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Identification of diterpenes in canvas painting varnishes by gas chromatography–mass spectrometry with combined derivatisation☆

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Abstract

A derivatisation method that combines the formation of ethyl esters from the carboxylic groups and trimethylsilyl ethers from hydroxyl groups of the components of diterpenic resins is presented in this paper. This methodology involves two experimental steps: (1) formation of ethyl esters using ethyl chloroformate; and (2) the esterified compounds are lead to react with trimethylsilylimidazole to form the corresponding trimethylsilyl ethers. The main advantage of the proposed method is the possibility of performing simultaneously the analysis of amino acids from proteins, fatty acids from drying oils, and diterpenic compounds from natural resins usually found in works of art. This methodology is of considerable interest due to the requirements of minimum sampling that usually involves the analysis of works of art. A chemometric study has been developed to adjust the optimal working conditions of the proposed derivatisation method in which chromatographic peak areas of the larixyl acetate derivative and the abietic acid derivative referred to *n*-hexadecane as internal standard have been compared. Samples of Venetian turpentine naturally aged have been used in this study. Finally, the efficiency of the proposed derivatisation method has been tested on other diterpenic resins and pigments commonly used in fine arts such as Strasbourg turpentine, Canada balsam, colophony, copper resinate and a sample from a Renaissance Altarpiece.

Keywords: Art analysis; Turpentine; Chemometrics; Diterpenes; Terpenes; Ethyl chloroformate; Trimethylsilylimidazole

1. Introduction

Characterisation of painting varnishes is of interest in the field of conservation and restoration of works of art due to the rapid alteration that involves them because of the combined action of different environmental agents (humidity, temperature, light, atmospheric pollutants, micro-organisms, etc.). Identification of the main components of the varnish is essential for the establishment of the suitable restoration treatment.

Studies dealing with the gas chromatography (GC) analysis of terpenic compounds are, in general, based on the formation of methyl esters, from the carboxylic groups in the compounds, using different types of derivatisation agents such as diazomethane, methyl chloroformate and direct methylation [1–5]. Other methods based on the formation of trimethylsilyl ethers of the carboxylic groups [6–8] have been proposed. Finally, a third category of methods based on the simultaneous formation of trimethylsilyl ethers of carboxylic and hydroxyl groups of the compounds using bis(trimethylsilyl)trifluoroacetamide (BSTFA) as derivatisation agent [9,10] have been developed.

A derivatisation method that combines the formation of ethyl esters from the carboxylic groups and trimethylsilyl ethers from hydroxyl groups of the components of diterpenic resins is presented in this paper that provides a rapid method of analysis in which the information obtained on the chemical composition of the diterpenic resin is increased. This methodology is based on the previously one developed by the authors [11–13] and involves two experimental steps: (1) formation of ethyl esters using ethyl chloroformate (ECF) in ethanol–pyridine medium; and (2) the esterified compounds are lead to react with trimethylsilylimidazole (TMSI) to form the corresponding trimethylsilyl ethers. The main advantage of the proposed method is the possibility of simultaneously

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performing the analysis of amino acids from proteins, fatty acids from drying oils, and diterpenic compounds from natural resins using only a sample of a few microgram. This methodology is of considerable interest due to the requirements of minimum sampling that usually involves the analysis of works of art. In particular, in the cases in which there is disposal of only a sample due to the difficulty in the separation of the different pictorial strata, a few μm width. Accuracy in the identification and quantitation of these compounds is achieved by means of the separation of amino acids from fatty acids and diterpenic compounds from natural resins that is included as a first step in the ECF derivatisation process. In this way, two chromatograms are finally obtained in which proteins are resolved separately from the fatty acids and diterpenic compounds avoiding overlapping problems. Additionally, fatty acids and terpenic compounds are also perfectly resolved due to their different retention time exhibited at the working conditions selected in the GC–mass spectrometry (MS) system.

A chemometric study has been developed to adjust the optimal working conditions of the proposed derivatisation method in which chromatographic peak areas of the larixyl acetate derivative and the abietic acid derivative referred to *n*-hexadecane as internal standard have been compared. Samples of Venetian turpentine naturally aged have been used in this study. Finally, the efficiency of the proposed derivatisation method has been tested on other diterpenic resins and pigments commonly used in fine arts such as Strasbourg turpentine, Canada balsam, colophony, copper resinate and a sample from a Renaissance Altarpiece.

2. Experimental

2.1. Solvents and reagents

The following reagents were used to treat the samples: ECF (purity >98%) and absolute pyridine (Fluka, Buchs, Switzerland). Hydrochloric acid at 37% for analysis (Scharlau, Barcelona, Spain). Chloroform (purity >98%) for GC (Acros, NJ, USA). Absolute ethanol and *n*-hexane for analysis (Fisher, Loughboruogh, UK) and sodium hydrogenocarbonate for analysis was purchased from Panreac (Barcelona, Spain). *n*-Hexadecane (minimum 99%) as internal standard and *N*-trimethylsilylimidazole (TMSI) (98%) (Sigma, Steinheim, Germany). Strasbourg turpentine, Canada balsam, copper resinate and linseed oil (Kremer, Farbmühle, D-88317 Aichstten/Allgäu, Germany) were supplied by AP Fitzpatrick (London, UK). Colophony and Venetian turpentine (Talens), were supplied by RCM (Productos de Conservación, Barcelona, Spain).

2.2. Test specimens

A series of test specimens were prepared in which Venetian turpentine, Strasbourg turpentine, Canada balsam, colophony and copper resinate were spread as a thin layer on glass slides. Then the test specimens were dried at room temperature (during 15 days) and kept in the fridge until the analysis. A second series of samples were prepared as mentioned earlier mixing linseed oil (75%) and Venetian turpentine (25%).

With the purpose of simulating the ageing conditions in which works of art samples are found, a series of test specimens of Venetian turpentine, prepared as mentioned earlier in 1994, were used to perform the chemometric study.

2.3. Experimental procedure

Resin samples (5 mg) are dissolved in 50 μl of an ethanol–pyridine mixture (4:1). Then, 25 μl of ECF are added. The reaction mixture is shaken for about 10 s and the derivatives are extracted in 50 μl of chloroform. After that, 50 μl of a saturated solution of $NaHCO_3$ are added and the mixture is shaken carefully. The organic phase is extracted and evaporated to dryness on a heating-block under N_2 atmosphere. The residue is treated directly with 1.4–2.8 μl of TMSI under N_2 atmosphere at 40–80 °C for 10–30 min. The excess of derivatisating reagent is eliminated adding 100 μl of water and the derivatives are extracted with 50 μl of chloroform containing *n*-hexadecane as internal standard. After shaking the mixture by ultrasons for 15 min, a 1 μl aliquot of the organic phase is injected for GC analysis.

2.4. Instrumentation

An Agilent 5973N mass spectrometer coupled to an Agilent 6890N gas chromatograph (Agilent Instruments, USA) was used. Agilent Chemstation software (MSD) was used for the integration of peaks and for the mass spectra evaluation.

GC separation was achieved in a chemically bonded fused-silica capillary column HP-5-MS (Agilent, USA), (stationary phase 5% phenyl–95% methylpolysiloxane, 30 m × 0.25 mm i.d., 0.25 μm film thickness). The chromatographic conditions were: temperature initial of the gas chromatograph 100 °C. Oven temperature was programmed with a gradient of 20 °C min^{-1} up to 295 °C. The carrier gas was He with inlet pressure of 72.5 kPa and 1:20 split ratio. The electronic pressure control was set to constant flow mode with vacuum compensation. Ions were generated by electron ionisation (70 eV) in the ionisation chamber. The mass spectrometer was scanned from *m*/*z* 20–800, with a cycle time of 1 s.

3. Results and discussion

3.1. Characterisation of Venetian turpentine components: preliminary probes

Stoichiometric calculations based on the results of Van den Berg and Pastorova [14], which indicate that natural

Table 1
Retention time and mass fragmentations of the different derivatives formed from the Venetian turpentine applying the proposed derivatisation method

Diterpenic acid	Derivative	t_R (min)	*m*/*z*
Pimaric acid	Ethyl ether	9.34	330, 315, *257*, 194, *121*
	Trimethylsilyl ether	9.37	374, 359, *257*, 241, *121*, 73
Sandaracopimaric acid	Ethyl ether	9.41	330, 315, *257*, 194, *121*
	Trimethylsilyl ether	9.43	374, 359, *257*, 241, *121*, 73
Isopimaric acid	Trimethylsilyl ether	9.49	374, 359, *256*, *241*
	Ethyl ether	9.55	330, *315*, 301, *257*, *241*
Levopimaric/palustric acids	Trimethylsilyl ether	9.56	374, *359*, *241*, 73
	Ethyl ether	9.59	330, *315*, 257, *241*
Dehydro-dehydroabietic acid	Ethyl ether	9.65	*326*, 252, *237*, 197
Dehydroabietic acid	Trimethylsilyl ether	9.68	372, 357, *239*, 73
	Ethyl ether	9.71	*328*, 313, *239*
Larixyl acetate	Trimethylsilyl ether	9.86	270, 255, *143*, 73, 43
Abietic acid	Trimethylsilyl ether	9.80	374, 359, *256*, *241*
	Ethyl ether	9.90	330, *256*, *241*, 213, 185
Abietatetraenoic acid	Ethyl ether	10.14	*326*, 311, *237*
Neoabietic acid	Ethyl ether	10.23	330, 239, 148, *135*, *121*
7-Oxodehydroabietic acid	Ethyl ether	10.75	342, 298, 269, *253*, *187*
15-Hydroxy-7-oxodehydroabietic acid	Ethyl ether	11.01	*401*, 385, 73

aged diterpenic resins have a content of hydroxylated resinic acids between 20 and 40%, led firstly to establish the amount of ECF and TMSI ranged between 5 and 8, and 1.4 and 2.8 μl, respectively, for accomplishing the derivatisation process of 5 mg of sample. Time of the silylation reaction of 10–30 min and silylation temperature of 40–80 °C were also determined on the basis of the previous results found in the literature [15–18].

A preliminary study focused on the estimate of the optimal values of physical parameters in the GC system was developed in which several samples consisting in binary systems drying oil–diterpenic resin, that reproduce the traditional recipes of art varnishes, were checked in order to assess the possible interference of fatty acids from the drying oils on the analysis of diterpenic compounds. The experimental conditions in this study were established as mentioned earlier. Two series of experiences were carried out differing in the inclusion of a previous step of hydrolysis. Different initial and final oven temperature and temperature ramp were also tested. Final value ramp was fixed at 20 °C min^{-1}, thus the chromatographic separation can be accomplished in 16 min.

The analysis of a binary sample of linseed oil (75%)–Venetian turpentine (25%) without previous hydrolysis step showed that the components of the Venetian turpentine appear at two different time intervals in the chromatogram. ECF and TMSI derivatives of sesquiterpenic compounds such as α-pinene, camphene, limonene, jupinene, α-terpinolene and α-terpineol, among others, are found from 2 to 6 min. Peaks corresponding to ECF derivatives of palmitic, oleic and stearic fatty acids from linseed oil can be observed in the range from 5 to 8 min. ECF derivatives of diterpenic components from Venetian turpentine, which appear in the time range from 9 to 12 min, are the main compounds found in the chromatogram. Table 1 summarises the retention time and main *m*/*z* values corresponding to the different derivatives of diterpenic compounds found. It should be noted that different derivatives are found for the main diterpenic components (i.e. pimaric ethyl ester at 9.34 min and TMS derivative of dehydroabietic acid at 9.68 min) evidencing that a competition between the two derivatisation reactions is taking place. These results suggest that an accurate optimisation of the variables directly influencing on the derivatisation reactions used in the proposed method is required.

Fig. 1 shows the chromatogram corresponding to the ECF and TMS derivatives of a binary sample of linseed oil (75%)–Venetian turpentine (25%) in which a previous hydrolysis step, adding 100 μl of 3 M HCl to the sample for 30 min at 110 °C under inert atmosphere, was included. Peaks corresponding to ECF derivatives of palmitic, oleic and stearic fatty acids from linseed oil appear in the range from 5 to 8 min. Diterpenic components of the Venetian turpentine appear, in the same way that the non-hydrolysed sample, after 9 min and only a significant decreasing in the peak area of larixyl acetate is observed in agreement with the bibliography [9]. No difference is observed in the peak area of other characteristic diterpenic compounds such as pimaric, isopimaric, sandaracopimaric, dehydroabietic, abietic and neoabietic acids, among others, that usually are found in diterpenic resins as main components. These preliminary experiences indicate that a satisfactory separation of the components from linseed oil and Venetian turpentine can be obtained and this result could be extended to the other diterpenic resins. The results obtained suggest that the proposed method can be used for studying more complex systems composed by drying oils and diterpenic resins,

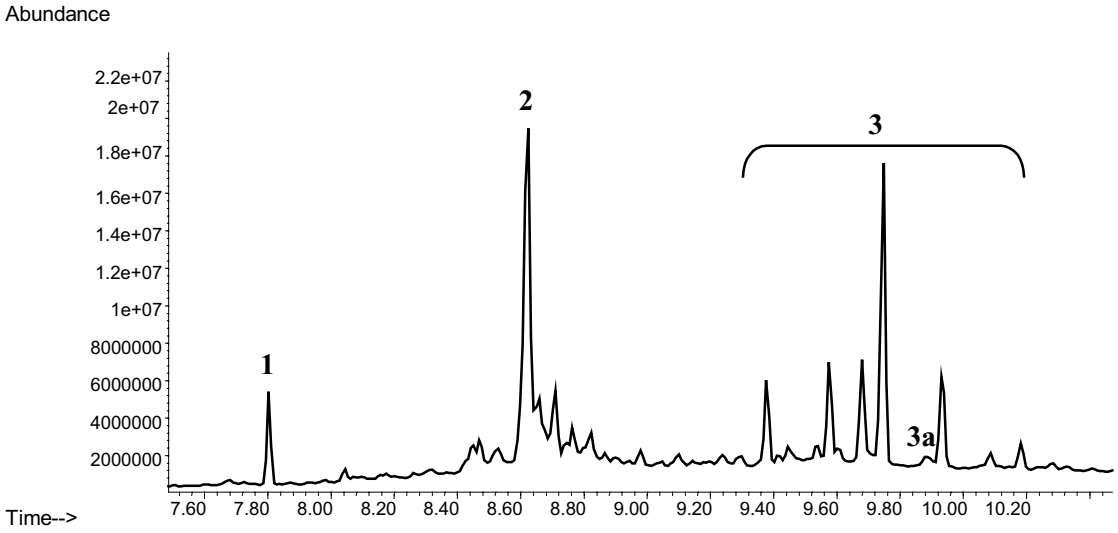


Fig. 1. Gas chromatogram of ECF and TMS derivatives of a binary sample linseed oil (75%)–Venetian turpentine (25%) of the series prepared with previous hydrolysis step. Peaks: (1) ethyl palmitate; (2) ethyl oleate; (3) diterpenic fraction; (3a) TMS derivative of larixyl acetate. Time scale in min.

which are commonly used in the preparation of traditional paint varnishes.

3.2. Chemometric experimental design for optimising the derivatisation procedure

A chemometric experimental design has been carried out in order to optimise the proposed analytical method. This procedure enables the examination and optimisation of each parameter in a predefined range by performing a series of experiments in each of which the values of the different parameters are changed at the same time.

A screening factorial design has been carried out with the aim of detecting the variables having the highest influence on the yield of both derivatisation reactions. The four variables that have been included in the proposed design are the amount of ECF (V_{ECF}), TMSI (V_{TMSI}), temperature (T) and time of silylation (t). The selected ranges are 5–8 μl for the amount of ECF, 1.4–2.8 μl for the amount of TMSI, 40–80 °C for the silylation temperature and 10–30 min for the silylation time. Thus, in the two-level design, 16 experiments (three replicates each) were performed. GC responses used for studying the effect of each screened parameter on the derivatisation reaction were the peak area ratios of the TMS derivative of larixyl acetate and the abietic ethyl ester to the *n*-hexadecane. This last compound was selected as internal standard in both cases. Response of these two components of the Venetian turpentine was considered necessary for assess accurately the effect of the different parameters influencing the derivatisation procedure. The first one was selected as the most representative compound found in the chromatogram that exhibits a hydroxyl group able to react with TMSI. The second one was also selected due to its high signal and the carboxylic group in its molecule that is able to react with ECF and TMSI. Both compounds and the molecular structure of their selected derivatives are depicted in Table 2.

The main effect of each variable was defined as the average change in the response value from the GC–MS when the design variable goes from its low to its high level. Table 3 shows the results obtained for the four parameters considered in this study. As can be seen in the diagram depicted, the amount of ECF is the only variable displaying negative main effect on the larixyl acetate response and silylation temperature is the variable with most significant positive

Table 2
Molecular structure of the larixyl acetate and abietic acid derivatives selected for the screening design

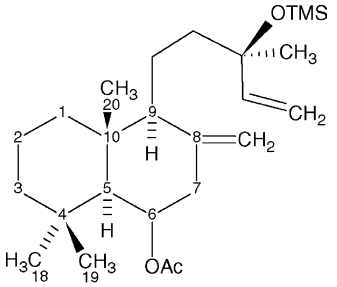
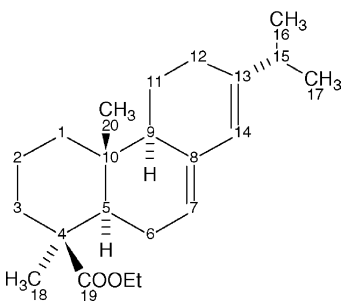
Compound	Molecular structure	Derivative
Larixyl acetate		Larixyl acetate trimethylsilyl ether
Abietic acid		Abietic acid ethyl ester

Table 3
Values of main effect and different interaction effects of the four screened variables for the abietic acid and larixyl acetate GC–MS responses

Variable	Effect on abietic acid response	Effect on larixyl acetate response
V_{ECF}	0.14	−0.12
V_{TMSI}	−0.06	0.07
t	−0.09	0.04
T	−0.12	0.20
V_{ECF}–V_{TMSI}	0	0.09
V_{ECF}–t	−0.10	−0.04
V_{ECF}–T	0.11	0.07
V_{TMSI}–t	−0.03	0.04
V_{TMSI}–T	0	0.02
t–T	−0.04	0
V_{ECF}–V_{TMSI}–t	0.03	−0.02
V_{ECF}–t–T	0.09	0.02
V_{TMSI}–t–T	−0.03	0.07
V_{ECF}–V_{TMSI}–t–T	0.07	0.03

main effect on this compound. Opposed results in the main effects for abietic acid response were found.

The two-variable interaction is also illustrated in Table 3. The interaction effect is defined as the average change in the response value from the GC–MS when the design variable goes from its low to its high level. The amount of ECF–silylation temperature has a significant positive effect on the yield of both selected derivatised larixyl acetate and abietic acid. This means that, in order to get the best response of the larixyl acetate TMS derivative and the abietic ethyl ester, both variables should be set to their higher or lower levels. In the same way, amount of ECF–silylation time have the highest negative effect, thus these variables should be set to opposite levels to obtain the best results. Similarly, interaction of three variables diagram indicates that amount of ECF–temperature–time of silylation is the only case in which positive effect is obtained in both response variables and they promote the highest change in the peak of abietic ethyl ester and a positive change on larixyl acetate TMS derivative. Finally, the four variables interaction indicates that those variables have positive effect on both products of reaction.

The results of the screening design can be summarised as follows:

(i) All variables studied have opposite main effect on both abietic ethyl ester and TMS–larixyl acetate derivatives.
(ii) Amount of ECF and silylation temperature are the variables that exhibit the highest main effect.
(iii) Amount of ECF–silylation temperature and amount of ECF–silylation time exhibit, respectively, the highest positive and negative two-variable interaction effect. It suggests that best results should be obtained if ECF amount and silylation temperature is set to high values and silylation time is fixed to the lowest value.
(iv) Three-variable interaction effect of amount of ECF–silylation temperature–silylation time is the only one that exhibits positive value on the both derivatisation products studied.
(v) Four-variable interaction effect exhibits positive value on both derivatisation products studied.

The results obtained in the experimental design lead to establish that optimum conditions can be allowed if a larger amount of ECF is selected and the silylation temperature is fixed at the higher value of 80 °C. Thus, the reaction time could be set at lower value of 10 min, taking into account the highly positive interaction effect of those two first variables. Similarly, amount of TMSI is maintained in the lowest value.

3.3. Minimisation of secondary derivatisation effects

The chemometric study carried out enables for the optimisation of the proposed method of derivatisation and the results obtained can be considered highly satisfactory. Nevertheless, the presence of secondary derivatisation products, as result of the use of two derivatisation reactives, is not completely avoided by applying the selected experimental conditions. For instance, two derivatisation products are formed from the pimaric acid, in which a carboxylic group is found, corresponding to the ECF and TMS derivatives (retention times: 9.34 and 9.36 min). Comparison of the peak area responses of both derivatisation products indicates that, in the experimental conditions proposed as result of the chemometric study, a notable decreasing in the analytical signal of pimaric ethyl ester is caused as result of the formation of the TMS derivative in a significant amount. Similar behaviour was observed in other diterpenic compounds.

Attempting to reduce the formation of these secondary derivatisation products a further step in the performed derivatisation method has been proposed. This final step consists in the extraction of the products of derivatisation with chloroform in which a small amount of ECF was added. Thus, an increasing in the ECF derivative response on that from the TMS derivative is achieved. The addition of 30 μl of 5% ECF solution in chloroform permits a reduction between 95% (for the pimaric acid TMS derivative) and 60% (for the dehydroabietic acid TMS derivative).

3.4. Analysis of diterpenic resins

Fig. 2 show the gas chromatograms of colophony (A), Strasbourg turpentine (B), Canada balsam (C) and copper resinate (D). Significant peaks of sandaracopimaric ethyl ester (retention time: 9.44 min), isopimaric ethyl ester (retention time: 9.54 min), levopimaric/palustric ethyl ester (retention time: 9.57 min), dehydro-dehydroabietic ethyl ester (retention time: 9.63 min), dehydroabietic ethyl ester (retention time: 9.71 min), abietic ethyl ester (retention time: 9.92 min), abietatetraenoic ethyl ester (retention time: 10.14 min), neoabietic ethyl ester (retention time: 10.23 min), 7-oxodehydrobietic ethyl ester (retention time: 10.75 min) and 15-OH-7-oxodehydroabietic ethyl ester–TMS ether (retention time: 11.01 min) are found in all of them. Peak of larixyl acetate TMS ether was found only

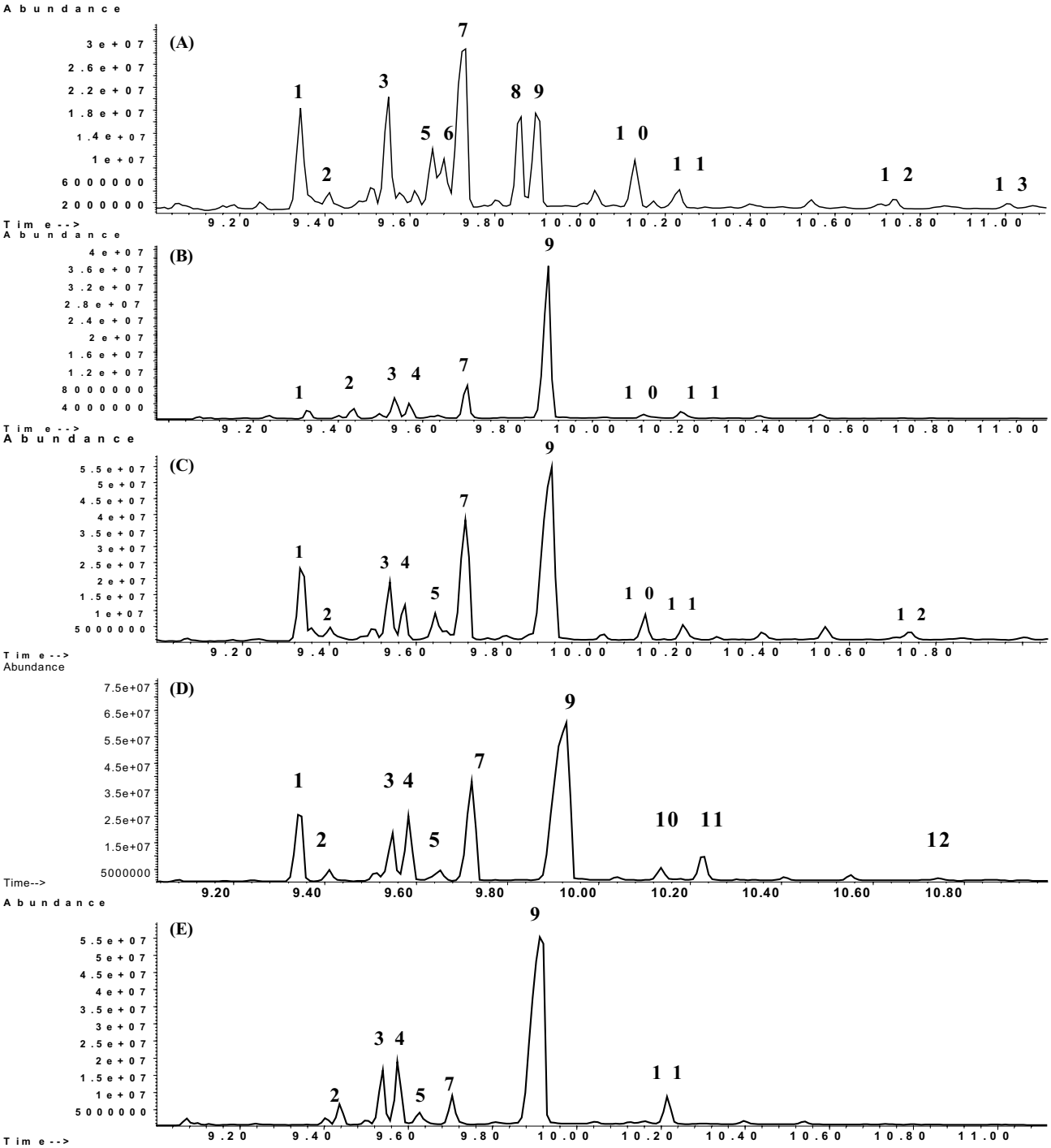


Fig. 2. Gas chromatogram of ECF and TMS derivatives of diterpenic compounds from: (A) Venetian turpentine; (B) Canada balsam; (C) colophony; (D) copper resinate; (E) Strasbourg turpentine. Peaks: (1) pimaric ethyl ester; (2) sandaracopimaric ethyl ester; (3) isopimaric ethyl ester; (4) levopimaric/palustric ethyl esters; (5) dehydro-dehydroabietic ethyl ester; (6) dehydroabietic trimethylsilyl ether; (7) dehydroabietic ethyl ester; (8) larixyl acetate trimethylsilyl ether; (9) abietic ethyl ester; (10) abietatetraenoic ethyl ester; (11) neoabietic ethyl ester; (12) 7-oxodehydroabietic ethyl ester; (13) 15-hydroxy-7-oxodehydroabietic ethyl ester–trimethylsilyl ether. Time scale in min.

in Venetian turpentine, which is in good agreement with the results obtained by other authors [9] and enables the accurate differentiation of this diterpenic resin from those others.

A statistical study has been performed using principal component analysis (PCA). Four quotients of the derivatisation products referred to the abietic ethyl ester (pimaric ethyl ester, isopimaric ethyl ester, dehydroabietic ethyl ester and neoabietic ethyl ester) have been used as variables. With PCA, it is possible to reduce the data obtained to two or three principal components that account for, at least, 99%

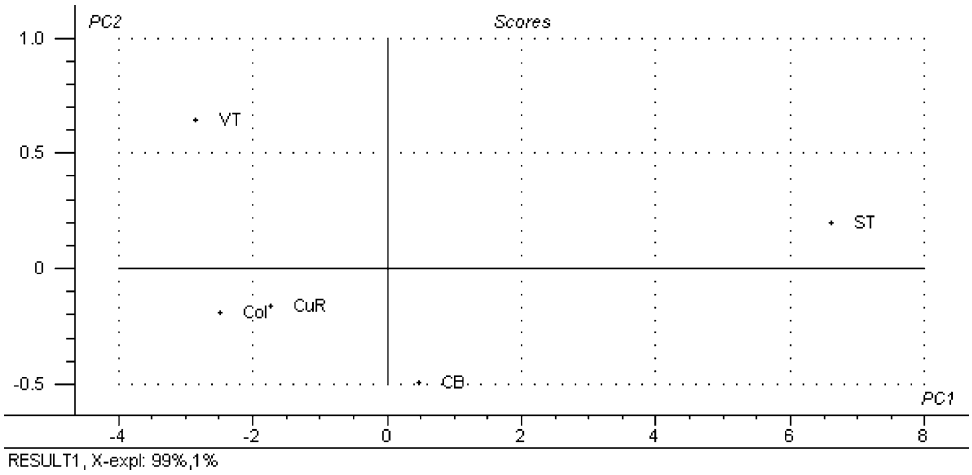


Fig. 3. Discrimination by principal component analysis (PCA). Diagram of colophony (COL); Strasbourg turpentine (ST); Canada balsam (CB); copper resinate (CUR); Venetian turpentine (VT).

of the variance. Fig. 3 shows a two-dimensional drawing of the principal components, where the different types of diterpenic resins can be seen grouped, according to their botanical characteristics, in four main categories: colophony and copper resinate (from *Pinus* species) that exhibits a high ethyl pimarate/ethyl abietate and an ethyl isopimarate/ethyl abietate ratios, Canada balsam (from *Abies* species) that exhibits the lowest ethyl pimarate/ethyl abietate ratio, Strasbourg turpentine (from *Abies* species) that exhibits the highest ethyl neoabietate/ethyl abietate ratio and Venetian turpentine (from *Larix* species) that exhibits a high larixyl acetate TMS derivative/ethyl abietate ratio. These results enable an adequate discrimination of these natural resins and pigment.

3.5. Analysis of real samples

A panel painting sample has been analysed using the proposed method in order to assess its capability for identifying and discriminating the type of diterpenic resin. The sample analysed was taken from a dark spot on the central panel of the St. Michel Altarpiece painted by Vicente Maçip in 1523. Analysis of this sample has been carried out in order to identify the chemical composition of this product deposed on the surface. Fig. 4 shows the gas chromatogram obtained in which significant peaks appear that are assigned to pimaric, dehydro-dehydroabietic and dehydroabietic acids. It evidences that a panacea resin, probably coming from the wood support, is the main component of this deposed

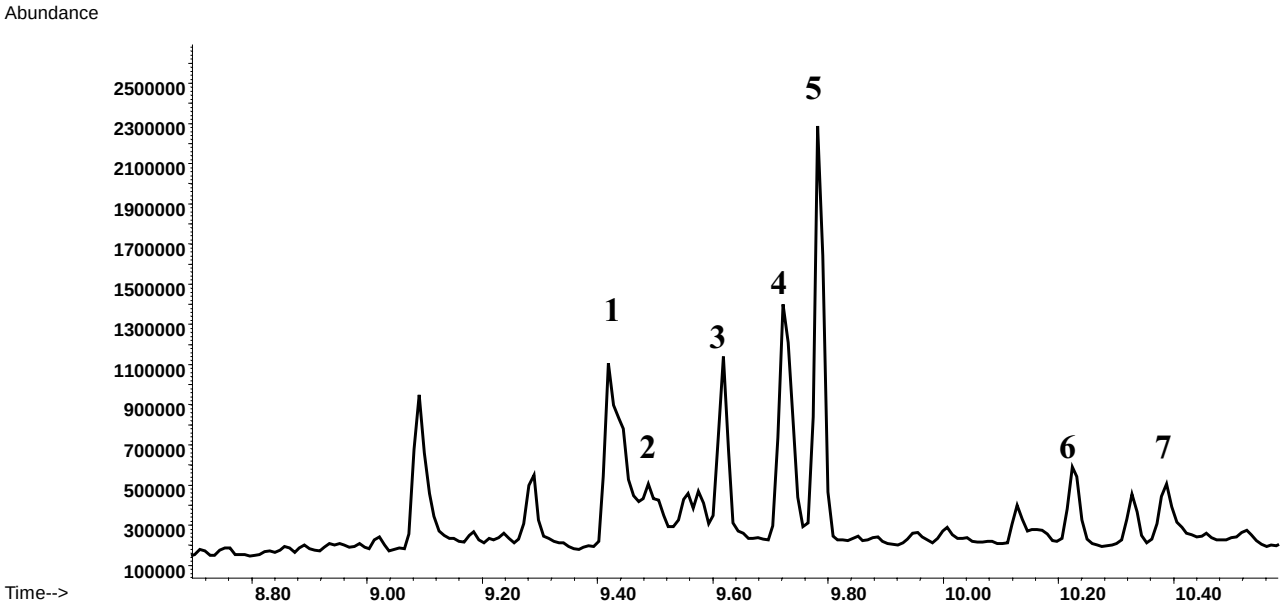


Fig. 4. Gas chromatogram of sample from a dark spot from St. Michel Altarpiece (1523) painted by Vicente Maçip (Valencia, Spain). Peaks: (1) pimaric ethyl ester; (2) sandaracopimaric ethyl ester; (3) isopimaric ethyl ester; (4) dehydro-dehydroabietic ethyl ester; (5) dehydroabietic ethyl ester; (6) neoabietic ethyl ester; (7) 7-oxodehydroabietic ethyl ester. Time scale in min.

product. Absence of larixyl acetate is consistent with the hypothesis mentioned earlier. The high content in dehydroabietic acid and the absence of abietic acid indicate a notable ageing and these results are in good agreement with these others obtained by other authors [9].

4. Conclusions

(1) A derivatisation method combining the formation of ethyl esters from the carboxylic groups and the trimethylsilyl ethers from hydroxyl groups from diterpenic resins used in artistic varnishes, that is consistent with these other proposed by the authors for analysing drying oils and proteins usually used as binders, has been developed.
(2) A chemometric experimental design carried out for optimising the parameters depending on the products of derivatisation lead to establish the use of 8 μl of ECF and 1.4 μl of TMSI, a silylation temperature of 80 °C and a silylation time of 10 min as the best analytical conditions.
(3) A final step of extraction with chloroform, in which a few μl of ECF have been added, has been suggested for increasing the efficiency of the proposed method.
(4) The proposed method enables the identification of the main components of the traditional diterpenic resins used in art works such as colophony, Venetian turpentine, Strasbourg turpentine, Canada balsam and the copper resinate pigment without interfering with the fatty acids from the drying oils.
(5) Analysis of a real sample in which pine resin has been identified evidence the applicability of this method in the analysis of diterpenic resins from art works.

Acknowledgements

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