

CHARACTERIZATION OF ORGANIC MATERIALS
IN WALL PAINTINGS IN BULGARIA:
A MULTI-ANALYTICAL APPROACH

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Abstract

The identification of organic materials in cultural heritage objects is crucial for elucidating historical artistic practices and for designing appropriate conservation strategies. This study presents an integrated analytical protocol for the characterization of organic materials in wall paintings through the combined application of complementary analytical techniques. The performance of the proposed approach is demonstrated through several case studies of Bulgarian monuments. The findings indicate that a combination of Fourier transform infrared spectroscopy (FTIR) with enzyme-linked immunosorbent assay (ELISA), thermogravimetric analysis (TGA) and analytical pyrolysis coupled with gas chromatography and mass spectrometry (Py-GC-MS) facilitates the reliable identification of organic materials, even in minimal samples.

Key words: organic materials, cultural heritage, FTIR, TGA, ELISA, Py-GC-MS

Introduction. The study and preservation of archaeological, historical, and artistic objects created over millennia in present-day Bulgaria have become a major priority for both the state and society. Knowledge of the materials used in

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the creation of cultural heritage objects is essential for the development of appropriate conservation and restoration strategies as well as for optimizing storage conditions [1, 2]. In artistic practice, organic materials are frequently employed, fulfilling multiple functional roles such as binders, adhesives, coatings, and modifiers. The identification of organic pictorial materials has been demonstrated to yield significant insights into historical artistic technologies, facilitate the differentiation of painting techniques, support the diagnosis of degradation processes, and facilitate the development of appropriate restoration procedures [3, 4]. Despite their importance, the identification of organic materials remains analytically challenging due to their typically low concentrations relative to inorganic components, such as pigments and plasters. Furthermore, the process of long-term ageing and degradation leads to a reduction in detectability and potential alterations to the chemical composition of the materials.

In recent decades, significant efforts have been directed towards the development of analytical techniques for the investigation of cultural heritage materials, including organic substance [5–7].

The aim of the present study is to establish an analytical protocol that defines the minimum number of analyses required to reliably determine the nature of organic materials found in cultural heritage objects. The applicability of the proposed approach is demonstrated through the analysis of selected archaeological and artistic samples from Bulgarian monuments.

Analytical techniques applied in the study of organic materials. *Fourier transform infrared spectroscopy (FTIR)* is a widely employed analytical technique in the field of cultural heritage research, particularly in the investigation of organic materials. The method is a comprehensive approach that affords molecular and structural information regarding natural materials, encompassing a wide range of forms, including solids, liquids, powders, and polymers [8–10]. A significant advantage of FTIR spectroscopy is its capacity to detect organic compounds, even when these are present in mixtures with inorganic components. Spectra can be collected through the absorption or reflection of infrared radiation from the sample. The utilisation of attenuated total reflection (ATR) facilitates the acquisition of spectra without the necessity for preliminary sample preparation. Moreover, the requirement of only small sample quantities (a few milligrams) renders it optimal for the analysis of precious samples from heritage objects. The ATR-FTIR spectra were measured in the middle IR region ($4000\text{--}600\text{ cm}^{-1}$) on Bruker Invenio R FT spectrometer equipped with a diamond crystal ATR accessory. The samples spectra were acquired by accumulating 64 scans at resolution of 2 cm^{-1} .

The enzyme-linked immunosorbent assay (ELISA) is a highly specific analytical method capable of detecting extremely small quantities of proteins, often in the nanogram range. It is particularly useful for identifying proteinaceous materials such as egg, casein, collagen-based animal glues, and blood.

This high specificity makes the method especially valuable when very small samples are available and spectroscopic techniques alone cannot provide reliable identification [11, 12]. For the enzyme-linked immunosorbent assay (ELISA), the sample solutions were loaded on a 96-well Nunc maxisorb immuno plate (ThermoScientific, Waltham, MA, USA) and were incubated overnight at 4 °C. In two subsequent steps, for the detection of egg white, a rabbit anti-chicken ovalbumin primary antibody (1:10 000) (Bio-Rad) and a goat anti-rabbit IgG labelled with an alkaline phosphatase secondary antibody (1:2000) (Bio-Rad) were added. For the detection of casein, a rabbit polyclonal antibody to casein (1:500) (Abcam, Cambridge, UK) and the same goat anti-rabbit IgG labelled with an alkaline phosphatase secondary antibody (1:1500) were used. The OD at 405 nm of the ELISA plate was read on a HiPo Microplate reader (Biosan, Warren, MI, USA). Control experiments without antigens were performed and were considered a negative control.

Analytical pyrolysis coupled with gas chromatography and mass spectrometry (Py-GC-MS) is among the most powerful analytical methods for identifying organic substances in cultural heritage materials [13]. Py-GC-MS allows complex macromolecular materials such as natural resins, waxes, and polymerized oils to be analysed directly. It is particularly valuable for studying organic substances that contain insoluble or chemically resistant fractions that cannot be analysed by conventional chromatographic techniques. Pyrolyses were carried out with a Pyroprobe 5000 platinum-heated filament pyrolyzer (CDS Analytical, Inc, US) interfaced to a GC7890A (Agilent Technologies, US) gas chromatograph coupled with a MSD (Mass selective Detector) 5975C inert XL EI/CI. A 30 m × 0.25 mm i.d. open tubular column (film thickness: 0.25 µm) HP-5MS (unpolar) (Agilent Technologies, US) was used. The mass spectra were recorded under electron impact ionization at 70 eV electron energy, in the range from 15 to 600 m/z.

Thermogravimetric analysis (TGA) is a technique that measures the change in mass of a sample during controlled heating, thereby providing information about the thermal stability and decomposition behaviour of materials. The method has been demonstrated to facilitate the identification of plant resins, waxes, proteins, and carbohydrates. Despite the fact that it is seldom employed as a standalone technique, it furnishes valuable complementary information that can corroborate results obtained by spectroscopic or chromatographic methods [14]. Thermogravimetric analysis (TGA) was performed in a Setaram TG92 instrument. The samples in the range of 12–40 mg were placed in a glass crucible and heated in an air flow (100 cm³/min) up to 773 K with a heating rate of 10 K/min.

Results and discussion. The wide array of materials that comprise cultural heritage objects necessitates a multidisciplinary approach to their study and identification, particularly in relation to organic materials and their components. The natural organic materials found in artworks and archaeological objects can be classified into the following main groups: proteins (e.g. egg, casein, blood, and

collagen-based animal glues); carbohydrates (e.g. sugars, honey and plant gums, as well as polysaccharide adhesives); lipids (e.g. vegetable oils and animal fats, including those found in egg yolk); and resins (e.g. colophony, sandarac, mastic and dammar, as well as shellac).

Given the generally minimal quantities of material available from artefacts, it is of particular importance to ascertain the minimum number of analytical techniques required to characterise their chemical composition.

Based on the practical experience in our lab, the following step-by-step procedure was developed for conducting the analysis.

Step 1. Detection of organic material. The utilisation of FTIR spectroscopy can provide a preliminary indication of the presence of organic substances. It is evident that organic compounds demonstrate characteristic absorption bands that deviate from those exhibited by inorganic materials, particularly within the region between 3100 and 2800 cm^{-1} , which correspond to C–H stretching vibrations. It has been demonstrated that FTIR analysis is capable of detecting organic matter even in mixtures where its concentration is lower than that of inorganic components. In mixtures where organic materials are present in minute quantities, microextraction employing suitable solvents can facilitate enhanced detection. The sequential extraction of organic substances utilising solvents of increasing polarity (e.g. hexane, acetone, and water) has been demonstrated to facilitate the separation of diverse groups of organic substances, thereby enhancing the efficacy of spectral identification.

Step 2. Distinguishing the type of organic material. The subsequent analytical step is aimed at determining the chemical class of the organic material. The presence of characteristic IR absorption bands is indicative of the material's molecular composition, namely whether it is a protein, lipid, wax, carbohydrate, or resin.

For instance, proteinaceous materials exhibit characteristic amide bands at:

- 3400–3200 cm^{-1} – N–H stretching vibrations (amide A)
- 1660–1600 cm^{-1} – C=O stretching vibrations (amide I)
- 1565–1500 cm^{-1} – N–H bending vibrations (amide II),

while lipids exhibit characteristic absorption bands at:

- 3000–2800 cm^{-1} – C–H stretching vibrations
- 1750–1720 cm^{-1} – C=O stretching vibrations
- 1480–1300 cm^{-1} – C–H bending vibrations
- 1300–900 cm^{-1} – C–O stretching vibrations
- 750–700 cm^{-1} – out-of-plane C–H bending vibrations.

It is evident that IR spectra may exhibit bands that are indicative of multiple classes of organic materials, thereby reflecting the presence of natural mixtures.

For example, egg contains both proteins and lipids, and exhibits typical lipid absorption bands at around 1730 cm^{-1} , as well as protein absorption bands.

FTIR spectroscopy also provides information on degradation processes. Protein binders may form calcium oxalates, while drying oils undergo polymerisation, hydrolysis or formation of metal carboxylates.

Step 3. Specific identification. FTIR spectroscopy alone is often not enough to identify specific organic substances precisely. For instance, it is difficult to distinguish between similar triterpene resins, such as mastic and dammar, or between different protein binders.

Consequently, additional analytical methodologies must be employed in such cases. ELISA is a particularly effective method for the identification of specific proteins, including egg ovalbumin, milk casein and animal collagen. The application of thermal analysis has been demonstrated to be a reliable method for distinguishing between various classes of compounds, including but not limited to carbohydrates, vegetable oils, resins, and waxes. The highest level of analytical specificity is achieved using chromatographic techniques such as pyrolysis–gas chromatography–mass spectrometry (Py-GC-MS) and high-performance liquid chromatography (HPLC).

Figure 1 provides a comprehensive summary of the recommended analysis techniques for investigating organic materials from cultural heritage.

Case studies of Bulgarian monuments. Identification of an egg binder. In the context of Byzantine and post-Byzantine church painting, both the fresco technique, in which the lime plaster incorporates the wet pigments during the painting process, and during carbonization the plaster binds the pigments into its structure, and some secco techniques, which use organic binders, were used. Technological studies of wall paintings have demonstrated that proteinaceous binders (egg, animal glue, or casein) were predominantly used.

Our FTIR analysis of red, white and orange microsamples (Fig. 2A) obtained from the wall paintings in the Chapel of Ivan Rilski (part of the Rila Monastery's catholicon) revealed the presence of calcite, together with absorption bands in the $3000\text{--}2800\text{ cm}^{-1}$ region, indicative of an organic component in the paint composition. The spectra further exhibited bands characteristic of proteins: $\sim 3300\text{ cm}^{-1}$ (Amide A), $1630\text{--}1660\text{ cm}^{-1}$ (Amide I), and $1500\text{--}1550\text{ cm}^{-1}$ (Amide II), observed as shoulders to the intense stretching band of calcite at $\sim 1400\text{ cm}^{-1}$. As illustrated in Fig. 2B, the positions and relative intensities of these bands are consistent with reference spectra of dried aged egg, suggesting the presence of an egg-based binding medium.

In order to verify this assignment, the analysis was complemented by an enzyme-linked immunosorbent assay (ELISA). The samples were tested using antibodies to egg white and casein. The results obtained are presented in Fig. 3, wherein the dashed line signifies the limit of the analysis (i.e. the lowest limit of detection). The results show that the optical density values of all the samples

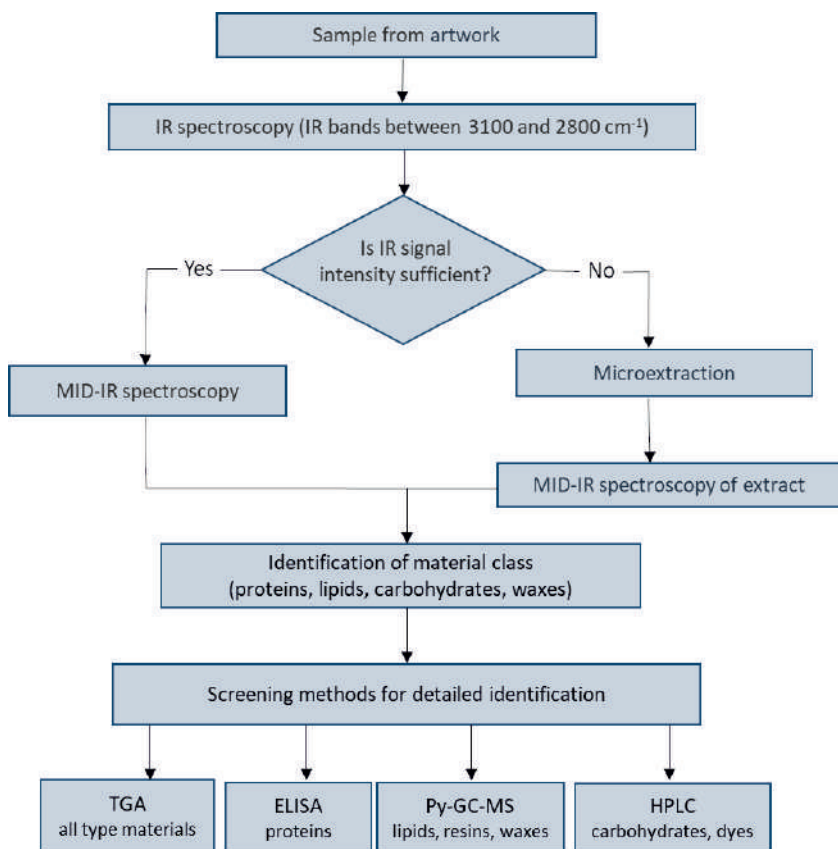


Fig. 1. Scheme of the analytical protocol for identification of organic materials in cultural heritage samples

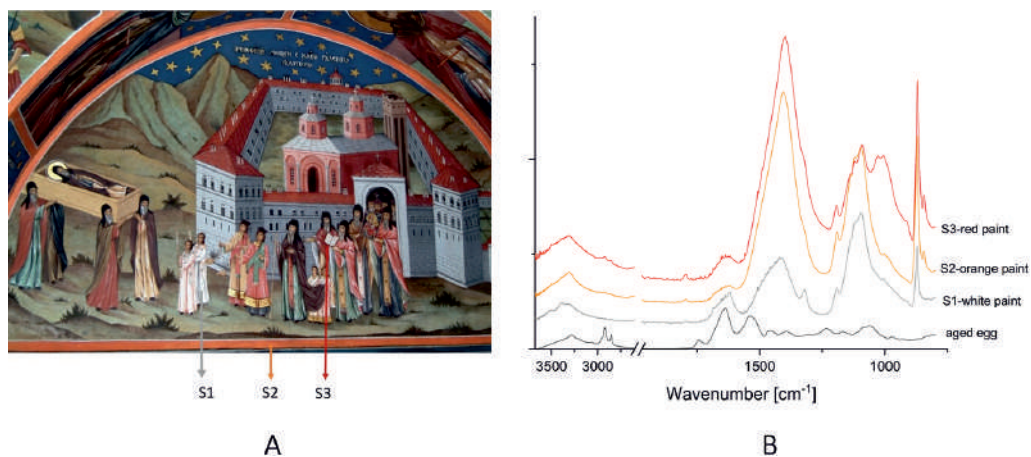


Fig. 2. Diagram showing the locations of the samples collected for analysis (A); ATR-FTIR spectra of the studied samples compared with the reference aged egg (B)

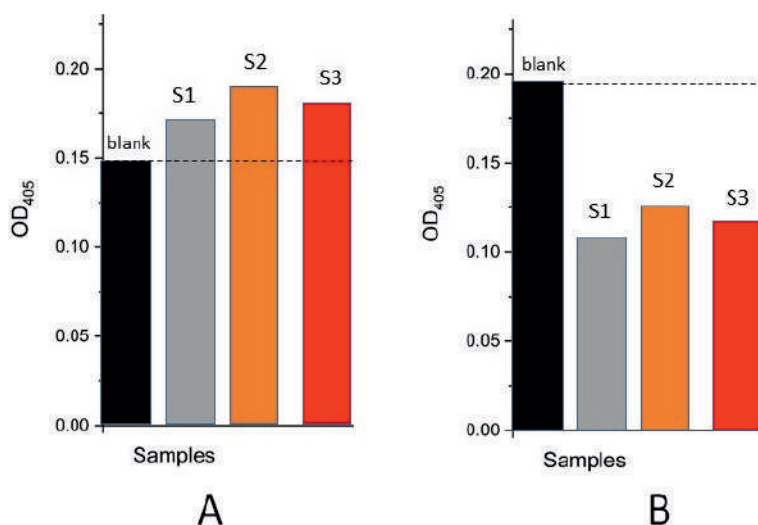


Fig. 3. Detection of egg white (A) and casein (B) by the measurement of the optical density at 405 nm using ELISA at optimized antibody dilutions. The dashed line represents the assay cut-off value

analysed exceed the established cut-off value for egg white (Fig. 3A), while remaining below the detection threshold for casein (Fig. 3B). This unequivocally confirmed the presence of egg white and excludes casein as a binding component. On the basis of the combined spectroscopic and immunochemical data, it was found that egg tempera was employed as the principal painting technique also in a significant proportion of the wall paintings in the main church [15] and the ossuary church of the Rila Monastery complex [16].

Identification of a carbohydrate binder. FTIR spectroscopic study of a blue paint sample from the Rila Monastery's main church wall paintings revealed the presence of pigment smalt together with an organic component. The FTIR spectra exhibited absorption bands at 3270 cm^{-1} (O–H str.), 2958 , 2922 and 2856 cm^{-1} (CH str.), 1637 cm^{-1} (OH bend.) and 1028 cm^{-1} (C–O str.), which are consistent with the presence of carbohydrate-type materials. Thermogravimetric analysis (TGA) showed thermal behaviour comparable to that of a reference wheat starch sample, including mass loss over a broad temperature range ($170\text{--}500^\circ\text{C}$) and two well-defined TG/DTG events. The combined FTIR and TGA data support the identification of a polysaccharide binder, most likely a starch-based adhesive such as wheat starch paste [17]. The utilisation of a starch-based binder in conjunction with smalt appears to diverge from the documented methodologies employed in many Greek and Cypriot monasteries, where egg has been identified as the binding medium for smalt pigment. This discrepancy may be of technological significance, as polysaccharide binders are less likely to alter the optical properties of smalt and may contribute to better preservation of its blue colour.

Identification of an adhesive for the gilding. As part of an extensive analytical study of the Rila Monastery's main church wall paintings, the composition of a mordant in the gilded saints' haloes was the object of close analysis. The bands observed in the FTIR spectrum of the samples supported the predominant organic content, as evidenced by strong absorptions for alkyl C–H, stretching vibrations in the region $2950\text{--}2830\text{ cm}^{-1}$, very strong bands at $1560, 1456\text{ cm}^{-1}$ and bands of moderate intensity at 1708 cm^{-1} and below 1300 cm^{-1} . The observed FTIR bands implied the possible presence of drying oil, fatty acid carboxylates and an additional organic component.

The use of Py-GC-MS analysis enabled the identification of a triterpene-type plant resin (dammar) along with the drying oil. Summarising the analytical data, it was concluded that the gilding mordant was made from drying oil, resin and siccative metal oxides [18].

Identification of beeswax. FTIR analysis of red paint collected from the monochrome zone of the Thracian tomb near Alexandrovo revealed the presence of red ochre, calcite and organic material. In view of the markedly small amount of the organic material, it was determined that chloroform extraction would be the most appropriate initial step in the analysis. The IR spectrum of the extract exhibited characteristic absorption bands at $2917, 2800\text{ cm}^{-1}$ (CH str.), 1738 cm^{-1} (C=O str.), 1472 cm^{-1} (CH def.), 1173 cm^{-1} (C–O str.) and 729 cm^{-1} (CH tor.) pointing out to beeswax. This identification was confirmed by thermal analysis of the sample, which indicated a melting temperature of approximately 64°C , consistent with reference beeswax sample [19]. This reliable method confirmed the use of wax in the ancient process of creating polished plasters not only in Alexandrovo tomb, but also in other Thracian tombs from the Hellenistic period, including the tombs of Maglish, Dolno Lukovo and Helvetsia [20].

Conclusion. The identification of organic materials in cultural heritage objects requires the combined use of complementary analytical techniques. FTIR spectroscopy provides rapid screening and preliminary classification, while ELISA, thermogravimetric analysis, and chromatographic techniques allow more specific identification.

The proposed analytical protocol enables reliable characterization of organic materials using minimal sample quantities. The presented case studies demonstrate that this multidisciplinary approach provides valuable information for understanding historical artistic technologies and supports the conservation of Bulgarian cultural heritage.

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