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Title

A round robin exercise in archaeometry: analysis of a blind sample reproducing a XVII century pharmaceutical ointment

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Abstract

Chemical analysis of ancient residues of pharmaceutical or cosmetic preparations as balms or ointments, is made problematic by the high complexity of these mixtures, composed of organic and inorganic materials. Consequently, a multianalytical approach and special caution in the interpretation of the results are necessary. In order to contribute to the improvement of analytical strategies for the characterization of complex residues, and to reconstruct ancient medical practices, a replica of a pharmaceutical formulation of the XVII century was prepared in the laboratory according to a historically documented recipe. In a round robin exercise, a portion of the preparation was analysed as a blind sample by 11 laboratories using various analytical techniques. These included spectroscopic, chromatographic and mass spectrometric methods.

None of the laboratories was able to completely reconstruct the complex formulation, but each of them gave partial positive results. The round robin exercise has demonstrated that the application of a multianalytical approach permits a complete and reliable reconstruction of the composition. Finally, on the basis of the results an analytical protocol for the study of residues of ancient medical and pharmaceutical preparations has been outlined.

1. Introduction

The chemical analysis of archaeological and historical residues of ancient medical preparations has a high potential to provide valuable information on ancient medical practices and on the development of scientific culture in past centuries. Revealing the secrets of past medicine also involves the study of natural substances that were traditionally used as ingredients of medicines and cosmetics: interest in the use of such natural compounds for therapeutic purposes has seen a revival in recent times attracting the attention of many pharmaceutical producers. Nevertheless, this kind of chemical analysis is complicated by factors such as: i) the complexity of the mixtures of organic and inorganic materials constituting pharmaceutical/medical preparations such as balms or ointments, ii) chemical transformations induced by ageing, iii) interferences in the analysis between the various components of the matrix. Not only are the samples complex in composition, they are often small and quite heterogeneous. In order to improve historical knowledge of the use of vessels, of trade routes, of ancient diets and manufacturing practices, multi-analytical approaches and special caution in the interpretation of the results are necessary. The availability of reference materials and the organization of Round Robin inter-laboratory tests are therefore necessary. Particularly, an evaluation of the ability of different analytical approaches to identify the ingredients in ancient formulations may be achieved by comparison of results from blind samples.

To date, few round robin tests have been performed in this field and very few reference materials have been produced for this purpose. Inter-laboratory exercises are common practice in the pharmaceutical, environmental and other fields of analytical chemistry, and within archaeometry there is a growing recognition of the need to improve analytical procedures by using reference materials and applying them to round robin tests. Thus, an inter-laboratory exercise has been recently reported by van Keulen [1], for a round robin conducted in the framework of the activity of (MASC, http://www.mascgroup.org/], where the test sample reproduced a paint layer from a historical artwork. Barnard et al. [2] conducted and reported a round robin in which the unknown

material was a simulated archaeological residue: a sherd from a pot in which camel milk had been cooked.

In order to contribute to the improvement of analytical strategies for the characterization of residues of complex mixtures of materials in archaeometry and to reconstruct ancient pharmaceutical practices, in late 2010 we organized a round robin inter-laboratory exercise. It consisted of analysis of a blind sample represented by a replica of a pharmaceutical formulation prepared in the laboratory following a recipe dating to the XVII century reported in "Nuovo Formulario Magistrale" by A. Bouchardat [7]. The round robin was organized in the context of an Italian National Research Project (MIUR Prin07) titled *"Colors and balms in antiquity-from the chemical study to the knowledge of technologies in cosmetics, painting and medicine"* (http://www.dcci.unipi.it/prin07/).

The composition of the blind sample was known only to the person who prepared the formulation. The participants in the round robin were asked to independently analyze the unknown material as if it was a residue of an ancient pharmaceutical or cosmetic preparation using analytical techniques and procedures that they deemed appropriate, and to report the results. Participation was invited from laboratories with demonstrated experience in the analysis of materials in historical, artistic or archeological objects. This is the first time that a round robin has been conducted on a material reproducing an old pharmaceutical formulation.

14 laboratories agreed to take part, and were sent a portion of the blind sample (RR), and a second portion of the same sample (RRoz) after an artificial ageing treatment based on exposure to ozone aimed to simulate oxidation of the organic matter due to ageing. The participants were informed that the two samples were the same pharmaceutical preparation with the only difference that one was treated with ozone. They were also informed that the pharmaceutical formulation could contain both organic and inorganic components and that it reproduced a pharmaceutical recipe reconstructed on the basis of the critical interpretation of historical pharmaceutical treatises. They were not provided with any additional information on the material, and they were not given a list of possible ingredients. The collection of reference materials used for the interpretation of the results by the various participant laboratories was expected to be various and different, reflecting their respective archaeometric research interests, some in ancient pharmaceuticals, some in archaeological residues, others in artists materials. In comparing results submitted by the participants we mainly looked for the capacity of the methods to disclose the minor and major

compounds used in the preparation and for the ability to evaluate the degradation state of the materials.

11 laboratories succeeded in producing a report prior to the meeting in which the composition of the blind sample was revealed [3]: they adopted analytical methods included spectroscopic, chromatographic and mass-spectrometric methods.

After a description of the composition and preparation of the sample and of the analytical methods adopted by the participants, the results obtained with the various analytical approaches used by the various laboratories are here described and critically compared. Conclusions follow, attempting to comment on the state of the art of analytical strategies for the study of residues of past medical or cosmetic preparations, and to suggest an analytical protocol for this kind of sample.

2. Preparation of the blind sample

For the preparation of the blind sample different ancient bibliographic sources were studied and interpreted [4,5,6,7,8,9]. After consideration of different formulations and recipes, the selected preparation was *Diachylon Bouchardat Plaster* described in "*Nuovo Formulario Magistrale*" (XVII century, A. Bouchardat) [7]. This formulation shared important similarities with recipes reported in other bibliographic sources, suggesting that it was a quite commonly used pharmaceutical preparation. About 600g. of the entire ointment was prepared. The ointment contained both organic and inorganic ingredients, and included in the ingredients the so-called *Simple Bouchardat Plaster* (the recipe is reported in the same text) which was also prepared in laboratory. Table 1 reports the ingredients used for reproducing the blind sample formulation with the corresponding amounts. Table 2 reports the ingredients used for reproducing the *Simple Bouchardat Plaster.*

The preparation procedures were as follows:

For the *Diachylon Bouchardat Plaster:*

- The Simple Plaster was heated at 70-80°C;
- Beeswax, cut into small pieces and galbanum resin were heated and stirred continuously until melted;
- Colophony and pine resin were blended in a mortar and then added to the heated mixture. The ointment was stirred at room temperature until cool.

For the *Simple Bouchardat Plaster:*

- Pig suet was dissolved at 60-70°C for 360 minutes.

- Olive oil, water and pig suet were separately heated at 70-80°C, mixed together and then silver litharge was added to the mixture;
- The mixture was heated for 30 minutes at 70-80°C with stirring.
- The mixture was kept under magnetic stirring at room temperature for at least 180 minutes.

A portion of the *Diachylon Bouchardat Plaster* (named sample RR) was submitted to an oxidation treatment based on ozone exposure, aiming to simulate organic matter oxidation during ageing (sample RRoz). For this purpose, 10 g of the material were suspended in 250 ml of water by sonication. Ozone was bubbled (5 ml/min) into the solution for 20 hours. At the end of the treatment, the sample was freeze-dried. This treatment turned out to be relatively mild, and produced only a limited oxidation of some of the components, as demonstrated by the analysis.

Portions of about 30 mg of the two samples were sent to the laboratories participating in the Round Robin exercise.

The fresh ointment had the following weight percentage composition: silver Litharge 16%, Pig suet 16%, Olive oil 16%, galbanum 3 %, beeswax 8%, pine resin 4%, colophony 4%, water 33%.

3. Participant laboratories and adopted analytical techniques

The participant laboratories were as follows:

Istituto CNR-ISTM Perugia (Italy);

LETIAM, IUT d' Orsay, Orsay (France);

British Museum, Department of Conservation and Scientific Research, London (UK);

Centro Interdipartimentale di Ricerca per le Scienze Ambientali, Microchemistry and Microscopy Art Diagnostic Laboratory (M2ADL), Università di Bologna (Italy);

Dipartimento di Scienze dell'Ambiente e del Territorio, Università di Milano Bicocca, Milan (Italy);

Dipartimento di Chimica e Chimica Industriale, Università di Pisa (Italy);

Dipartimento di Chimica, Materiali e Ingegneria Chimica, Politecnico di Milano (Italy);

Department of Pharmaceutical Science and Chemistry Department, University of Modena and Reggio Emilia (Italy);

Doerner Institut, München (Germany);

Ca' Foscari, Venezia (Italy);

Dipartimento di Chimica, Università di Torino (Italy).

The 11 participant laboratories adopted the analytical techniques listed in Table 3.

4. Results and discussion

Anticipating that the composition of the unknown sample was mainly organic in nature, many of the participants adopted analytical strategies focused mainly on chromatographic techniques such as gas chromatography coupled with mass spectrometry (GC/MS), pyrolysis coupled with gas chromatography and mass spectrometry (Py-GC/MS) and liquid chromatography coupled with mass spectrometry (LC/MS). Chromatographic/mass spectrometric techniques were chosen by nine participating laboratories, showing the importance of these methods in revealing the nature of complex organic samples. However, exclusive use of such techniques does not permit the determination of the inorganic components of the sample.

Infrared and Raman spectroscopy were used by eight laboratories to gain information on both organic and inorganic components.

NMR spectroscopy is currently rarely applied to the characterization of organic residues in archaeology due to the difficulty in the interpretation of NMR signals deriving from complex mixtures of molecules. Interestingly, NMR analysis was chosen by only one laboratory: they acquired ^{1}H-NMR and ^{31}P-NMR spectra after sample extraction. Elemental analysis by XRF was applied by only one laboratory for the identification of inorganic components.

The approaches adopted and the results are described in the following paragraphs.

4.1 Spectroscopic methods

FTIR spectroscopy

Five laboratories used FTIR analysis to achieve information on the nature of the ointment ingredients, by recognizing the specific characteristic absorption bands.

The sample was generally not pre-treated; KBr pellets or a diamond cell were used to acquire spectra. µFTIR-RAS (reflection/ absorption spectroscopy) analysis was also performed. In this case spectra were acquired applying the sample on a gilded support. Figure 1 reports the spectrum of RR sample and Table 4 summarizes the possible peak assignment.

Although the spectra of both RR and RRoz are quite complex, a good matching was found with a non siccative oil (olive oil or seed oil: 3005, 1746 (shoulder) cm^{-1}) and beeswax (2919, 2850, 1739,

728-720 cm^{-1}). The ozone treated sample appears similar to the untreated one, but with a marked reduction of the double bonds associated with the non siccative oil.

The spectra are characterized by typical absorption of lead carboxylates [10] (ν_{as}CO at about 1520 cm^{-1} and ν_s CO at about 1410 cm^{-1}) that are present both in the unaged and artificially aged sample. A lead salt, possibly lead carbonate (about 1400 cm^{-1}) was also hypothesized by all but one of the laboratories. The presence of lead was confirmed by XRF measurements. Thus, lead carboxylates seem to have been formed by the interaction of a lead compound and the vegetable oil present in the sample. These findings suggested to the participants that the unknown ointment was a lead plaster. forming lead soaps and glycerol.

Other organic compounds (like proteins or vegetable gums) as well as pigments or fillers were ruled out. However, one laboratory suggested also the presence of a natural resin, probably containing an aromatic component.

Raman spectroscopy

Two laboratories employed Raman spectroscopy to study the inorganic composition of the samples. Micro-Raman spectra identified lead monoxide litharge present in small reddish yellow crystals, and after extraction of the samples with a mixture of chloroform/ethanol (2:1), the remaining insoluble white residue was recognized as lead carbonate.

Micro-Raman and SERS (Surface-Enhanced Raman Scattering) techniques, applied by another laboratory, highlighted the potential of these techniques in identifying various organic and inorganic materials in the mixture.

The Raman spectra of the RR samples exhibited a fluorescence background, nevertheless the presence of PbO as massicot was determined on the basis of strong peaks at 288 and 141 cm^{-1} , and as litharge by the peaks at 145, 289, and 339 cm^{-1}.

The spectrum of RRoz sample was extremely similar to that of the non-treated sample, except for a reduction in the fluorescence, and for the fact that the ozone treatment (sample RRoz) seems to favor the transformation of the lead oxide into cerussite [11]. On the basis of peaks at 1058, 1054 and at 1051 cm^{-1} the participant hypothesized the presence of pig fat, KNO_3 and $PbCO_3$.

For the application of the SERS technique the silver colloidal suspension was prepared according to Lee and Meisel's method [12], on a microscope glass. Figure 2 shows the SERS spectra acquired for the RRoz compared to those of some reference materials. The oxidation treatment noticeably

reduces the background fluorescence. From the comparison spectra, pig suet is recognized. The signal at 1659 cm^{-1} shows that some unsaturated bonds are still present highlighting that the ageing process was indeed quite mild.

As reported by the literature [13], SERS is particularly suitable for the analysis of resinous materials. SERS produced a decrease of fluorescence that enabled galbanum resin to be identified by the bands at 1555, 1350 and 760 cm^{-1}. The band at 1652 cm^{-1} has been explained by the presence of colophony or pine resin[14]. The peak width of the 1440 and 1300 cm-1 bands could be indicative of an oil presence, although generally in the micro Raman spectrum it is quite difficult to distinguish an oil signal in presence of pig suet and to distinguish different kind of oils.

The interpretation of Raman results thus suggest a mixture with the following composition: PbO (litharge and massicot) and $PbCO_3$, animal fat (pig suet), natural resins: galbanum and colophony (or Pinaceae resin).

NMR

^{1}H-NMR and ^{31}P-NMR spectra were collected for the organic fraction separated in polar (water:ethanol) and apolar (hexane) fraction by solvent extraction. Unguent samples (20-30 mg) were refluxed with a mixture of chloroform/methanol 2:1 for 2 hours. After filtering and evaporation to dryness, the residue was suspended in hexane and the polar fraction was then extracted with water/ethanol 1:2. The dried fractions were re-dissolved in $CDCl_3$ for ^{1}H-NMR analysis and derivatizated with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane for ^{31}P-NMR analysis, respectively .

^{31}P-NMR technique allows for the detection and quantification of labile -OH groups such as alcohols and carboxylic acids. The ^{31}P nucleus is sensitive to the chemical neighbor created by the specific hydroxyl functionality with which it is reacted. This property, along with the extended dynamic range of ^{31}P-NMR (200, -20 ppm), the isotopic abundance and the high gyroscopic ratio of the ^{31}P nucleus, is a particular strength of the technique compared to other analytical approaches.

The interpretation of spectra was based on comparison with the spectra of the following reference materials: pine resin, beeswax, almond oil, olive oil, palm oil, suet fat, galbanum resin, red wine.

The ^{1}H-NMR spectrum of the polar fraction (5 % w/w of the starting unguent) is reported in Figure 3. The residual water was observed at 4.91 ppm. The peaks in the aromatic region assigned to the pine resin are related to the presence of dehydroabietic acid. The other peaks between 6.0 and 5.0 ppm are related to the presence of abietic, palustric and pimaric acids. Glycerol has been assigned

to the multiplet (m) at 3.67 and the other two doublet of doublet (dd) at 3.60 and 3.53 ppm. Due the composition complexity of the galbanum resin, peak assignment has not been performed and the interpretation is based on comparison with the reference sample.

On this basis, the participant laboratory reported that it was possible to recognize peaks corresponding to signals characteristic of pine resin, galbanum resin and glycerol. They hypothesized the presence of wine on the basis of the presence of glycerol.

In the apolar fraction (60 % w/w of the starting unguent), the participating laboratory reported the presence of beeswax, an oily and/or fat material, and pine resin. It was not possible to reach further conclusions on the type of lipid materials.

On the basis of all the analyses, a proportional composition was suggested: oil/fat, 42-48 %; beeswax, 9-12 %; pine Resin, 5 %; galbanum and wine, 5 %; inorganic part, 12 %; water, 23 %.

4.2 CHROMATOGRAPHY/MASS SPECTROMETRY METHODS

Py(THM)-GC/MS

Analytical pyrolysis was adopted by only one group, using in-situ thermally assisted hydrolysis and methylation with tetramethylammonium hydroxide (TMAH) [15]. The resulting chromatogram for sample RR is shown in Figure 4, with peak identification listed in Table 5. The pyrolytic profile shows the presence in the sample of lipid and terpenic materials. In particular, a plant oil was hypothesized, on the basis of the high amount of fatty acid methyl esters and 1,2,3-trimethoxy-propane (permethylated glycerol). It was reported that the oil present in the sample is not a siccative one, because the only unsaturated acid observed in the unaged sample was the oleic acid (9-octadecenoic acid methyl ester) and because dicarboxylic acids were not observed in significant amounts in the artificially aged sample. No significant differences were observed between the unaged and artificially aged sample. The presence of animal fats was excluded on the basis of the absence of odd carbon atom chain fatty acids. The 1-alkenes/alkanes series together with the presence of long chain fatty acid methyl esters and alkanes, was considered a fingerprint for the presence of beeswax. The pyrograms also show diterpenoid compounds, typical biomarkers of a diterpenic resin.

GC-MS

Gas chromatography /mass spectrometry (GC/MS) was the technique adopted by the majority of the participating laboratories: 5 out of 10 laboratories applied GC/MS to obtain information on the organic components of the samples.

The 5 participants adopted various chemical pre-treatment methods for the sample, which aimed to separate components with different polarity by selective extraction, to hydrolyse triglycerides and to free fatty acids, and to derivatise them in order to obtain volatile silyl or methyl esters. One of the laboratories applied specific treatments aimed at the analysis of sugars and amino acids after hydrolysis of polysaccharides and proteins, respectively. Another laboratory completed the analysis of the bulk material with the analysis of volatile components by mean of headspace solid phase microextraction GC/MS.

The adopted wet-chemical pretreatments that the various laboratories performed before injection into GC/MS were:

- hydroalcoholic alkaline saponification and extraction of the organic neutral and acidic fractions, followed by trimethysilylation of carboxylic and alcoholic moieties [16];
- acidic methanolysis followed by trimethylsilylation of hydroxylic moieties [17];
- trimethysilylation of carboxylic and alcoholic moieties after extraction in CH2Cl2 [18];
- extraction with solvents at increasing polarity (isooctane, methanol, chloroform/methanol and anhydrous oxalic acid in methanol) followed by hydrolysis/methylation with trimethyl sulfonium hydroxide (TMSH, 0.2 N in methanol) [19];
- amino acid analysis after acid hydrolysis [19,20]
- methanolysis, mercaptlation and silylation for the analysis of sugars [20]
- head space solid phase micro extraction (SPME) to detect volatile compounds deriving from essential oils or terpenic resins [18,21];

Although a variety of pre-treatments were applied to the samples, the round robin showed a high degree of concordance of results obtained by this technique: all the laboratories obtained coherent information and identified the same materials. The identified materials and the molecular biomarkers used for the GC/MS characterization of the mixture are reported in Table 6. Figure 5 shows the chromatograms of the acidic and neutral fractions of sample RR after saponification and silylation, obtained by one of the participating laboratories.

All the participants agree that the major compounds observed in the GC/MS chromatograms are fatty acids, with oleic acid as the most abundant, accompanied by palmitic, stearic, palmitoleic acids and others. They also succeeded in identifying by GC/MS the specific molecular fingerprint of beeswax: long chain fatty acids and long chain alcohols, together with alkanes in the neutral fraction. The only significant difference observed between the RR sample and the artificially aged RRoz sample is a decrease of the amount of the unsaturated fatty acid oleic acid.

The evaluation of the fatty acid profile played an important role in the reported discussions of the results, and all the laboratories recognized the presence of a glycerolipid material in addition to beeswax. The low quantity of azelaic acid in the sample was used to conclude that the glicerolipid material is not a drying oil. Concerning the origin of the lipid, there were discrepancies between the hypotheses made by the participants: some hypothesized a plant oil, while others hypothesized an animal fat (lard) or a mixture of plant oil and animal fat. The presence of a plant oil was confirmed by the detection of sitosterol in the neutral fraction.

The presence of animal fat was inferred on the basis of the presence of fatty acids with odd carbon number chains (pentadecanoic acid and heptadecanoic acid) and of cholesterol in the neutral fraction.

Also the presence of a diterpenoid resin originating from conifer of the Pinaceae family was determined by all the participants on the basis of the presence of characteristic diterpenoid compounds with abietane skeleton (abietic acid and isomers, dehydroabietic acid). One of the laboratories reported the presence of unidentified triterpenoids, which origin was not attributed but could derive from the insaponifiable fraction of olive oil.

GC/MS analysis did not show any evidence for the presence of proteins and polysaccharidic materials.

The chromatograms obtained for RR and RRoz after SPME-GC/MS are presented in figure 6. SPME GC analysis of volatile compounds highlighted the presence of mono and sesquiterpenes including longifolene (peak 18), and due to the abundance of this compound the participant laboratory suggested that the pine resin was from *Pinus sylvestris,* whose resin is particularly rich in this compound. In RRoz, numerous terpenes were present in relative lower amount with respect to RR. Longifolene was still particularly abundant. New compounds (A-I) appeared, corresponding to oxidation products of terpenes or fatty matter.

HPLC-MS

Three different procedures and instrumental assets were adopted for LC/MS analysis (Table 7).

The LC/MS Q-TOF analysis of the extracts of the sample in various solvents gave information not dissimilar from that obtained from GC/MS analysis. The presence of fatty acids with prevalence of oleic acid was highlighted in the isopropanol extract after hydrolysis. Beeswax components were recognized in the hexane extract. Diterpenoids were detected in the acetone extract, even if their origin could not be clearly attributed (pine resin, Venice turpentine or colophony and sandarac resin were hypothesized).

The laboratory which performed the analysis on the acetonitrile extract highlighted the presence of piretrine compounds (cinerine and jasmoline), carotenoids (violaxantine and norbixine) and β-carophyllene. A clear origin was not hypothesized for these compounds, which can derive from many resins and essential oils.

HPLC-APCI-MS analysis aimed to determine triglycerides, obtaining the TAG profile shown in Figure 7. The predominance of triolein (OOO), together with the presence of higly unsaurated tryglicerides suggests a plant oil: almond and/or olive oils are hypothesized. The presence of palmitoyl-oleyl-stearyl glycerol (POS), palmitoyl-stearyl-linoleyl glycerol (PSL) and dipalmitoyl stearyl glycerol (PPS) showed that an animal fat is also present [22,23,24].

5. Conclusions

A Round robin exercise has been carried out on a pharmaceutical ointment "*Diachylon Bouchardat Plaster"* prepared according to the "*Nuovo Formulario Magistrale*" (XVII century, A. Bouchardat), containing PbO, pig suet, olive oil, galbanum resin, beeswax, and pine resin/colophony 8%.

Eleven laboratories participated in the exercise analysing two samples, one fresh and the other aged by an ozone treatment.

The majority of participants reported that the aged sample had a composition very similar to the unaged one, demonstrating that the ageing treatment used was mild and not appropriate to effectively model the changes that take place during prolonged natural aging. Most of the ingredients in the ointment were recognised, but none of the eleven laboratories was able to give a full description of the preparation.

Table 8 summarises the ingredients of the ointment identified by the diverse laboratories together with the analytical techniques used.

The identification of inorganic components by FT-IR and Raman analysis, seems to present some problems: only one laboratory detected the occurrence of both PbO and $PbCO_3$. Given that $PbCO_3$ formed during the heating of the mixture, as reported in the literature [10], and because the organic material was present in a much higher amount, it is conceivable that these inorganic compounds, present at low concentration, are not uniformly distributed in the bulk.

Unravelling the complex mixtures of natural organic substances in the samples was a challenge for all the participants. Significantly no single technique proved completely satisfactory in the source identification of glycerolipids: plant oil origin is only hypothesised, animal fat is seldom recognised. The identification has been complicated by the superposition of several materials with similar components.

Non destructive techniques (FTIR and Raman) give a fast screening for discriminating between inorganic and organic materials, and to determine the class of organic compounds: the SERS technique shows the potential for better recognition of ingredients due to the lowering of the background fluorescence. Because these techniques require no sample pre-treatment or a limited one, and because use a very small amount of sample (few micrograms), they should be performed in all the cases.

Most of the chromatographic/mass spectrometric techniques successfully determined the presence of animal fat, pine resin, non siccative oil and beeswax but failed in the identification of galbanum resin. The reason may be due to the absence of the reference material in the laboratory, to low efficiency of extraction in the pre-treatment used, or to interferences from the matrix. Moreover the amount of galbanum resin was quite low (3%) compared to the other materials.

It is extremely interesting to compare the hypothesis made on the basis of NMR analysis with the results obtained with more classical techniques, as FTIR and GC/MS: the potential of NMR technique in the identification of resinous materials is interesting. However, an ingredient not used (wine) was also suggested. The identification was based on the recognition of glycerol, however, its source was not wine but most probably partially saponified plant oil. In fact, the interpretation of the NMR chemical shifts is very complex when similar materials like vegetable oils and animal fats are present together. Moreover, NMR analysis, like chromatographic techniques, requires the pre-treatment of samples, but the necessary sample size (>>10 mg) is much higher than that generally available when sampling archeological or museum objects.

The results highlight the need for a laboratory to have comprehensive collection of reference materials in order to construct a database of NMR, FTIR, Raman, MS spectra of materials used in old pharmaceutical preparations and to compare results. This needs to be informed by the study of ancient books, the interpretation of formulations and reliable sourcing of the relevant materials. Additionally, to detect stable biomarkers, properly aged materials should be also available. This last point is problematic because there are no standardized ageing procedures used across the field: there is a huge amount of research still to be done in understanding how best to reproduce natural ageing using laboratory methods.

The laboratories which adopted multi-analytical procedures achieved identification of the greatest number of ingredients. Thus, an analytical scheme like that described in Figure 8 is recommended for a reliable recognition of compounds and the reconstruction of the formulations. After observation of the sample under the optical microscope, subsamples would be investigated by the following analytical techniques:

1. Infrared and Raman spectroscopy. Infrared and Raman investigation permits the identification of inorganic components , and can sometimes achieve a good level of detail in the identification of organic components. These investigations are highly recommended before deciding to adopt other techniques.

2. If organic components are hypothesized, the next step is to apply chromatographic techniques coupled with mass spectrometry (GC/MS, Py-GC/MS, LC/MS). The sample pre-treatment procedures to be used should be selected on the basis of the results of infrared and Raman analysis.

3. NMR spectroscopy is to be considered a useful complement, if a sufficient amount of sample and a suitable collection of reference materials are available.

As highlighted by the round robin exercise, the data collected should be critically evaluated and carefully compared with old recipes which in turn requires the interpretation of historical documents such as ancient scientific treatises.

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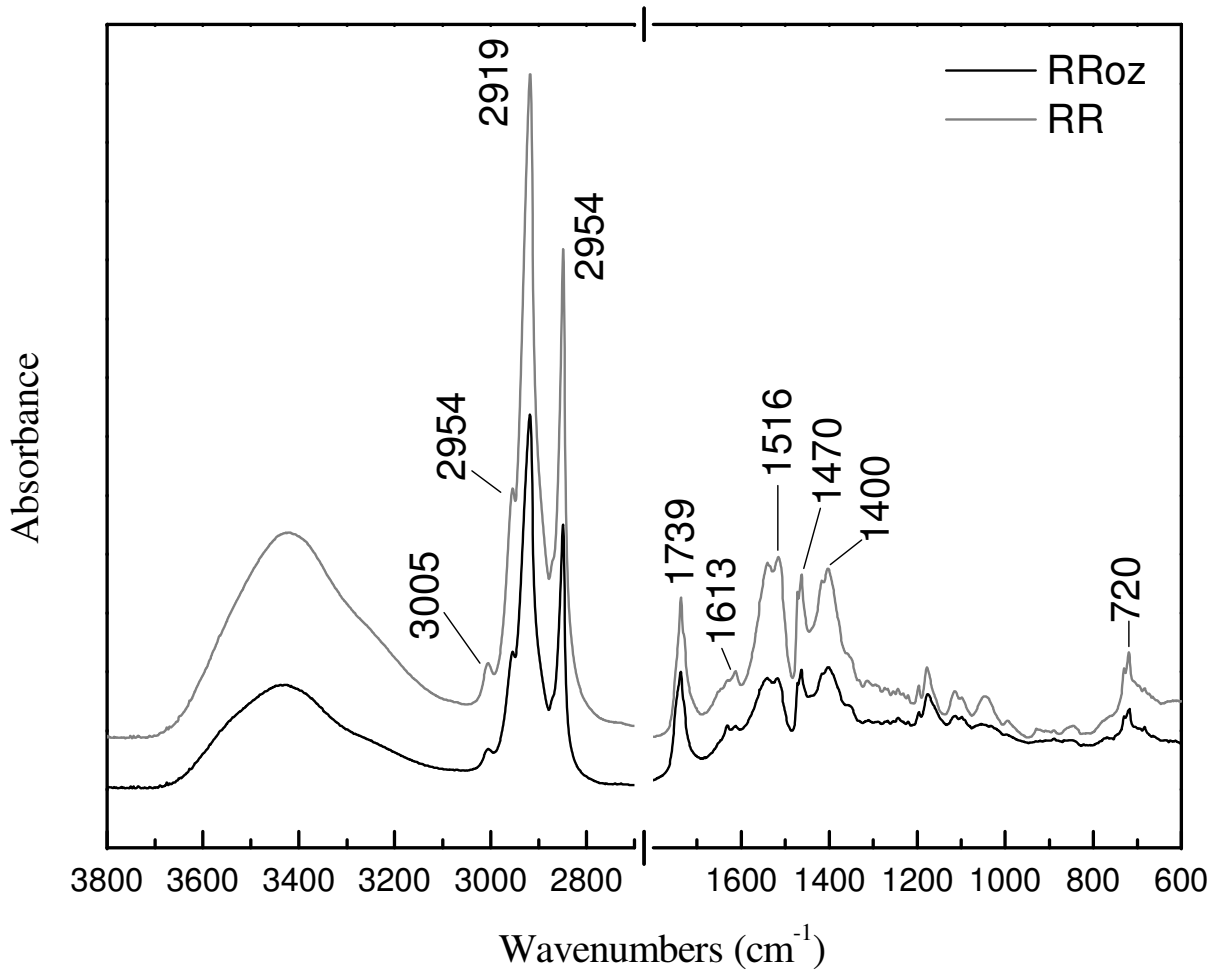

RRoz
RR
2919
2954
2954
3005
1739
1613
1516
1470
1400
720
Absorbance
3800 3600 3400 3200 3000 2800
1600 1400 1200 1000 800 600
Wavenumbers (cm-1)

Figure 1

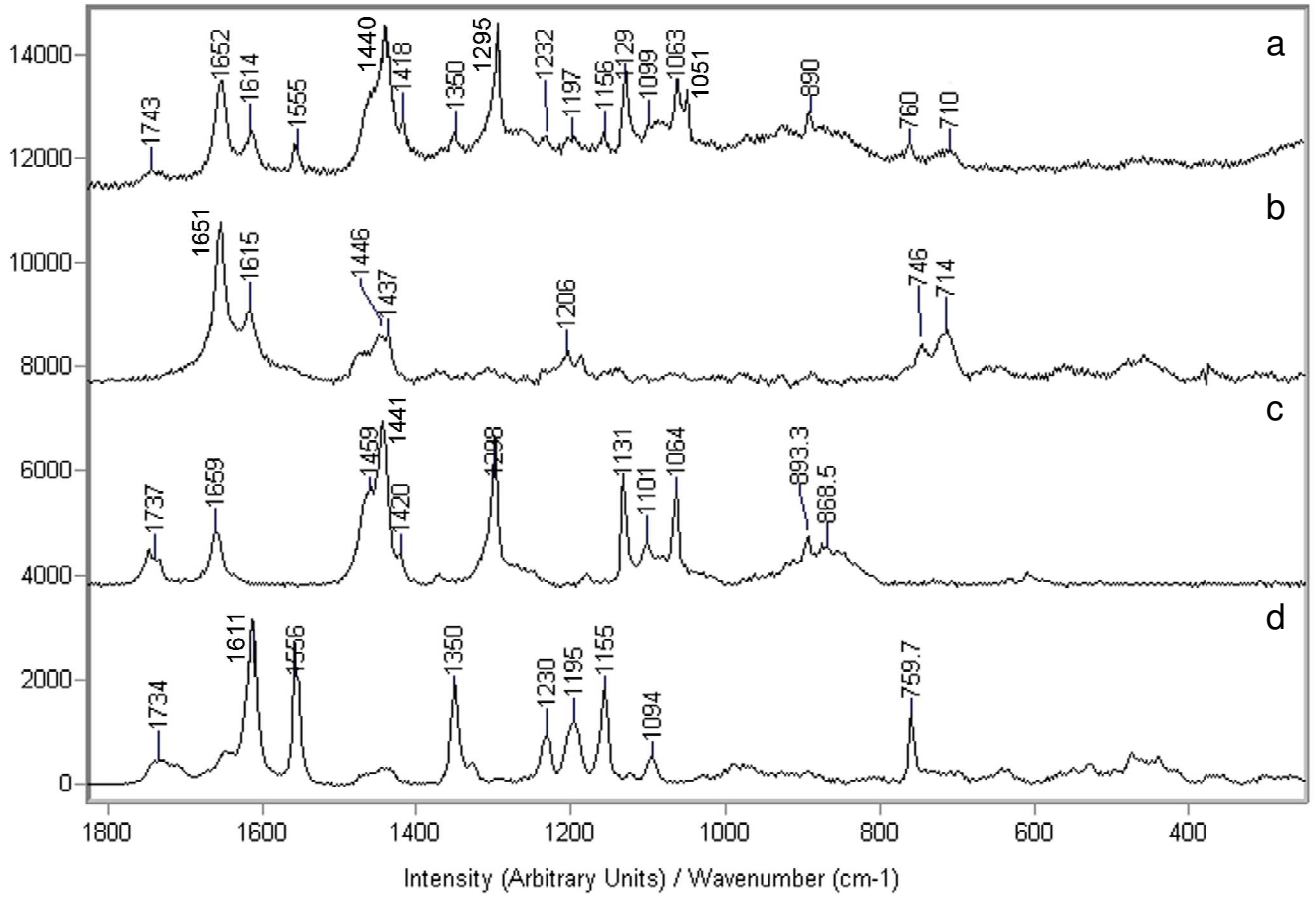

a
b
c
d
1743
1652
1614
1555
1440
1418
1350
1295
1232
1197
1156
1129
1099
1063
1051
890
760
710
1651
1615
1446
1437
1206
746
714
1737
1659
1459
1441
1420
1131
1101
1064
893.3
868.5
1734
1611
1556
1350
1230
1195
1155
1094
759.7
14000
12000
10000
8000
6000
4000
2000
0
1800
1600
1400
1200
1000
800
600
400
Intensity (Arbitrary Units) / Wavenumber (cm-1)

Figure 2

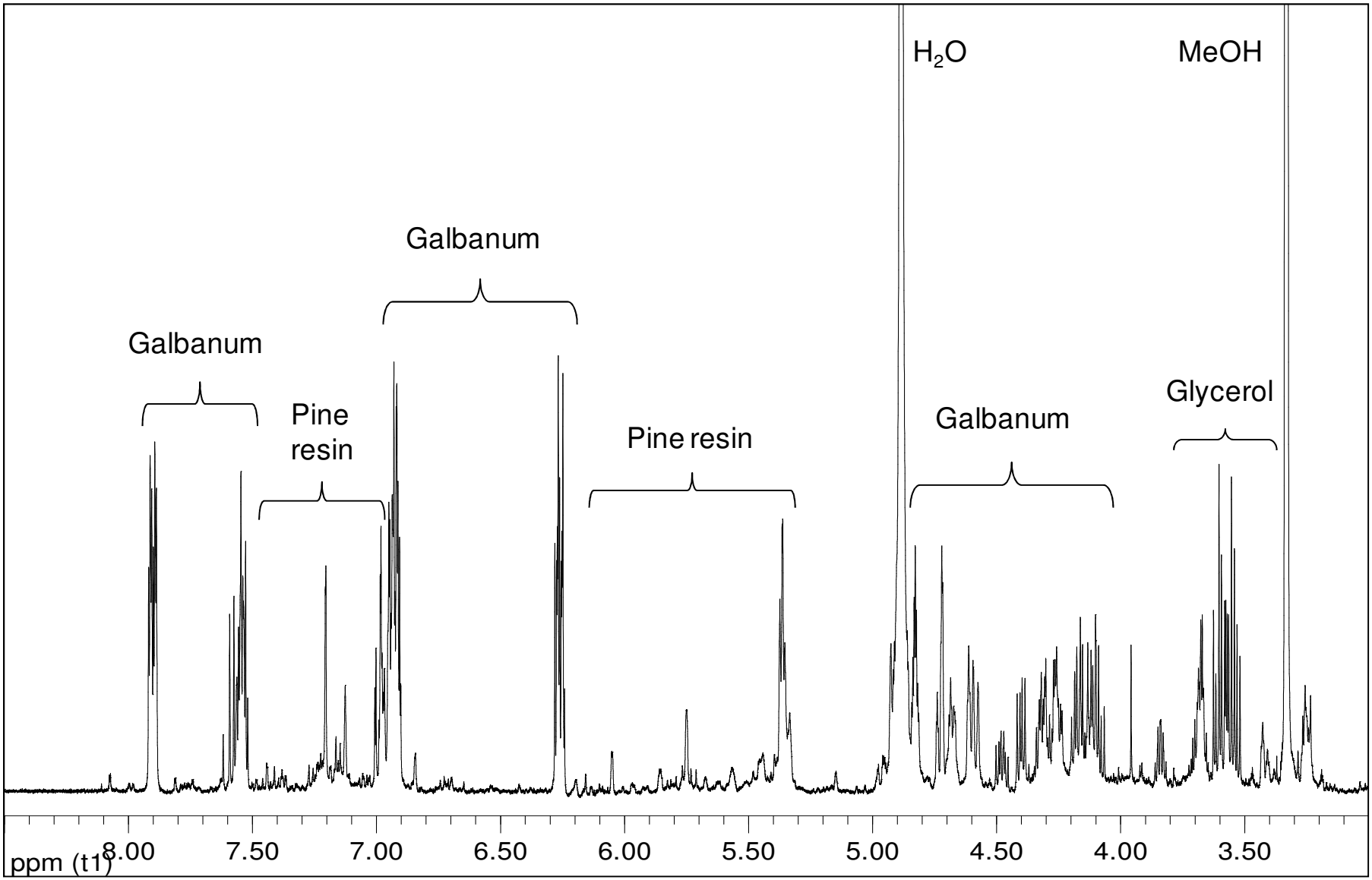
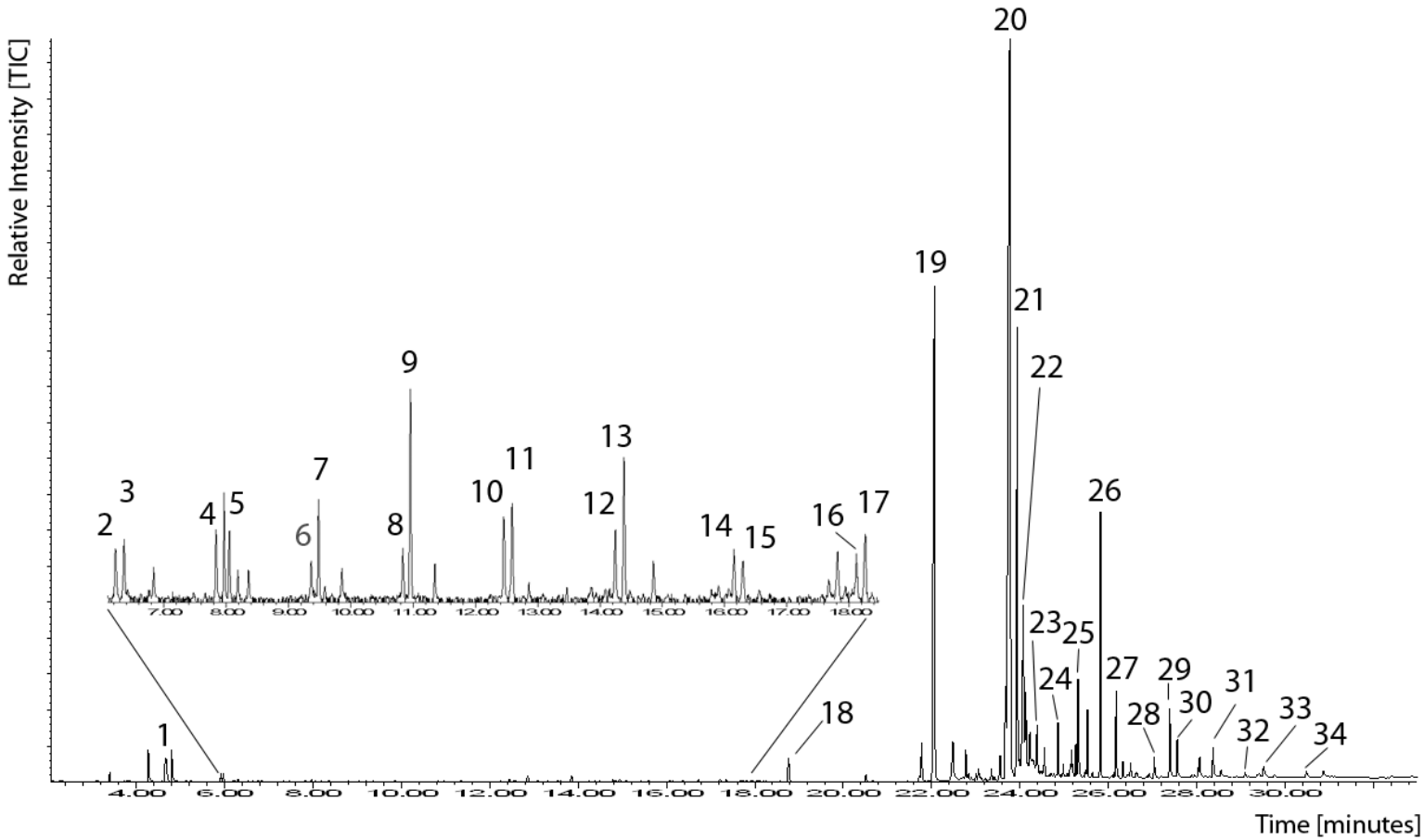

H2O
MeOH
Galbanum
Galbanum
Pine resin
Pine resin
Galbanum
Glycerol
ppm (t1)
8.00
7.50
7.00
6.50
6.00
5.50
5.00
4.50
4.00
3.50

Figure 3


Relative Intensity [TIC]
Time [minutes]
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
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32
33
34

Figure 4

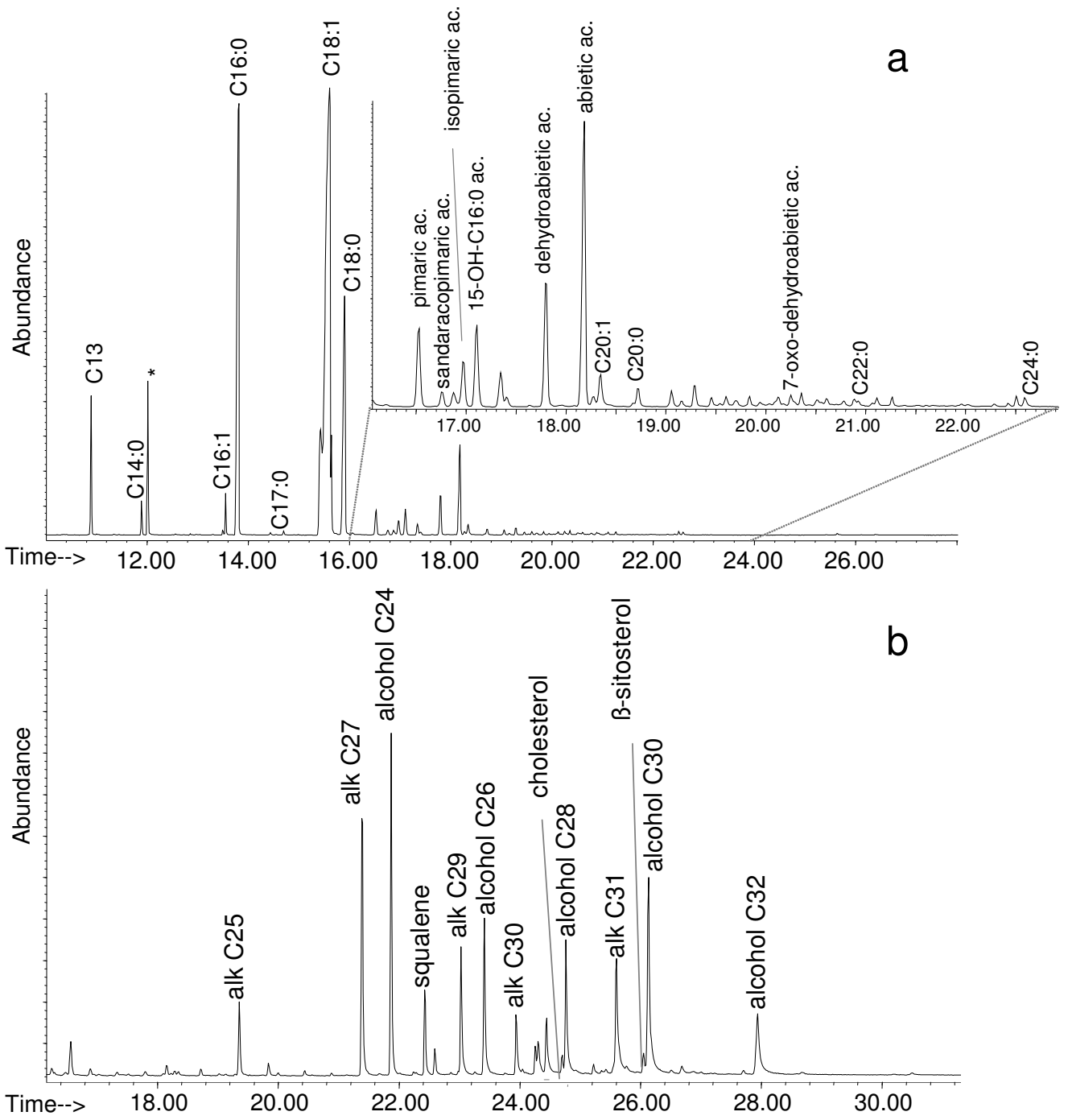
a
Abundance
C13
C14:0
*
C16:1
C16:0
C17:0
C18:1
C18:0
pimaric ac.
sandaracopimaric ac.
isopimaric ac.
15-OH-C16:0 ac.
dehydroabietic ac.
abietic ac.
C20:1
C20:0
7-oxo-dehydroabietic ac.
C22:0
C24:0
17.00
18.00
19.00
20.00
21.00
22.00
Time-->
12.00
14.00
16.00
18.00
20.00
22.00
24.00
26.00
b
Abundance
alk C25
alk C27
alcohol C24
squalene
alk C29
alcohol C26
alk C30
cholesterol
alcohol C28
ß-sitosterol
alk C31
alcohol C30
alcohol C32
Time-->
18.00
20.00
22.00
24.00
26.00
28.00
30.00

Figure 5

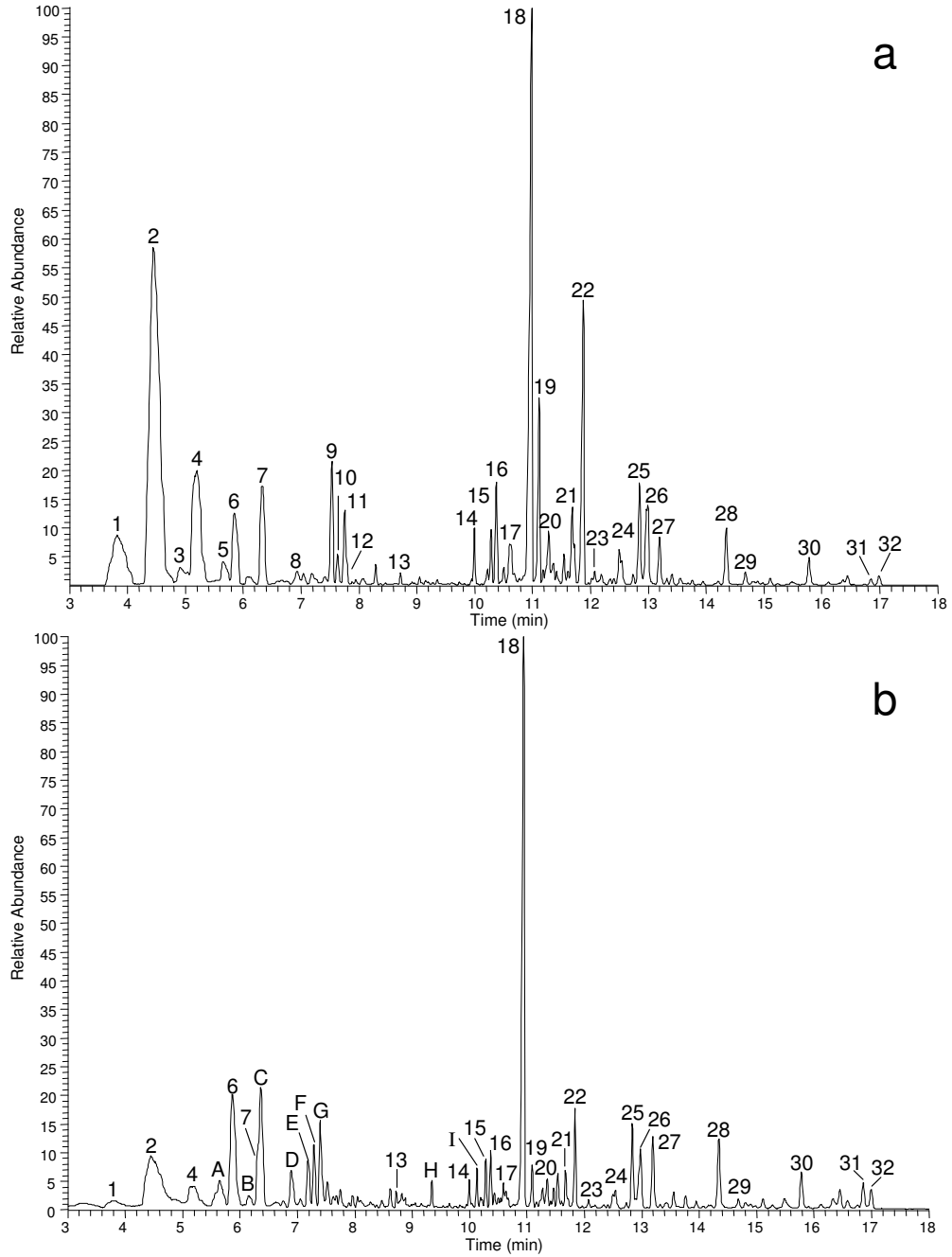
a
b
Relative Abundance
Time (min)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
A
B
C
D
E
F
G
H
I

Figure 6

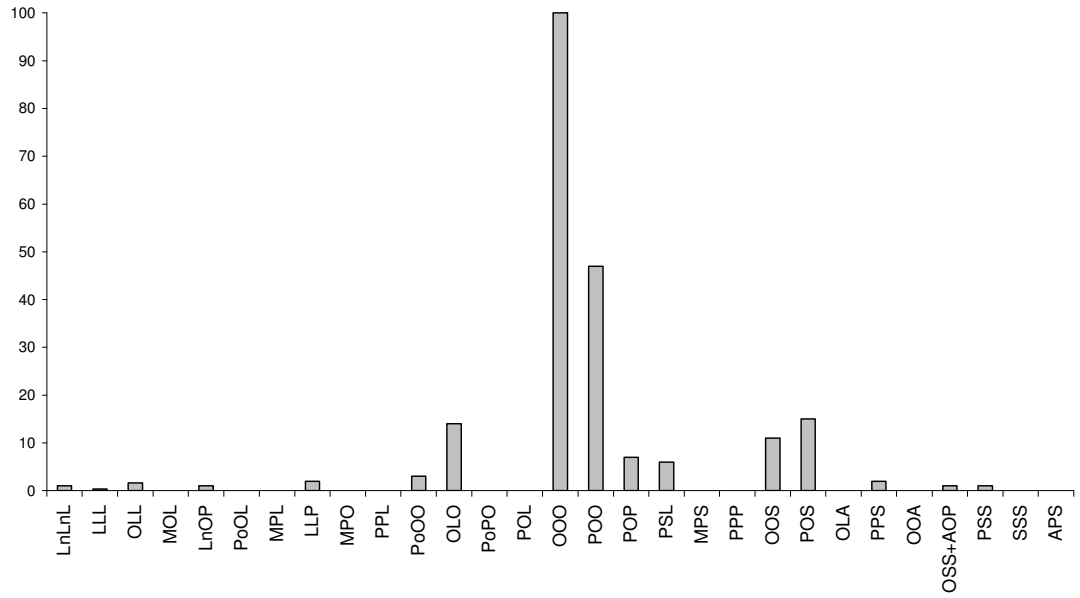
100
90
80
70
60
50
40
30
20
10
0
LnLnL
LLL
OLL
MOL
LnOP
PoOL
MPL
LLP
MPO
PPL
PoOO
OLO
PoPO
POL
OOO
POO
POP
PSL
MPS
PPP
OOS
POS
OLA
PPS
OOA
OSS+AOP
PSS
SSS
APS

Figure 7

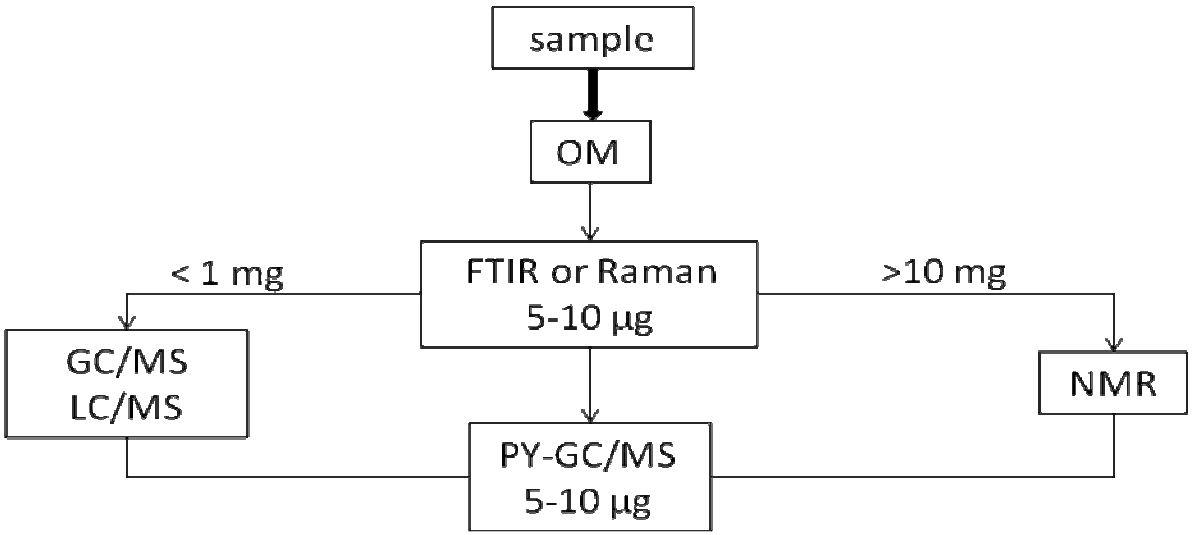
sample
OM
FTIR or Raman
5-10 µg
< 1 mg
>10 mg
GC/MS
LC/MS
NMR
PY-GC/MS
5-10 µg

Figure 8

Figure 1. μ-FTIR Spectrum of the samples acquired with diamond cell in transmission mode between 3800 and 600 cm^{-1}

Figure 2: SERS Raman spectra of sample RRoz (a) compared with Raman spectra of colophony (b), pig suet (c) and galbanum (d).

Figure 3. ^{1}H-NMR spectrum of the polar fraction of RR. The total number of scans for every experiment was 64 and the acquisition time was set at 1.60 s. All ^{1}H-NMR chemical shifts reported are relative to the peak of residual CH_4OH which has been observed to give sharp signals at 3.31 ppm.

Figure 4. Pyrogram of RR sample obtained by Py(TMAH)-GC/MS.

Figure 5: GC/MS chromatograms of of the acidic (a) and neutral (b) fraction of sample RR after saponification and trimethylsilylation. Cx:y linear monocarboxylic acid with x carbon atoms and y-insaturations; Alk Cx linear alkane with x carbon atoms; alcohol Cx linear alcohol with x carbon atoms. Carboxylic acids and alcohols are separated as TMS-derivatives.

Figure 6: SPME-GC/MS chromatograms of the sample a) RR and b) RRoz. 1: α-pinene; 2: β-pinene; 3: 3-carene; 4: o-cymene; 5: menthadiene; 6: cis-β-terpineol; 7: trans-β-terpineol; 8: trans-pinocarveol; 9: 4-terpineol; 10: p-cymen-8-ol + cryptone; 11: p-menth-1-en-8-ol; 12: methyl-chavicol; 13: 2-decenal; 14: α-longipinene; 15: cyclosativene; 16: longicyclene + α-copaene; 17: β-elemene + sativene; 18: longifolene; 19: Z-caryophyllene; 20: α-gurjunene; 21: α-caryophyllene; 22: muurola-4(14), 5-diene; 23: γ-muurolene; 24: α-muurolene + β-bisabolene; 25: γ-cadinene; 26: δ-cadinene + trans-calamenene; 27: 10-epi-cubebol; 28: non identified; 29: guaiol; 30: epi-α-cadinol; 31: muurol-5-en-4-one; 32: acorenone; A: ethylcyclohexenone; B: 2-nonanone; C: nonanal; D: norinone; E: sabina ketone; F: non identified; G: nonanol; H: non identified; I: γ-nonalactone.

Figure 7: Relative abundance of TAGs determined in sample RR. M: myristic, P: palmitic, Po: palmitoleic, S: stearic, O: oleic, L: linoleic, Ln : linolenic, A: arachidic. The positional distribution of the fatty acids was determined only for few TAG and it is here not taken into account.

Figure 8. Example of a multi-analytical approach for the analysis of historical and archaeological residues of pharmaceutical or cosmetic preparations.

Table 1. Ingredients used in the preparation of the *Diachylon Bouchardat Plaster* [7], used as the blind sample.

Diachylon Bouchardat Plaster		
Ingredient	**Amount**	**Supplier**
Plaster Simple	250 g.	
Galbanum	8 g.	*www.laviadellincenso.it*
Beeswax	24 g.	Local producer, Pisa, Italy
Pine resin	12 g.	*www.laviadellincenso.it*
Colophony	12 g.	*Zecchi (Florence-Italy)*

Table 2: Ingredients used in the preparation of the *Simple Bouchardat Plaster* [7] used as an ingredient of the Diachylon Bouchardat Plaster.

Simple Bouchardat Plaster		
Ingredient	**Amount**	**Supplier**
Silver Litharge (PbO)	100 g.	Sigma Aldrich, Milano, Italy
Pig suet	100 g.	Butcher's Shop, Venice, Italy
Olive oil	100 g.	"La Giara" oil producer, Trapani, Italy
Deionised Water	200 g.	

Table 3: Analytical techniques employed by the participating laboratories

Analytical technique	**Number of laboratories**
FTIR:	5
micro-Raman	3
XRF	1
NMR	1
Py-GC/MS	1
GC/MS	5
LC/MS	3

Table 4. Possible assignment of the peaks obtained from FTIR spectra.

Wavenumber (cm^{-1})	**assignment**
3005	νC=C-H (cis)
2954 (shoulder)	νCH_3
2919	νCH_2 asymmetric
2850	νCH_2 symmetric
1739	νC=O
1613	νC=C
1540-1520	$\nu C{=}OO^-$ asymmetric or νC=C
1470-1460	δCH_3 asymmetric
1460-1410	$\nu C{=}OO^-$ symmetric
1417	C-H rocking
1387	δ CH3
1540-1515	νC=O asymmetric
1471	δ CH_3 asymmetric
1460-1410	νC=O symmetric
1417	C-H rocking
1387	δ CH
1242	νC-O
1172	νC-O
1116	νC-O
1097	νC-O
728-720	δ $(CH_2)_n$

Table 5. Peak assignment in the pyrogram shown in Figure 1.

Peak n°	RT [min.]	Assignment	m/z	MW
1	4,69	1,2,3 -trimethoxy-propane	45,58,**59**,89,102	134
2	6,22	1-decene	41,43,55,**56,**70	140
3	6,36	decane	41,43,**57**,71,85	142
4	7,84	1-undecene	41,**43**,55,56,70	154
5	8,05	undecane	41,43,**57**,71,85	156
6	9,36	1-dodecene	**41**,43,55,56,69	168
7	9,48	dodecane	41,43,**57**,71,85	170
8	10,83	1-tridecene	41,**43**,55,56,57	182
9	10,96	tridecane	41,43,**57**,71,85	184
10	12,46	1-tetradecene	41,**43**,55,56,57	196
11	12,60	tetradecane	41,43,**57**,71,85	198
12	14,24	1-pentadecene	**41**,43,55,57,83	210
13	14,40	pentadecane	41,43,**57**,71,85	212
14	16,14	1-hexadecene	41,**43**,55,57,69	224
15	16,31	hexadecane	41,43,**57**,85,71	226
16	18,11	1-heptadecene	**41**,43,55,57,83	238
17	18,27	heptadecane	41,43,57,85,71	240
18	18,78	tetradecanoic acid, methyl ester	41,43,55,**74**,87	242
19	22,06	hexadecanoic acid, methyl ester	41,43,55,**74**,87	270
20	23,70	9-octadecenoic acid, methyl ester	**55**,69,74,83,97	296
21	23,94	octadecanoic acid, methyl ester	43,55,**74,75,**87	298
22	24,22	octadecanoic acid	**43**,55,57,60,73	284
23	24,86	sandaracopimaric acid, methyl ester	91,**121**,257,301,316	316
24	25,31	pimara-8,15-dienoic acid, methyl ester	119,**241**,257,301,316	316
25	25,52	dehydroabietic acid, methyl ester	43,141,**239**, 240,299	314
26	25,82	abietic acid, methyl ester	41,43,121,256,**316**	316
27	26,17	neoabietic acid, methyl ester	91,121,**135**,148,316	316
28	27,03	1-docosanol , methyl ether	43,45,57,**83**,97	340
29	27,39	heptacosane	41,43,**57**,71,85	380
30	27,55	tetracosanoic acid, methyl ester	55,**74**,75,87,382	382
31	28,37	octacosane	41,43,**57**,85,71	394
32	29,08	1-octacosanol	43,55,**57**,83,97	410
33	29,50	hentriacontane	43,55,**57**,71,85	436
34	30,47	1-triacontanol	43,55,**57**,97,83	438

Table 6: GC/MS methods results: identified compounds and identified materials.

Identified biomarkers	**Interpretation of the origin**
Even-numbered, saturated fatty acids, C12-C26, mainly palmitic (C16:0) and stearic acid (C18:0), oleic acid, dicarboxylic acids, chain length C7-C11	Glicerolipid: non-drying plant oil and/or animal fat
Glycerol	
Sitosterol	Plant oil
Cholesterol	Animal fat
Odd-numbered hydrocarbons (paraffins), chain length C25 to C33, main C27	Beeswax
Long-chain wax esters of palmitic acid, typical m/z 257	
Long-chain alcohols, chain length C26-C30	
Sesquiterpenoids, e.g. longifolene, caryophyllene, cubebene, cadinene, calamenene; MW 204 + 220	Pinaceae resin
Diterpenoid resin acids, e.g. dehydroabietic acid, abietic acid, pimaric acid, isopimaric acid, sandaracopimaric acid, hydroxy-abietic acid	

Table 7: Analytical methods adopted for LC/MS analysis.

HPLC system	Column	Eluition gradient	Sample treatment
HPLC-ESI-HR ToF MS	C18	H_2O/MeOH	Extraction in various solvents and separate analysis of the fractions: isopropanol, acetone, hexane and isopropanol after acidic hydrolysis (to determine the fatty acid distribution)
HPLC- Q-ToF MS	C18	acetonitrile/H_2O	Extraction in acetonitrile
HPLC-APCI-MS	C18	isopropanol/MeOH	Extraction in chloroform:methanol 2:1 to obtain the organic fraction, then extraction in hexane to isolate the apolar lipid fraction containing TAGs

Table 8. Summary of the results of the round robin exercise. In *Italic*: materials not present in the unknown ointment.

Laboratory	Analytical technique	Identified or hypothesized materials
1	FT-IR	Plant oil (olive oil), beeswax, aromatic compounds
2	FT-IR, XRF	Plant oil, beeswax, aromatic natural resin, $PbCO_3$
3	Micro-Raman, SERS	Animal fat (pig suet), colophony or pine resin, Galbanum resin, PbO (Litharge and Massicot), $PbCO_3$
4	NMR, HPLC-APCI-MS	Plant oil (*almond* or olive oil), animal fat, beeswax, Pinaceae resin, galbanum resin, *wine*
5	Py-GC/MS	Plant oil, beeswax, diterpenoid resin
6	GC/MS, SPME-GC/MS	Animal fat (pig lard), beeswax, Pinus sylvestris resin, *triterpenoid compounds*
7	GC/MS	Plant oil, animal fat, beeswax, Pinaceae resin
8	GC/MS, FTIR	Plant oil, beeswax, Pinaceae resin, $PbCO_3$
9	GC/MS, micro-Raman	Plant oil (*almond* or olive oil), animal fat, beeswax, Pinaceae resin, $PbCO_3$
10	GC/MS, HPLC-Q-ToF MS, FT-IR, micro-Raman	Plant oil, beeswax, diterpenoid resin, PbO
11	HPLC-ESI-HR ToF MS	Plant oil (olive oil), beeswax , Pinaceae resin, *sandarac resin*