

MOLECULAR TRACING OF THE BIOLOGICAL ORIGIN OF DRYING OILS USED IN WORKS OF ART

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Abstract

Until recent times, artists have used a variety of binding media obtained from biological materials of animal or plant origin. Seed or nut oils capable to form a protective film when drying were an essential component of paints. Current methods such as gas chromatography coupled with mass spectrometry allow identifying biological origin of a specific drying oil, but chemical composition is a highly variable factor and can change with plant cultivar, soil, season or weather conditions of harvesting increasing the possibility of error. In addition, mixing oil from different biological species can mislead the analyses because it alters the composition of chemical compounds used in species identification. We report the development of a protocol for the identification of the biological source of drying oils used to prepare binding media for paints. Chloroplast DNA sequences were successfully amplified from tiny oil samples, both fresh and from aged pictorial models. Sequencing of the trnL-trnF intergenic spacer allowed to unequivocally identify the plant species from which the drying oils were derived. Our method shows high specificity and sensitivity and can provide new insight for studying ancient artwork by tracing the origin of the drying oils used by the artists.

Keywords: PCR, Chloroplast DNA, Binding media, Drying oils

Introduction

For millennia, artists have used a variety of biological materials of animal and plant origin, selecting them from those at hand, in nature or in agricultural products, and according to their technological ability to extract and use them in a satisfactory way, considering both the aesthetical outcome and the durability of the artwork [1, 2]. Therefore, a close relationship exists between the biological origin of a material used in a work of art and the period and place in which the work was created. Data on biological materials can be very useful to ascertain the authenticity of the artwork and the trade routes of the materials used to achieve them. Detailed knowledge of the materials is also important for a correct conservation of the artworks. From the fourteenth to the twentieth century, across Europe, the binders were obtained from biological substances such as animal glues, egg, milk and casein, or drying oils extracted from plant seed [1, 3]. Drying oils were also used in the Americas but they were extracted mainly from insects [4].

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The biological and chemical characterization of the natural organic substances in paints has been the object of increasing interest in the last decade thanks to the development of new analytical approaches operating in a totally noninvasive or micro-destructive way [5, 6]. Many efforts have been undertaken to develop combined analytical procedures capable to provide detailed information on the organic components used in artworks. However, the identification of binding media is still challenging because of their intrinsic chemical complexity, generally a mixture of organic substances containing the same common elements, and their pronounced tendency to degrade [1, 5].

The most traditional techniques for the analysis of binders in works of art is molecular spectroscopy, particularly the vibrational ones, and chromatographic methods. The most applied spectroscopy method has been the Fourier transforms infrared (FTIR) technique [7-10], that gives information on the chemical composition of the binder, allowing distinguishing different classes such as protein, polysaccharide and lipid components, due to its ability to recognize various functional groups. This technique allows to quickly analyzing very low amount of samples scratched off artworks (less than 0.5mg) [8]. By coupling FTIR spectroscopy with microscopy it is also possible to perform a stratigraphic localization of binders in the cross-sections of micro-samples taken from the paintings [9]. The main drawback of this technique is lack of specificity since the discrimination among proteins (egg, milk, and animal glue) and lipids (egg yolk and siccative oil) is not achievable.

The Micro-Raman spectroscopy, so as FTIR, allows analyzing, in a very fast way, micro-samples, both untreated or in cross-section, distinguishing only the various classes of binders [11, 12]. The application of chemometric techniques to Raman spectra of lipid paint binders proved to be a valid tool for their identification and characterization [13].

Currently, chromatographic analysis appears to be the best method to obtain detailed information on the large class of organic compounds used in artworks [14-16]. Gas chromatography coupled with mass spectrometric (GC-MS) detection and high performances liquid chromatography (HPLC) with either mass spectrometric or spectroscopic detection (UV-Vis absorption or fluorescence) have been developed to distinguish among different proteins (egg, animal glue, milk or casein) on the basis of quantitative determination of their amino acidic profile [14, 16, 17]. A complementary technique to chromatographic analyses is Matrix-Assisted Laser Desorption/Ionization-Time of Flight-MS (MALDI-TOF-MS) that recently allowed to determine the presence of animal glue in paint, by coupling this technique with tandem MS (MS/MS) experiments (TOF/TOF analysis) for identification of selected peptides [18, 19]. A newly developed LC-ESI-MS method is applied for the study of aged drying oils and oil paints [20].

In recent years, proteomics procedures have been developed for the characterization of proteinaceous binders in cultural heritage samples [18, 21-29] but they need further improvement for routine applications. Recently, immunochemical approaches have been successfully applied to characterization of proteinaceous binders as simple, cost-effective and versatile alternative method for recognizing different proteins and their biological source in paint materials [30-37].

At present, the best analytical tool for identifying drying oils is GC-MS, that exploits either the palmitic-to-stearic acid ratio [1, 16, 13], or eight fatty acids markers [13, 18], or combination with chemometrics [38]. By these methods it is possible to distinguish the three main plant species used as source of drying oils in use in European painting: linseed (*Linum usitatissimum L.*), poppy (*Papaver rhoeas L.*) and walnut (*Juglans regia L.*). The main drawback of these methods is the need of complex sample pretreatment processes to perform chromatographic analyses that partially reduce the information content related to their biological sources. Very recently, MALDI-TOF-MS was applied to lipid fingerprints in micro-samples taken from artworks. The presence of specific markers allowed unambiguous

discrimination between egg- and siccative oil-based binders and a simultaneous identification of lipid and proteinaceous binding media [39].

In an effort to develop more specific analytical methods for the identification of organic binders, DNA analysis has been proved to be suitable for the identification of animal glue origin [40, 41]. However, DNA- based identification of drying oils has not been reported to date.

Since the works of art are often subjected to various biotic and abiotic processes resulting in fragmentation of DNA it is crucial to select genomic sequences that are both easy to detect and capable to discriminate the species of interest. Generally, plastid DNA is considered a good candidate due to very high copy number in plant cells, which partly offsets the problems of small sample size and DNA degradation, as is the case for ancient art specimens [42]. In addition, due to their low mutation rate and prevalent maternal inheritance, chloroplast DNA markers are phylogenetically very informative at the plant family level.

Several studies have shown that the non-coding regions between genes are the most rapidly evolving sequences of the chloroplast genome and are therefore suitable for identifying phylogeny, even at the subfamily level [43-45].

In this work, we developed a protocol for the molecular identification of the biological origin of drying oils from micro-samples of fresh and aged pictorial oil standards, by analyzing intergenic sequences of the plastid genome. This bio-molecular method is compared with the traditional ones, such as infrared spectroscopy and GC-MS, for the analysis of drying oils.

Experimental

Plant seeds and oils

Seeds of red poppy (*Papaver rhoeas*) and flax (*Linum usitatissimum*) (Table 1) were provided by the Institute of Plant Genetics and Crops Plant Research (IPK Institute, Leibniz, Germany). Seeds were germinated and plantlets grown on a soil mixture in a growth chamber grown at 22°C with a light/dark cycle of 16/8h, respectively. Walnut leaf samples were provided by Institute of Biosciences and Bioresources (IBBR, Perugia, Italy).

Linseed, walnut and poppy oils were purchased from Zecchi (Firenze). Drying oils were spread on a Petri dish in daylight to prepare laboratory pictorial samples. After drying, the samples were aged 1 year at room temperature (20°C) and relative humidity (50%RH) before analyses.

Table 1. Accession of *L. usitatissimum* and *P. rhoeas* used in this work

Accession	Name	Origin
LIN 1816	<i>Linum usitatissimum</i> L. subsp. <i>angustifolium</i> (Huds.) Thell.	Italy
PAP 351	<i>Papaver rhoeas</i> L. - 'Pierrot'	Sweden
PAP 363	<i>P. rhoeas</i> L.	Spain

Infrared spectroscopy ATR-FTIR and Gas chromatography-Mass Spectrometry

The portable infrared spectrophotometer ALPHA (Bruker Optik GmbH), equipped with a Global IR source, an interferometer (RockSolid)TM, insensitive to external vibrations and able to work in any spatial orientation), a DLATGS room temperature detector and an ATR accessory with a diamond crystal was used. The IR spectra were acquired in the spectral range 4000-375cm⁻¹ with a spectral resolution of 4cm⁻¹ and 128 interferograms. The sample to be analyzed does not require preparation and was placed directly into the sample probe. The GC-MS data were extracted from the literature in particular from the work of *Lluveras et al.* [15].

PCR and DNA sequencing

The genomic DNA was isolated from leaves of walnut, poppy and flax using the DNeasy Plant Kit (Qiagen NV, Venlo, Netherlands) according to manufacturer's instructions using 20 mg of leaf tissue. A modified NucleoSpin Food Kit (Macherey Nagel) was employed for isolating DNA from either 1mL of commercial siccative oil or 200mg of aged laboratory

pictorial-like samples. PCR reactions were performed in a volume of 25 μ L containing 15 ng/ μ L template DNA, 1 \times PCR buffer (Invitrogen, Carlsbad, CA), 50 mM MgCl₂, 2 μ M dNTPs, 1 μ L each of 10 μ M For and Rev primers, and 1 U Taq DNA polymerase (Invitrogen). Amplification was performed with the following thermal profile: 94°C for 180s, followed by 37 cycles of 94°C for 20s, 59°C for 30s, and 68°C for 20s.

Six universal primers amplifying intergenic cpDNA regions were derived from *Taberlet et al.* [46] (Table 2). To design species-specific primers for each species, amplicons of the three species compared with the *trnL-trnF* intergenic spacer sequences of 38 cpDNA sequences retrieved from the GenBank database using ClustalW in MEGA 4.0 software [47]. Similarities for all DNA amplified and sequenced fragments of the three-different species were analyzed with the VECTOR NTI Suite 8 AlignX software (InforMax) for multiple sequence alignments to highlight conserved and variable regions.

Table 2. Sequences of six universal primers for the amplification of three non-coding regions of cpDNA [46]

Code	Name	Sequence 5'-3'
<i>a</i>	<i>TrnT</i> -a-For	CATTACAAATGCGATGCTCT
<i>b</i>	<i>TrnL</i> -b-Rev	TCTACCGATTTCGCCATAC
<i>c</i>	<i>TrnL</i> -c-For	CGAAATCGGTAGACGCTACG
<i>d</i>	<i>TrnL</i> -d-Rev	GGGGATGAGGGACTTGAAAC
<i>e</i>	<i>TrnL</i> -e-For	GGTTC AAGTCCCTCTATCCC
<i>f</i>	<i>TrnF</i> -f-Rev	ATTTGA ACTGGTGACACGAG

For each species, one specific primer pair was developed using the online tool Primer3 [48]. Once verified on DNA isolated from fresh leaves, primer pairs were first used for PCR on DNA isolated from pure oils and then on DNA extracted from aged pictorial models. To avoid contamination, the steps taken from the DNA isolation to PCR reactions were carried out in an isolated sterile hood and replicated [49]. To maximize the specificity and quantity of amplicons, all the DNA amplifications were performed using a high-fidelity polymerase (Advantage 2 PCR Enzyme System, Clontech, Mountain View, CA). PCR amplification was performed in a total volume of 50 μ L containing 5 ng of genomic DNA, 0.2 μ M each dNTPs, 10 μ M of For and Rev primers, and 1 μ L of Advantage 2 Polymerase Mix (50 \times). Amplifications were performed with the following thermal profile: 95°C 60s, followed by 37 cycles of 95°C for 15s, 59°C for 20s, and 68°C for 20s. Two rounds of amplifications were performed for the amplification of DNA isolated from aged pictorial samples, using 5 μ L of the first reaction as a template for the second.

Two μ L of the amplified products were ligated into the TOPO TA Cloning vector using the TOPO TA Cloning kit for sequencing (Invitrogen) following the manufacturer's instruction. Colonies were grown overnight and amplified with specific primers (Table 2) to test for the presence of inserts. Plasmids from ten positive colonies for each transformation were purified and Sanger-sequenced as reported in *Albertini et al.* [40].

Results and discussion

Infrared spectroscopy ATR-FTIR

Dried pictorial films of the three oils were analyzed by ATR-FTIR spectroscopy and the infrared spectra are shown in figure 1. No characteristic peaks for any of the three species were observed, and their spectra perfectly overlapped. They shared all the typical functional groups of lipid compounds: the methylenic (CH₂) asymmetric and symmetric stretching at 2920 and 2950 cm⁻¹ respectively, carbonyl stretching between 1750-1700 cm⁻¹ and the ester linkage at 1170 cm⁻¹ [50]. The absence of 3010 cm⁻¹ signals and the presence of a broad band between 1750-1700 cm⁻¹ together with the signals at 1170 cm⁻¹ reveals the happened polymerization process in all samples [50, 51]. Therefore, infrared spectroscopy is not a suitable technique for

discriminating the three types of drying oils, and it can only be used to verify the presence of lipidic materials.

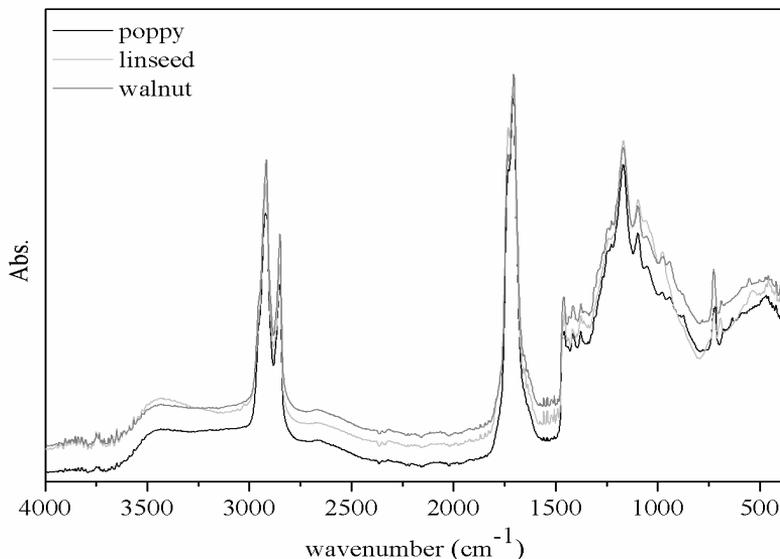


Fig. 1. Infrared spectra of dried films of oil standards

Gas chromatography Mass Spectrometry GC-MS

It is not possible to analyze polymerized drying oils directly by GC-MS. A wet chemical pretreatment that include chemolyses and derivatization reactions is needed to provide more suitable molecules for gas chromatography, transforming compounds containing polar functional group, such carboxylic, increasing their volatility. The identity and biological origin of drying oils may be determined from quantitative determination of specific monocarboxylic and dicarboxylic fatty acid [1]. Azelaic to palmitic acid (2C9/C16) ratio and the total dicarboxylic acid content (azelaic, suberic and sebacic) also can be used to identify drying oils used in paintings [13, 52]. The most popular approach is calculating palmitic to stearic acid (C16/C18) ratio, which is not affected by the ageing process [13, 52-54]. Analytical procedures used to calculate the quantitation limit (LOQ) at a statistical significance level of 0.05 is described in *Andreotti et al.* [55] and LOQ of palmitic acid of 1.478µg and stearic acid of 2.833µg are derived from ancient pictorial samples [15].

Considering that palmitic and stearic acid are used for identification, and adopting average carboxylic acids composition reported by *Mills and White* [1], the minimum sample size of seed oil containing an amount of carboxylic acid equal to the technical limit of quantitation (LOQ) can be calculated for each plant species (Table 3). If the species is unknown, the minimum amount of oil necessary for identification is around 566µg. This is confirmed by sample sizes 0.5 – 1 mg used by *Lliveras et al.* [15].

Table 3. Minimum samples size for seed oils from ancient pictorial samples

Species	Palmitic acid (%)	Stearic acid (%)	Minimum samples size palmitic (µg)	Minimum samples size stearic (µg)
Linseed	6-7	3-6	24.6-21.1	94.3-47.1
Poppy	10	2	14.8	141.5
Walnut	3-7	0.5-3	49.3-21.1	566-94.3

Species-specific Primer design

Six universal primers amplifying intergenic cpDNA regions were derived from Taberlet *et al.*, [46] (Tab. 4). Nine combinations of these primers were tested on genomic DNA extracted from leaves of the three species (not shown).

Table 4. Species-specific primer pairs for the amplification of the *trnL-trnF* intergenic spacer

Name	Sequence 5'-3'	Amplicon (bp)
<i>Walnut For</i>	TGAGCCCAAATCCTGTTTTCT	221
<i>Walnut Rev</i>	CAAGTCGTCATTAATTTATTTGAG	
<i>Linum For</i>	GATAGGTGCAGAGACTCG	184
<i>Linum Rev</i>	GGTTATACATTAATCATTGATA	
<i>Poppy For</i>	GGATTTCTGAAAGCGAGAA	190
<i>Poppy Rev</i>	AATAGAATCTTTGATATTTCCGGT	

Some combinations gave no amplifications or did not amplify in all species. In some cases multiple amplicons were obtained (not shown). The combination *trnL-c-For/trnF-f-Rev* (primers c and f of Table 4) gave the most reliable amplification results in all samples (Fig. 2) and was chosen for further analyses.

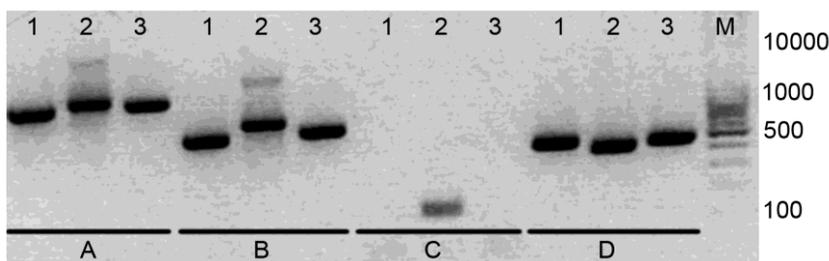


Fig. 2. Electrophoresis of PCR products obtained from genomic DNA extracted from leaf tissue showing the performances of four combinations of the primers reported in Table 4

Amplicons of the three species were sequenced and compared by performing a multiple alignment which included the *trnL-trnF* intergenic spacer sequences of several congeneric species retrieved from GenBank. Several single nucleotide or indel polymorphisms were found (Fig. 3), which permitted to design species-specific primer pairs for the three species of interest (Table 4).

Species-specific primer pairs were then used for amplifying linseed, poppy and walnut DNA extracted from leaves and confirmed to be specific: PCR amplifications were successful only in the expected species, while non-specific amplification products were absent (Fig. 4).

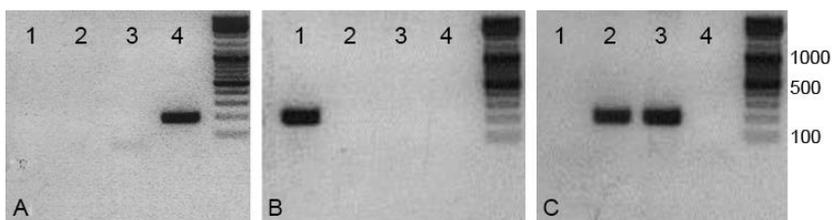


Fig. 4. Amplification of genomic DNA isolated from leaves with species-specific primer pairs. A: linseed-specific primers, B: walnut-specific primers, C: poppy-specific primers. 1: walnut DNA; 2, 3: poppyd DNA; 4: linseed DNA

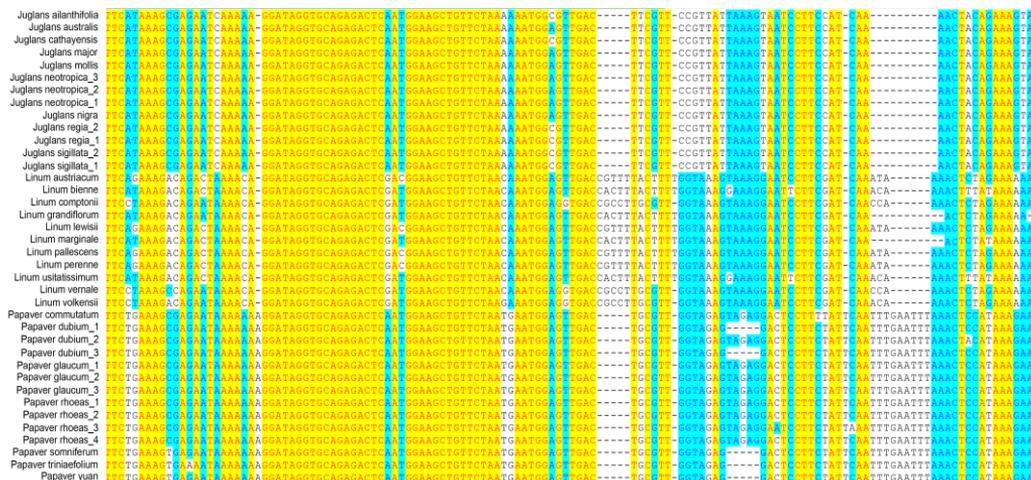


Figure 3. Multiple alignments of 41 *trnL-trnF* intergenic spacer sequences present in GenBank, belonging to the genera *Juglans*, *Linum* and *Papaver*

Amplification of DNA isolated from fresh oils and from aged pictorial models

Next, amplification of DNA isolated from fresh seed oils with species-specific primer pairs was attempted. As shown in figure 5, amplification was successful, and specificity was confirmed.

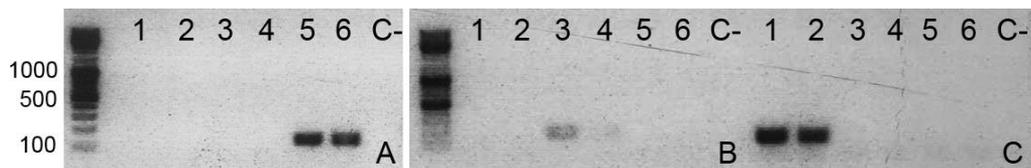


Fig. 5. Amplification of genomic DNA isolated from fresh seed oils. PCR reactions were carried out with species-specific primers (A: linseed, B: walnut, C: poppy). 1, 2: poppy oil DNA; 3, 4: Walnut oil DNA; 5, 6: linseed oil DNA

Finally, DNA isolated from aged pictorial films was used as template. Two rounds of PCRs were needed to get a good amplification product. Figure 6 shows PCR products from DNA samples obtained from poppy oil aged pictorial models. Direct sequencing of these amplicons (not shown) confirmed the specificity of amplification. A further step may be possible with our DNA-based method. The *trnL-trnF* intergenic spacer present a significant intraspecific sequence variability that lies between 5.9 and 55.0% [56].

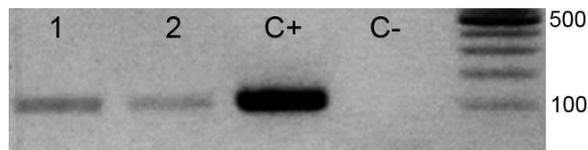


Fig. 6. Example of amplification of genomic DNA isolated from aged pictorial models. PCR reactions were performed with poppy-specific primers using two DNA samples from aged pictorial models (1, 2). C+: DNA from fresh tissues; C-: no template. M: molecular weight marker. The amplicon is 190 bp, as expected

This variability has been exploited to evaluate local diversity and identify cultivars in some plant species [57, 58]. Limited genetic data on different cultivars of plant species used for drying oil production are available, but with increasing accessible information it is reasonable to

assume that DNA based techniques will provide much more detailed knowledge compared to traditional chemical analysis, allowing to trace more geographical and chronological information. A limitation of this method may be the larger amount of sample needed for analysis (Table 5) however, we have not tested the method with reduced quantities, so we cannot exclude that significantly smaller samples can be used in practice.

Our results show that DNA analysis can provide new insights into the artists' *modus operandi*, unattainable using traditional chemical methods, as well as a new perspective for studying trade route of artist materials and the social context in which they operated.

Table 5. Comparison between analytical techniques for amount of sample needed and specificity for drying oils

Analytical technique	Quantity of sample (mg)	Specificity
Infrared spectroscopy (FTIR) [5-10]	< 0.5	Identification of the class of chemical compound (lipid)
GC-MS [13-17]	0.5 -1.0	Identification of plant species (linseed, walnut or poppy)
DNA	200.0	Identification of plant species and potentially of cultivars (linseed, walnut or poppy)

Conclusions

Chemical analysis can provide a way to empirically test scientific assumptions on ancient materials, but information regarding the biological origin of materials is difficult to obtain by chemical tools. Spectroscopic methods do not allow identifying the biological origin of drying oils. Traditional chromatographic methods under optimum conditions allow to trace biological species used to provide materials for artworks, but with some significant limitations. Chemical composition of a specific drying oil can vary with plant cultivar, soil, season or weather conditions of harvesting increasing the possibility of error. In addition, mixing oils from different biological species can mislead the analyses because it alters the composition of carboxylic acids used in oils identification.

Our experimental approach was successful in both extracting and amplifying DNA from aged pictorial models. Sequence analysis allowed discriminating the plant species from which the oils were extracted, and achieve the same information provided by the reference method based on GC-MS.

A further step may be possible with our DNA-based method. The *trnL-trnF* intergenic spacers present a significant intraspecific sequence variability that lies between 5.9 and 55.0% [56]. This variability has been exploited to evaluate local diversity and identify cultivars in some plant species [57, 58]. Limited genetic data on different cultivars of plant species used for drying oil production are available, but with increasing accessible information it is reasonable to assume that DNA based techniques will provide much more detailed knowledge compared to traditional chemical analysis, allowing to trace more geographical and chronological information. A limitation of this method may be the larger amount of sample needed for analysis (Table 5) however, we have not tested the method with reduced quantities, so we cannot exclude that significantly smaller samples can be used in practice.

Our results show that DNA analysis can provide new insights into the artists' *modus operandi*, unattainable using traditional chemical methods, as well as a new perspective for studying trade route of artist materials and the social context in which they operated.

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