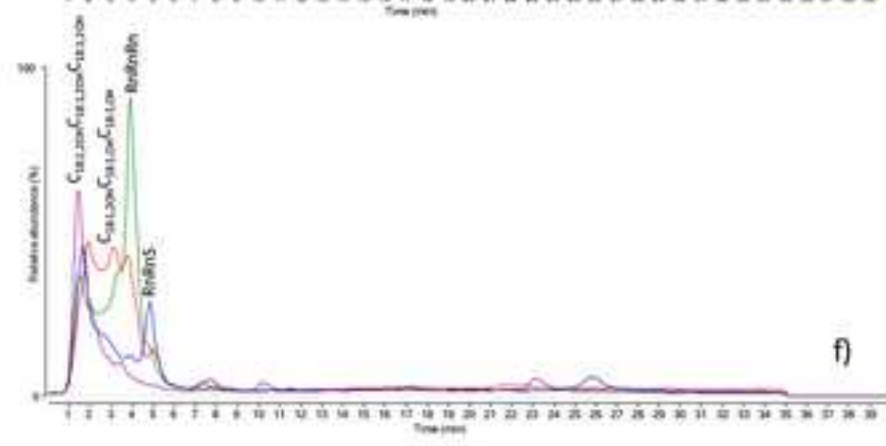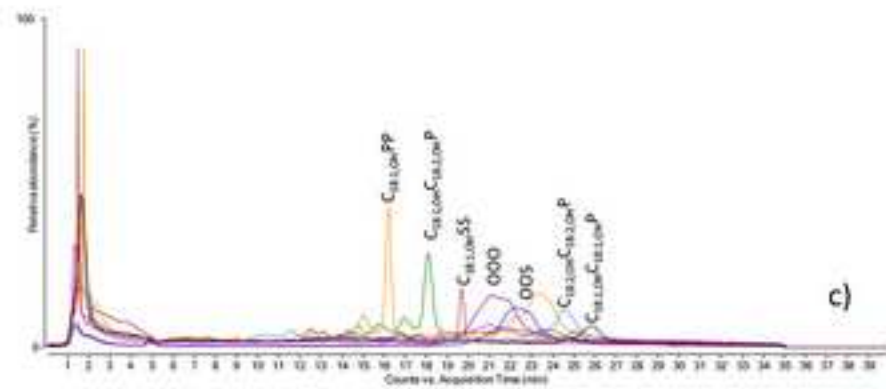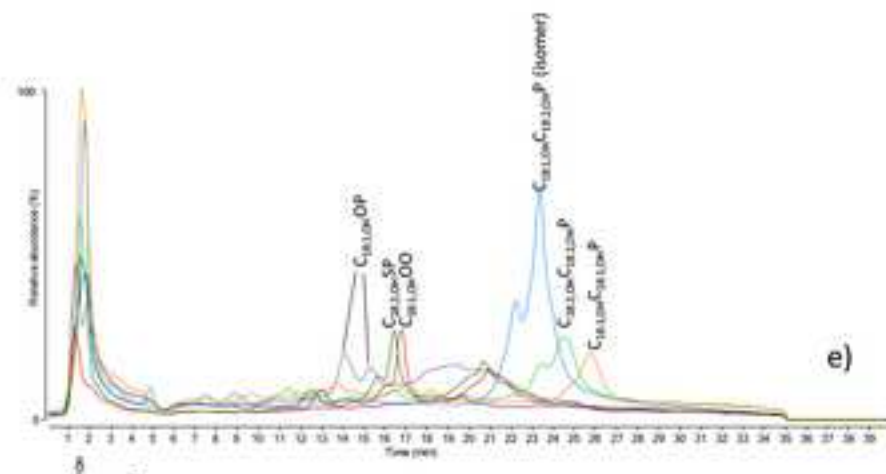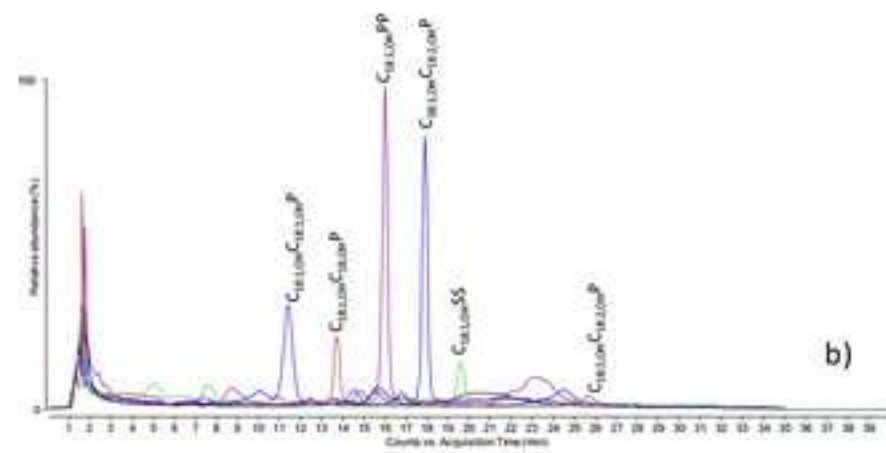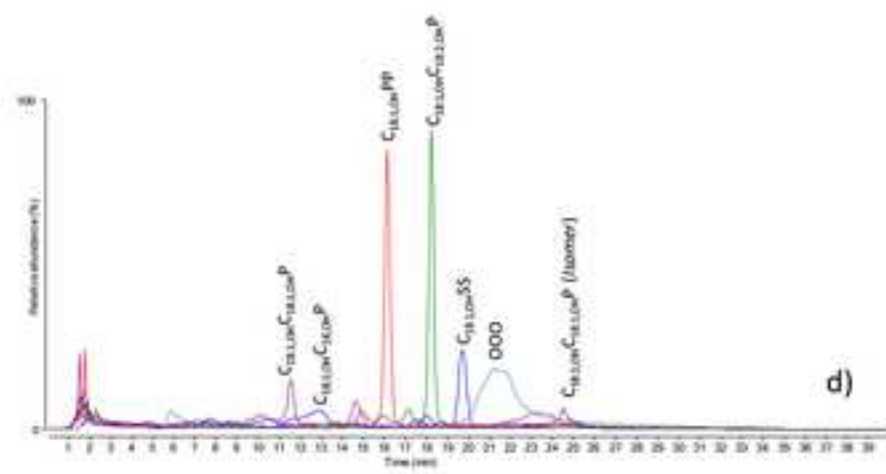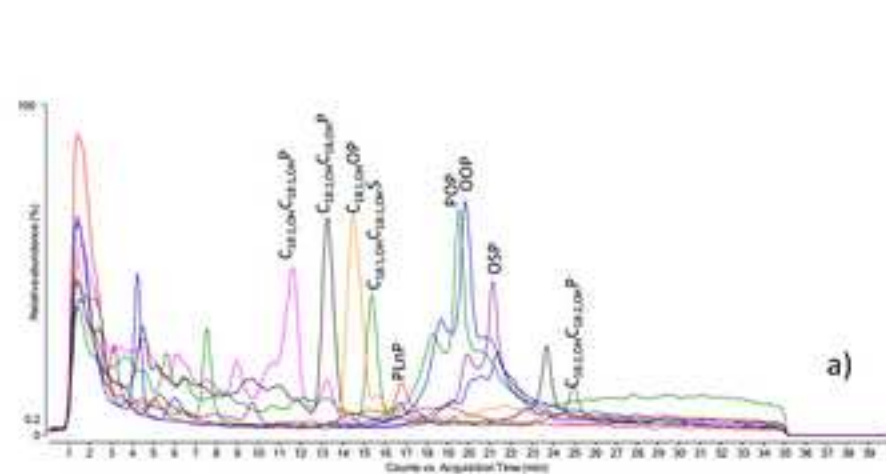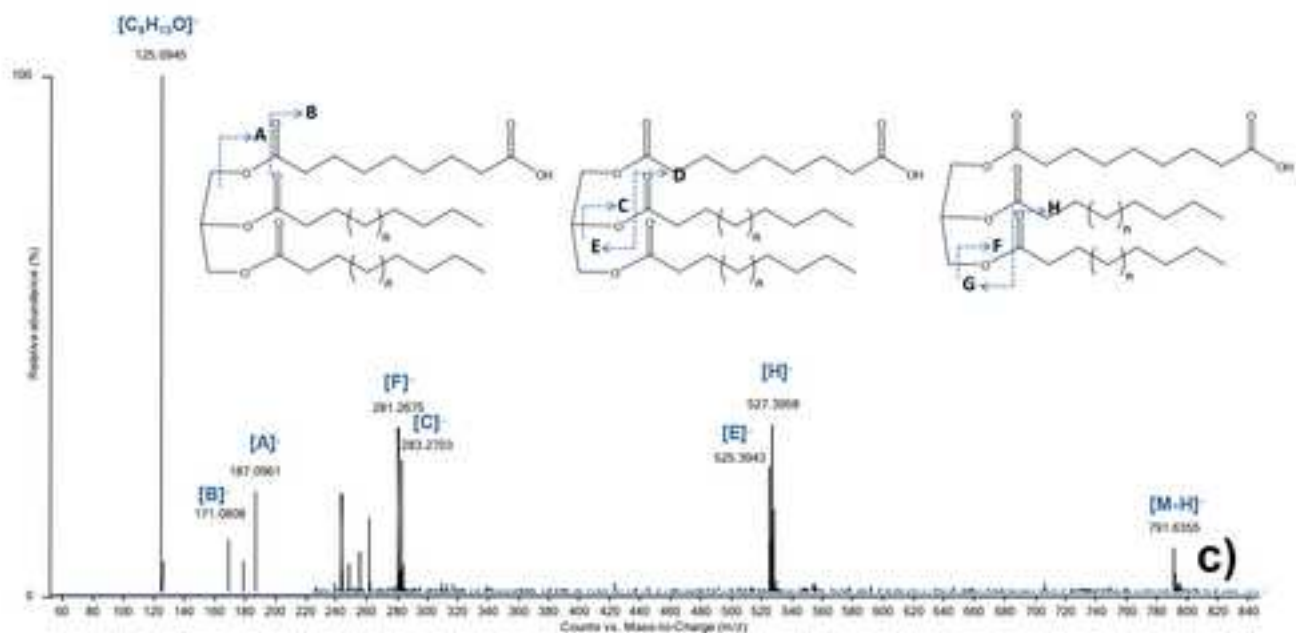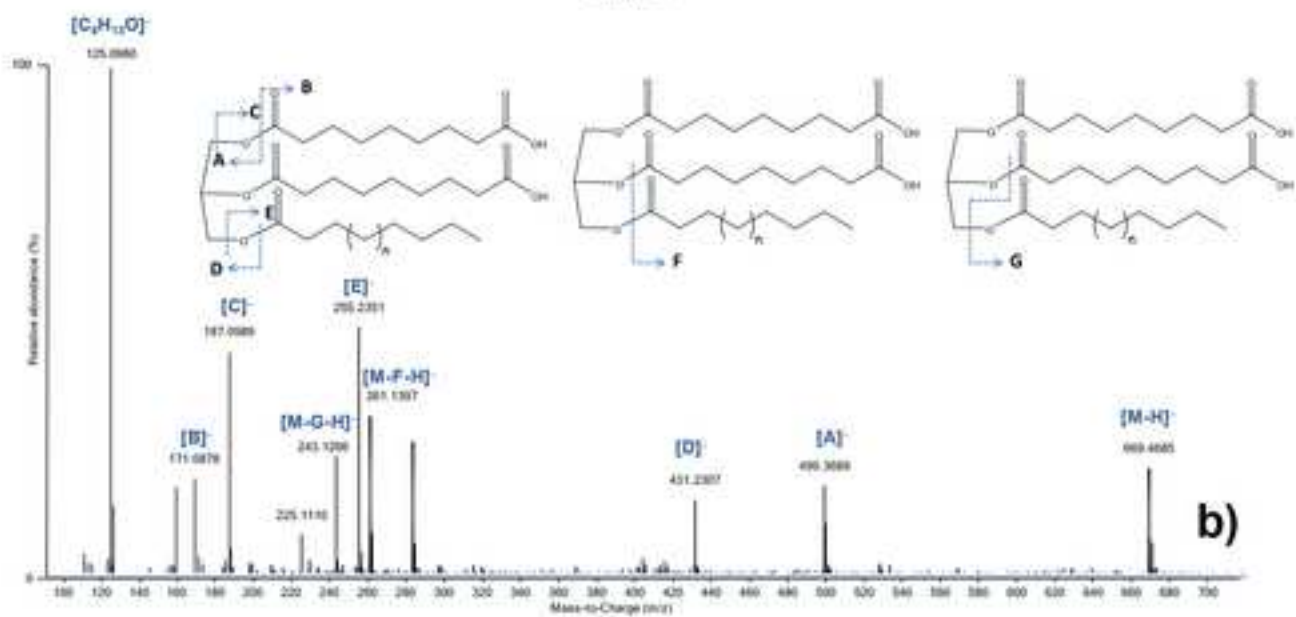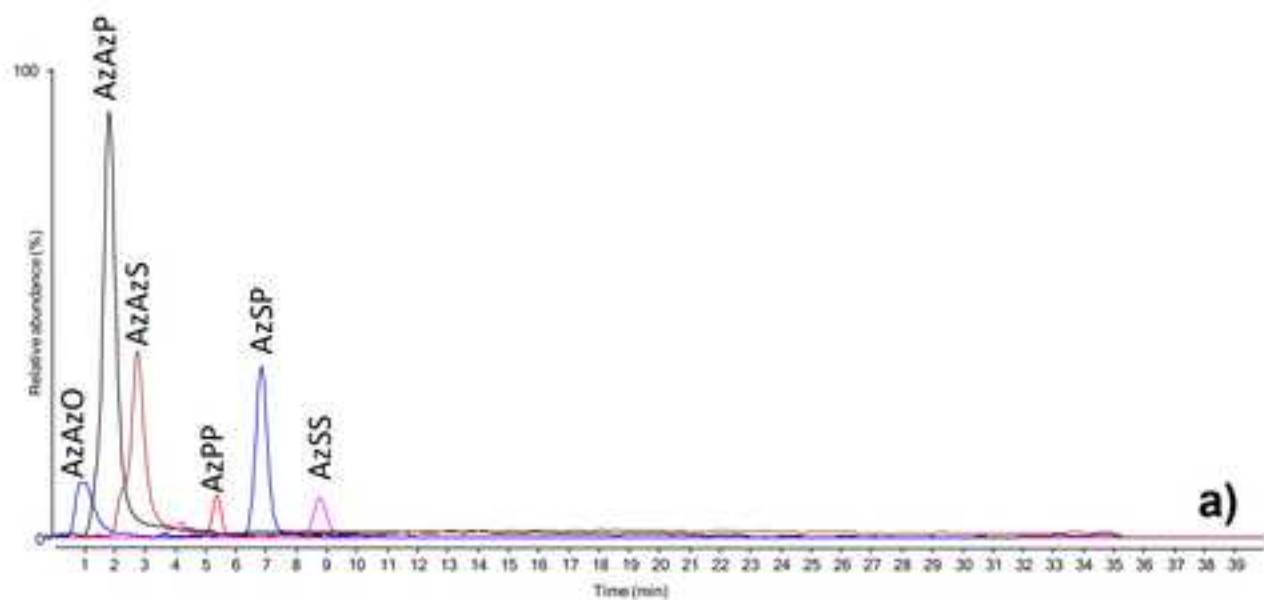
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