MOLART Report 10

Analysis of diterpenoid resins and polymers in paint media and varnishes

with an atlas of mass spectra

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MOLART and MOLART Reports

MOLART - Molecular Aspects of Ageing of Painted Art - was a 5-year cooperative project between art historians, conservators, analytical chemists and technical physicists funded by the Dutch Organisation for Scientific Research (NWO). Technical support and advice was given by Shell-SRTCA (Amsterdam), AKZO-NOBEL (Arnhem), Instituut Collectie Nederland (ICN, Amsterdam, at present called Cultural Heritage Agency RCE) and the Dutch art museums. The project was launched on 1 February 1995 and ended early 2003. The object of MOLART was to contribute to the development of a scientific framework for the conservation of painted art on the molecular level. The focus of MOLART was the determination of the present chemical and physical condition of works of art produced in the period from the 15th to the 20th century. Studies of historical paint manufacturing and workshop practice must give insight into the nature of the painters' media and the painting technique used originally. Fundamental studies on varnishes, paint, and colorants are undertaken to understand the molecular aspects of ageing since this is thought to be a main cause for the continued need to treat paintings.

This report is the tenth in a series of MOLART reports that summarise all research results obtained in the course of the project. Information about MOLART and its follow-up De Mayerne can be obtained from the project co-ordinator Prof. Dr. J.J. Boon, through http://www.jaap-enterprise.com.

1. *Molecular studies of fresh and aged triterpenoid varnishes*, Gisela A. van der Doelen, 1999. ISBN 90-801704-3-7
2. *A mathematical study on craquelure and other mechanical damage in paintings*, Petri de Willigen, 1999. ISBN 90-407-1946-2
3. *Solvent extractable components of oil paint films, Kenneth R. Sutherland*, 2001. ISBN 90-801704-4-5
4. *Molecular changes in egg tempera paint dosimeters as tools to monitor the museum environment,* Oscar F. van den Brink, 2001. ISBN 90-801704-6-1
5. *Discoloration in renaissance and baroque oil paintings*, Margriet van Eikema Hommes, 2002. Archetype Publications, London.
6. *Analytical chemical studies on traditional linseed oil paints*, Jorrit D.J. van den Berg, 2002. ISBN 90-801704-7-X
7. *Microspectroscopic analysis of traditional oil paint*, Jaap van der Weerd, 2002. ISBN 90-801704-8-8
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11: *Binding medium, pigments and metal soaps characterised and localised in paint cross-sections*, Katrien Keune, 2005. ISBN 90-77209-10-7
12. Paints quantified: image analytical studies of preparatory grounds used by Van Gogh. Beatrice Marino, 2006. ISBN-10 90-77209-19-6
13. Reporting Highlights of the De Mayerne Program, Research Program on Molecular Studies in Conservation and Technical Studies in Art History. Jaap J. Boon and Ester S.B. Ferreira (editors), 2006. ISBN 90-77875-14-X
14. Color changes and chemical reactivity in seventeenth-century oil paintings. Annelies van Loon, 2008. ISBN/EAN 978-90-77209-17-2

PDFs versions of the reports except nr 2, 3, 5 and 10 can be downloaded from www.amolf.nl (see publications). Printed books can be ordered from JAAP-Enterprise for Molart Advice email: boon@jaap-enterprise.com, as long as supply lasts. Nr 6 and 11 are

sold out. Cost 22 euro including postage. MOLART 10 only available as downloadable from www.jaap-enterprise.com and the RCE website, via www.cultureelerfgoed.nl.

Contents

Abbreviations

AMOLF	FOM Institute for Atomic and Molecular Physics
a.m.u.	atomic mass units
APCI	Atmospheric Pressure Chemical Ionisation
Da	Dalton units (= a.m.u.)
DHA	Dehydroabietic acid
DTMS	Direct Temperature resolved Mass Spectrometry
FOM	Fundamenteel Onderzoek der Materie
GCMS	Gas Chromatography/Mass Spectrometry
HPLC	High Performance Liquid Chromatography
IR	Infrared
MOLART	Molecular aspects of ageing in painted art
MS	Mass Spectrometry
MW	Molecular Weight
NMR	Nuclear Magnetic Resonance (spectroscopy)
NWO	Nederlandse Organisatie voor Wetenschappelijk Onderzoek
OH-DHA	Hydroxy-dehydroabietic acid
Py-	Pyrolysis
SEC	Size Exclusion Chromatography
SRAL	Stichting Restauratie Atelier Limburg
TIC	Total Ion Count
TMAAc	Tetramethylammonium acetate
TMAH	Tetramethylammonium hydroxide
TMS	Trimethylsilyl (functional group)
TMTFTH	Trimethyl-trifluoro-toluyl ammonium hydroxide

Preface – Rationale, summary and credits

Rationale

This MOLART report describes part of the research on diterpenoid resins and polymers in natural resins carried out within MOLART between 1995 and 2000. The report is not a PhD thesis like most MOLART reports, but an overview of the research done by the author as one of the project leaders in MOLART, extended with research by Dr. Ivana Pastorova (part of Chapter 1) and Dr. Inez van der Werf (Chapter 3) supervised by the author. This report is aims at compiling and making accessible a number of articles written on the subject. The papers are not reproduced *verbatim* but extended with previously unpublished results obtained up to 2003. References to publications are made up to 2003.

The editing and publication of this report was unfortunately delayed for many years. It was decided to make the content available on-line finally, on the occasion of prof. Jaap Boon's retirement from AMOLF in January 2012.
Since 2003, many more work on the topic of diterpenoid resins in works of art and archaeology has been published. Nevertheless, we hope and trust that the contents of this report will prove useful still to the conservation and conservation science community.

Introduction

Throughout the ages, natural resins have been important constituents of paint media [Mills and White 1977, 1994]. The resins, which are exudates from trees, were used, for example, to change the working properties of paints, and their high refractive index would add transparency to the paints to make them suitable for glazing. In combination with waxes they make excellent adhesives and as such they have been used often by conservators for lining, consolidation of paint flakes and facing. Natural resins are very useful in varnishes, either in spirit or in oil varnishes, because of their glossy appearance, ability to form a protective layer and saturate the colours in the painting itself. The volatile fraction of balsams, in particular the relatively cheap oil of turpentine, were popular as solvent and diluent for paint media.

In the 20th century a number of synthetic alternatives have become available as varnishes for paintings, although natural resins such as dammar and mastic are still used on a large scale by paintings conservators. Despite their limited stabiblity, many conservators believe there is no modern synthetic alternative that matches the workability and the optical properties of these natural resins.

Ageing of the resins applied in or on paintings has often lead to a change in the optical and mechanical properties of the materials in which they were used or applied, such as darkening, embrittlement, and loss of adhesive properties. These changes are due to the oxidation, polymerisation and other reactions that have taken place. A better knowledge of these molecular changes could in turn lead to a better understanding of the physical changes that occur in the course of time.

Published studies indicate that some resins are known to develop almost completely into new structures which are difficult to recognise using the usual analytical techniques. This is for example the case for copaiba balsams and copal resins. However it can be very important for a conservator to be aware of the use of these materials since it may give a better idea of the original appearance of the work of art. In addition, it may better explain the sometimes peculiar behaviour of the paint or the varnish, and may help in the choice of a conservation treatment. The development of analytical methodology for the recognition of copaiba balsams and copals is described in chapters 3 and 4.

Resins contain varying polymeric fractions in addition to the low molecular weight compounds. These fractions contribute for example in varnishes to their specific properties in terms of applicability. The polymers may react further in the course of time, serving as an anchor point for condensation of other small molecules.

Summary

The purpose of this MOLART report is to summarise our present knowledge of the fate of resins in works of art in general. It describes the research done predominantly by applying mass spectrometric techniques, and an atlas of mass spectra is disclosed to facilitate the identification of natural resin derived compounds.

In the first two chapters, the products of oxidation of abietic acids from *Pinaceae* resins in different media are investigated, and different analytical approaches compared. In addition, the index for the degree of oxidation (IDOX) is introduced, which may be used as a measure of the degree of ageing of the medium in which the resin is present. Chapter 3 deals with many aspects of the application of copaiba balsam and its fate in works of art, and it describes a method for analytical detection of traces of this resin. In chapter 4 the reactions of copal resin in the course of manufacturing, drying and ageing are briefly described, and a new analytical approach is presented to detect these resins in minute paint samples. Chapter 5 describes the search for and the discovery of the structure of the hitherto unknown polymeric fraction of mastic resin.

References

Mills, J.S. and R. White (1977), “Natural Resins of Art and Archaeology. Their Sources, Chemistry, and Identification.” *Studies in Conservation* 22: 12-31.

Mills, J.S. and R. White (1994), *The Organic Chemistry of Museum Objects*. London, Butterworth-Heinemann (2nd edition).

Articles in this report

The chapters are based on the following articles:

Chapter 1: *(1)* Van den Berg, K.J., J.J. Boon, I. Pastorova and L.F.M. Spetter, (2000) "Mass spectrometric methodology for the analysis of highly oxidised diterpenoid acids in Old Master paintings", *Journal of Mass Spectrometry* 35: 512-533. *(2)* Van den Berg, K.J., I. Pastorova, L.F.M. Spetter and J.J. Boon, (1996) "State of oxidation of diterpenoid *Pinaceae* resins in varnish, wax lining material, 18th century paint and copper resinate pigment", in *Preprints ICOM Committee for Conservation 11th Triennial Meeting, Edinburgh, Scotland, 1-6 September 1996*, Vol. II, p. 930-937. James & James, London. *(3)* Pastorova I., K.J. van den Berg, J.J. Boon and J.W. Verhoeven, (1997) "Analysis of oxidised diterpenoid acids using thermally assisted methylation with TMAH", *J. Anal. Appl. Pyrolysis* 43: 41-57.

Chapter 2: *(1)* Van den Berg, K.J., J.J. Boon, I. Pastorova and L.F.M. Spetter, (2000) "Mass spectrometric methodology for the analysis of highly oxidised diterpenoid acids in Old Master paintings", *Journal of Mass Spectrometry* 35: 512-533; *(2)* Van den Berg, K.J., J. van der Horst, J.J. Boon, N. Shibayama and E.R.de la Rie, (1998) "Mass spectrometry as a tool to study ageing processes of diterpenoid resins in works of art: GC- and LC-MS studies", in *Advances in Mass Spectrometry.* ed. by E.J. Karjalainen, A.E. Hesso, J.E. Jalonen and U.P. Karjalainen Vol. 14, Ch.23, p. 563-573. Elsevier, Amsterdam; *(3)* unpublished results obtained in the framework of the MOLART 'wax/resin lining' sub-project, carried out with Mireille te Marvelde (See te Marvelde, M. "The conservation-restoration history of the wax/resin lining procedure", in *Molart Final Report and Highlights 1995-2002*, p. 48, AMOLF).

Chapter 3: Van der Werf, I.D., K.J. Van den Berg, S. Schmitt and J.J. Boon, (2000) "Molecular characterisation of copaiba balsam as used in painting techniques and restoration procedures", *Studies in Conservation* 45: 1-18.

Chapter 4: *(1)* Van den Berg, K.J., J. van der Horst and J.J. Boon, (1999), "Recognition of copals in aged resin/oil paints and varnishes", in *Preprints ICOM Committee for Conservation 12th Triennial Meeting, Lyon, France, 29 Aug.-3 September 1999*, Vol. II, p. 855-861. James & James, London; *(2)* previously unpublished results.

Chapter 5: Van den Berg, K.J., J. van der Horst, J.J. Boon, and O.O. Sudmeijer, (1988), "*Cis*-1,4-poly-β-myrcene; the Structure of the Polymeric Fraction of Mastic Resin (Pistacia lentiscus L.) Elucidated", *Tetrahedron Letters*, 39: 2645-2648.

Credits

Many colleagues have made to the research presented in this report. MOLART-project co-ordinator Prof. Dr. Jaap Boon has been an inspirational driving force and catalyst for much of this work. Inez van der Werf wrote Chapter 3, which reports on only a small part of her research on copaiba balsams. Ivana Pastorova and Leo Spetter contributed extensively to Chapters 1 and 2. Jerre van der Horst did a great deal of analytical work for Chapters 4 and 5. Other co-authors of the papers on the basis of which this Report has been written are Nobuko Shibayama and René de la Rie, involved in Chapter 2, Sibylle Schmitt (Chapter 3), and Olof Sudmeier, (Chapter 5); their involvement is acknowledged.

Mireille te Marvelde (MOLART, presently at Frans Halsmuseum, Haarlem) for providing most of the lining adhesive samples (in the framework of the MOLART-wax-resin lining project). The nice co-operation within the wax-resin project with Mireille and René Hoppenbrouwers (Stichting Restauratie Atelier Limburg (SRAL), Maastricht) is acknowledged.

My other collegues from within and outside MOLART and AMOLF (Jorrit van den Berg, Gisela van der Doelen, Oscar van den Brink, Jos Pureveen, Ken Sutherland, Alan Phenix, Margriet van Eikema Hommes; Karin Groen, Muriel Geldof, René Boitelle, Georgiana Languri, Ron Heeren and Jaap van der Weerd, Filomena Bento; Leslie Carlyle and Joyce Townsend, Barbara Berrie, Arie Wallert, Petria Noble and Jørgen Wadum, J.J. van Asperen de Boer and many others) were good colleagues and pleasant company; their co-operation in other projects, helpful and stimulating and inspiring discussions in general was very much enjoyed.

Rene de la Rie, head of the scientific research group as well as Nobuko Shibayama, Barbara Berrie, Lesley Stevenson, Jill and all other colleagues at the National Gallery of Art are thanked for a very pleasant two months' stay in Washington, DC. The people at Shell Research and Technology Centre, Amsterdam (Wim Genuit, Wouter Koot, Olof Sudmeier, Graham Smith, Cees Mensch) were very generous with analytical support and discussions. Several museums ands institutions and their staff provided kind access to their paintings: Rijksmuseum, SRAL, Frans Halsmuseum, Van Gogh Museum, Mauritshuis, Tate Gallery, London.

Annebeth Kraij-Kerkhoff is thanked for her help putting the Atlas together, and Donna Mehos and Jaap Boon for editorial comments. Mark Clarke is gratefully acknowledged for his extensive editorial work.

The work described in this report was performed at AMOLF (FOM Institute for Atomic and Molecular Physics), present address Science Park 104, 1098 XG Amsterdam, The Netherlands. It was part of the Priority Program MOLART (Molecular aspects of Ageing in Painted Works of Art) supported by NWO (Nederlandse Organisatie voor Wetenschappelijk Onderzoek) and of the research program no. 28 (Mass Spectrometry of Macromolecular Systems) of FOM (Stichting voor Fundamenteel Onderzoek der Materie). Shell Research and Technology Centre, Amsterdam provided facilities for analytical research. The National Gallery of Art financially supported a study period in Washington, DC.

Klaas Jan van den Berg, october 2003

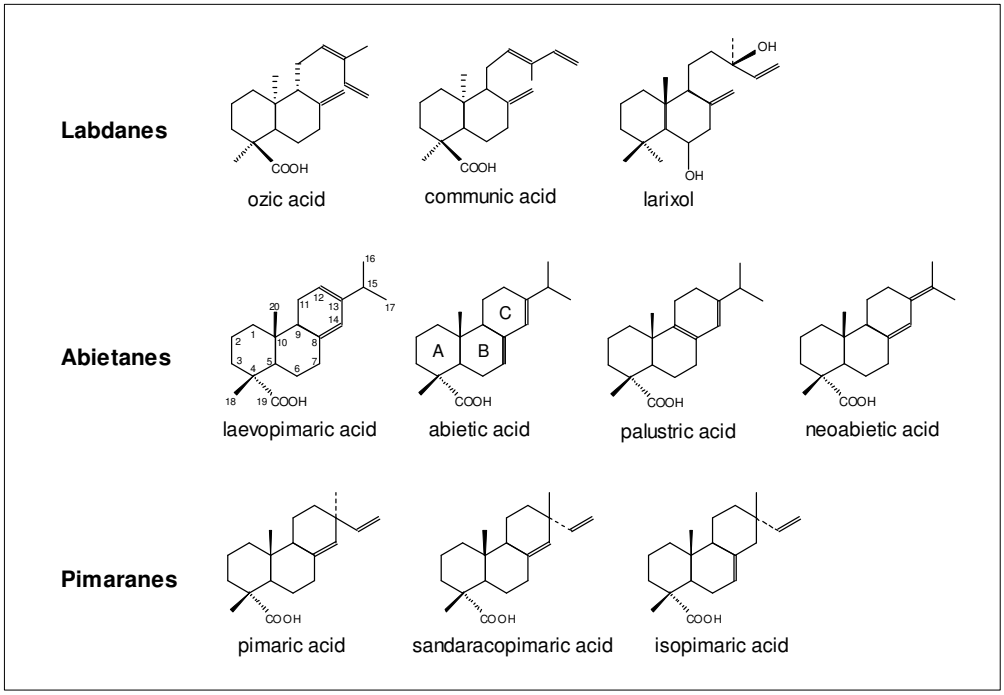
1. Mass spectrometric methodology for the analysis of highly oxidised diterpenoid acids in Old Master paintings. Part 1. GCMS studies

Abstract

Diterpenoid resins from larch and pine trees and the corresponding fractions in a more than 100 year-old wax-resin adhesive and varnish, and a 200 year-old resin/oil paint sample, were analysed with GCMS using several off-line *and* on-line *derivatisation methods. The main resin compounds were highly oxidised abietic acids. Important products found are hydroxy-dehydroabietic acids (OH-DHAs), 7-oxoDHA, di-OH-DHAs and 15-OH-7-oxoDHA. The latter two compounds have not been reported to occur in works of art before. Larixyl acetate, an important marker from larch resins, was found to be still present and preserved in high amounts in the adhesive. A large number of mass spectra of the different oxidation products as well as larixol and larixyl acetate are presented and their fragmentation behaviour under electron ionisation conditions discussed. Py-TMAH-GCMS is presented as a reliable and fast technique for the analysis of diterpenoid resins.*

Introduction

Diterpenoid resins are exudates from a large variety of trees throughout the world [Mills and White, 1977; Langenheim, 1995]. Most of these resins originate from conifers (*Coniferae*) and tropical flowering plants from the *Leguminosae* family. These families are known to produce amber-forming resins predominantly. Dicyclic diterpenoids (labdanes, Scheme 1) with a side chain containing a conjugated double bond are responsible for polymerisation and hardening of these resins. An important exception is the conifer subfamily *Pinaceae* that has been renowned throughout the ages for their resin production and comprises such species as pine (*Pinus*), larch (*Larix*), spruce (*Picea*) and fir (*Abies*). These trees produce non-amber-forming balsams, as a result of the large amounts of diterpenoid acids with abietane and pimarane structures which are known not to polymerise spontaneously (Scheme 1).



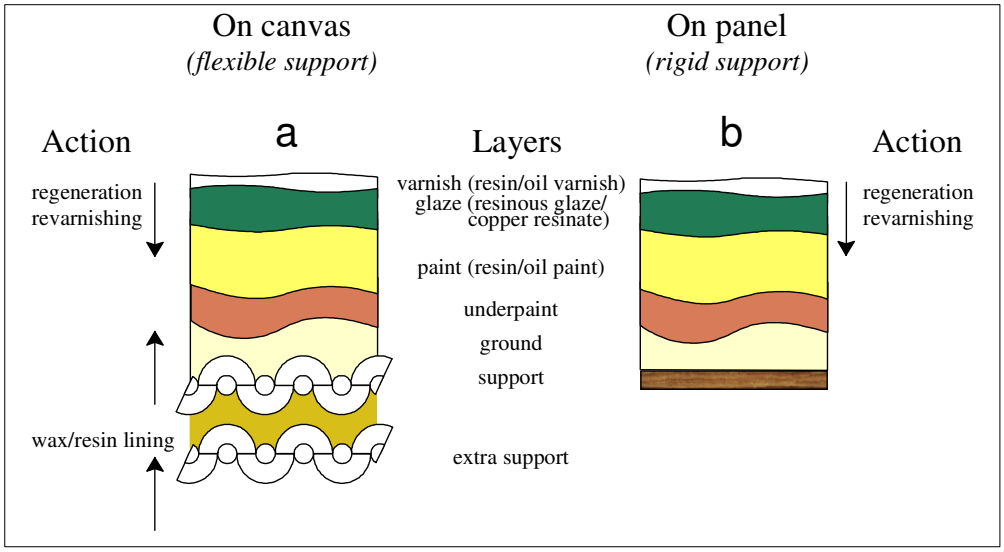
Scheme 1. Diterpenoid labdanes, pimaranes and abietanes from Pinaceae resins. Carbon numbers are depicted for one compound according to common nomenclature.

Diterpenoid *Pinaceae* resins have been known and applied through the ages, as adhesives, coatings etc. The resins exude from trees as balsams, which are rather viscous solutions of diterpenoid components in monoterpenes. Evaporation, of the volatile monoterpene fractions results in colophony (also referred to as rosin). Pine colophony predominantly contains diterpenoid acids and is obtained in the distillation process from the balsam exuded from the tree (gem rosin), by aromatic solvent extraction from wood (wood rosin) or as a by-product of the manufacture of cellulose (tall oil rosin) [Sandermann, 1960]. Strictly, colophony can also be obtained from other tree balsams but this is quite uncommon. At present, pine colophony is still a commonly used raw material for industrial purposes. Its properties and durability are often improved by chemical modification of the conjugated diene moiety in the

abietic acids through cyclo-addition or reduction reactions [McGuire and Suchanec, 1994].

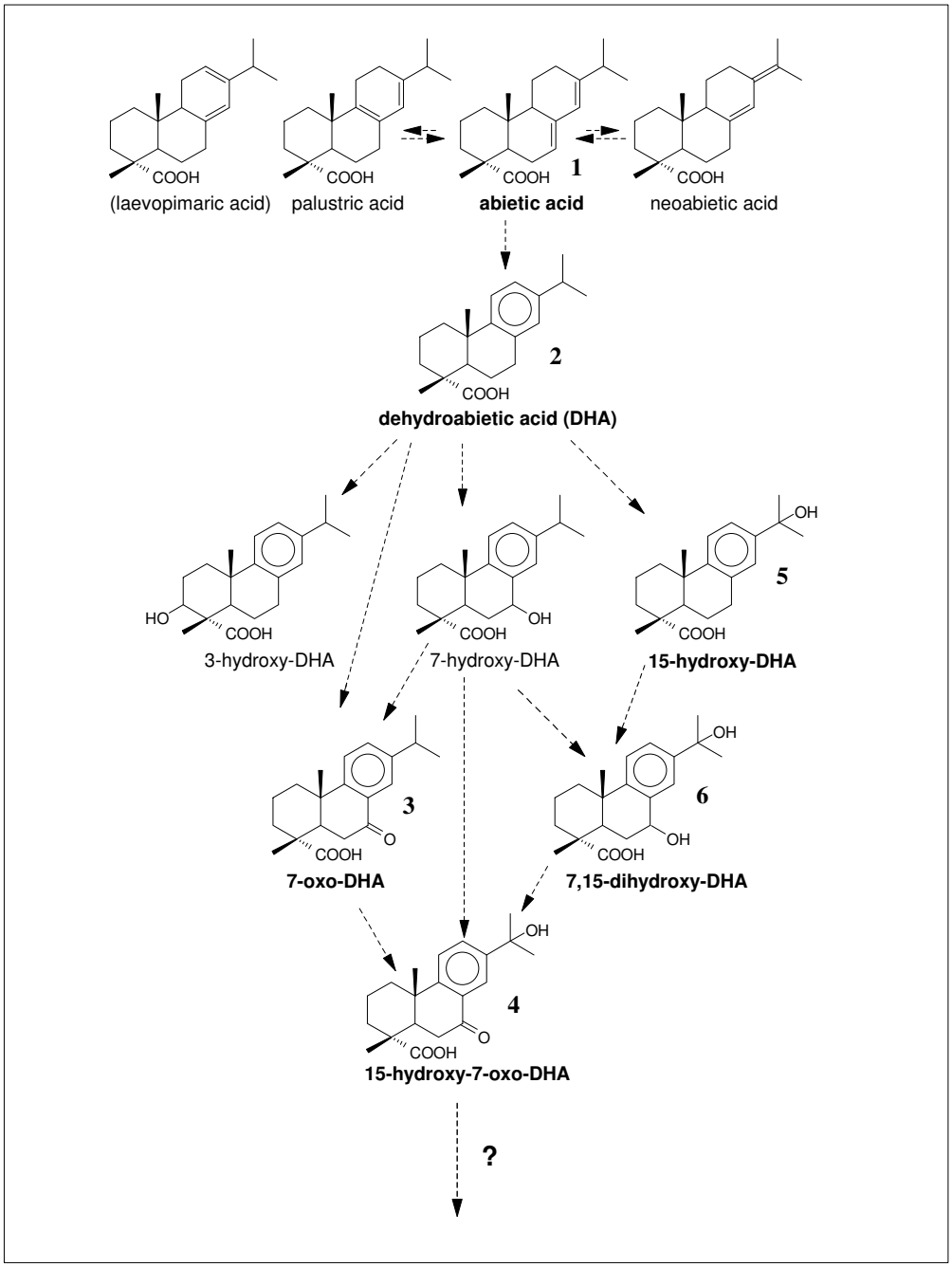
Diterpenoid resins have been used extensively in and on works of painted art, both by artists and conservators in virtually all parts of paintings. Some balsams, such as Venice Turpentine (larch), or Strassbourg Turpentine (fir) were most popular. Pine resins, especially pine colophony has the notoriously bad reputation of darkening and becoming brittle. Though these ageing effects must have kept painters away from its use as painting varnish, sources suggest that pine colophony-drying oil mixtures were used frequently as *e.g.* lacquers. The low price of pine colophony and its balsam common turpentine has also led to serious adulteration of more expensive resins such as Venice turpentine [Koller *et al*., 1997], Strasbourg turpentine [Van den Berg, unpublished] as well as of copals and copaiba balsams (See Chapter 3).

In Scheme 2, a schematic drawing of a cross section of a typical Old Master painting is shown, illustrating the origin of the diterpenoid resins that may be present in the complex layer structure. Painters used the resins as varnish, as additive to paints (e.g. glazes, lacquers), as pigment (copper resinates) and mordant (adhesive for *e.g.* gold leaf). Furthermore, resins may have been introduced by restoration procedures, both on the front as varnish or with other balsams (copaiba balsams) for regeneration purposes and at the reverse of the painting as wax/resin lining paste for repairing and strengthening of the support.



Scheme 2. Cross section of a typical Old Master painting a) on canvas and b) on panel and the possible source of diterpenoid resins which may be found in the different layers.

Once applied on or in a painting, oxidative conditions are expected to lead to the formation of a number of resin oxidation products. Metal-mediated oxidation is expected to play an important role inside the painting, whereas photochemical oxidation will be the predominant process in the varnish.



Scheme 3. Proposed isomerisation and oxidation reaction paths of abietic acids. The degree of oxidation of an acid (A-E) is represented by the vertical position of the compound.

The ageing of *Pinaceae* diterpenoid resins on the molecular level is predominantly the result of the oxidation of the conjugated diene element in abietane diterpenoid acids [Mills and White, 1977, 1994]. These acids are oxidised to form dehydroabietic acid (DHA), which in turn may be oxidised further by incorporation of oxygen. The precise mechanisms involved in this oxidation process are unclear, but may be similar to *e.g.* the oxidation of cholesterol, which is known in some detail [Das *et al.*, 1994]. Oxidation processes resulting in the incorporation of oxygen are expected to occur by formation of a peroxide functionality next to a double bond. Loss of water or reduction of this peroxide may form the oxo- or hydroxy compounds, respectively. In DHA, two likely reactive positions are the 7- and the 15-position [Proefke and Rhinehart, 1992]. In addition, 3-OH-DHA has been reported [Anderson and Winans, 1991]. In Scheme 3, possible isomerisation and oxidation pathways of three abietic acids are presented.

2 (DHA) and **3** (7-oxoDHA) have been found in paintings as reported by Mills, White and co-workers (see for example [Mills and White, 1982; White, 1986; White, 1994]). Oxidation products other than **2** and **3** are scarcely discussed in literature, with few exceptions related to fossil resins and Egyptian mummies [Anderson and Winans, 1991; Proefke and Rhinehart, 1992]. Krohn et al. synthesised and characterised a number of highly oxidised abietic acids as part of a study on their allergenic properties [Krohn *et al.*, 1992]. Some oxidised dehydroabietic acids have also been found in extracts from plants [Ohmoto *et al.*, 1987; Escudero *et al.*, 1983]. The most highly oxidised abietic acid known to date, **4** (15-OH-7-oxoDHA) was not previously reported to occur in works of art [Van den Berg, 1996; Pastorova *et al.*, 1997]. The four most important abietanes, **1** (abietic acid), **2**, **3** and **4**, are proposed as useful marker compounds to assess oxidising environments in paintings (Chapter 2).

In this chapter we present results on the identification of abietane skeletal marker compounds in typical oxidised larch and pine resin-containing samples from Old Master paintings. This is achieved using different derivatisation methods in combination with GCMS. In this way the most prominent structures can be elucidated providing a basis for confident study of minute and complex samples with Py-TMAH-GCMS. This technique is employed to analyse a 200 year-old small resin/oil paint sample. New EI mass spectra of several (highly oxidised) abietic acids are presented and their fragmentation patterns are discussed.

In Chapter 2, LCMS and DTMS are discussed as alternative methodologies for the identification of diterpenoid (abietic) acids.

Materials and methods

Materials

The Venice Turpentine balsam was obtained from Schmincke, Erkrath, Germany. The pine colophony was purchased from “Verfmolen de Kat”, Zaanstad, The Netherlands.

The varnish sample was taken during the restoration (at SRAL, Maastricht) of a painting by Ferdinand Bol (1618-1680) (on loan from the Rijksmuseum to the Provinciehuis, ‘s Hertogenbosch). The painting had been neglected for a long time and the varnish is thought to be at least 100 years old and could only be removed from the painting with acetone. The lining wax/resin sample was from the painting “*De zieke dame*” (C230, Rijksmuseum) by Jan Steen (1626-1679). The actual age of this

lining adhesive is not known, but must be at least 50 years old. The resin oil paint sample was taken from "*Jagers in de Duinen*", (*ca*. 1790, Frans Hals Museum) painted by Pieter Barbiers (1749-1842).

The chemicals used for sample preparation were commercially available and used as obtained.

Sample Preparation

Off-line derivatisation for on-column injection

Samples of typically 50 µg were used in the experiments. Methylation (**a**) was performed with 2 M TMSdiazomethane in hexane in a methanol/benzene solution at room temperature for 15 min. The reaction mixture was then evaporated to dryness and redissolved in methylene chloride. Trimethylsilylation (**c**) was carried out with o-bis (trimethylsilyl)trifluoracetamide (BSTFA) in benzene/pyridine, for 1 h. at 70 ^{o}C. The dried sample was dissolved in methylene chloride. Trimethylsilylation was also performed in procedure (**b**) after methylation with TMSdiazomethane.

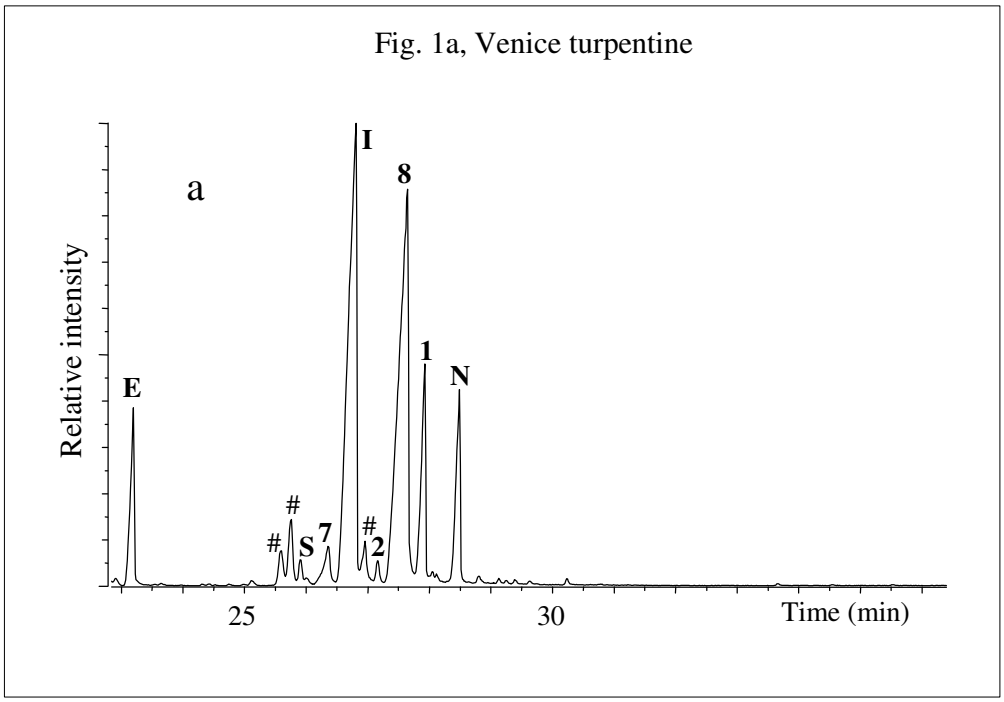
On-line: thermally assisted methylation

For thermally assisted methylation experiments (**d**, Py-TMAH-GCMS), the sample (typically 5-10 µg) was homogenised in a mini glass mortar and made into a suspension with a few µl of a 2.5% solution of tetramethylammonium hydroxide (TMAH) in water. Aliquots of the suspension were applied to the analytical filament (nickel, Curie point 358 ^{o}C) and dried *in vacuo*. Subsequently, the sample wire was inserted into the glass liner, flushed with argon and placed in the pyrolysis chamber (190 ^{o}C) [Van Loon and Boon, 1994]. The more volatile (methylated) components evaporate under these conditions; they were cold-trapped in the first part of the column. Inductive heating (358 ^{o}C) with a 1 MHz radiofrequency field to the Curie point temperature was applied to evaporate part of the remaining sample.

Instrumentation

Gas Chromatography-Mass Spectrometry

Gas-chromatographic separation of the compounds was achieved using a fused silica SGE BPX5 column (25 m, 0.32 mm i.d., 0.25 mm film thickness) in a Carlo Erba gas chromatograph (series 8565 HRGC MEGA 2) coupled directly to the ion source of a JEOL DX-303 double focusing (E/B) mass spectrometer. Helium was used as the carrier gas at a constant linear flow velocity of 26 cm/sec. The oven temperature of the gas chromatograph was programmed from the initial 50 ^{o}C with a ramp of 6 ^{o}C/min to 320 ^{o}C. Ions were generated by electron impact ionisation (70 eV) in the ionisation chamber, accelerated to 3 keV, mass separated and postaccelerated to 10 keV before detection. The mass spectrometer was scanned from m/z 40-700, with a cycle time of 1 s. A JEOL MP-7000 data system was used for data acquisition and processing.



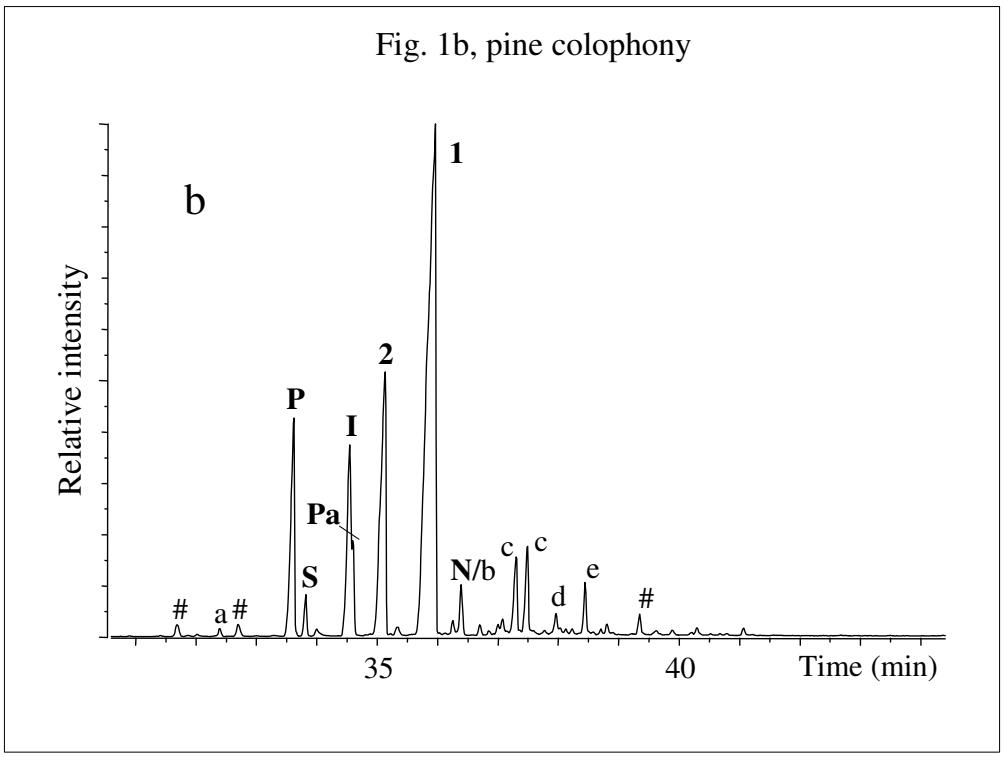
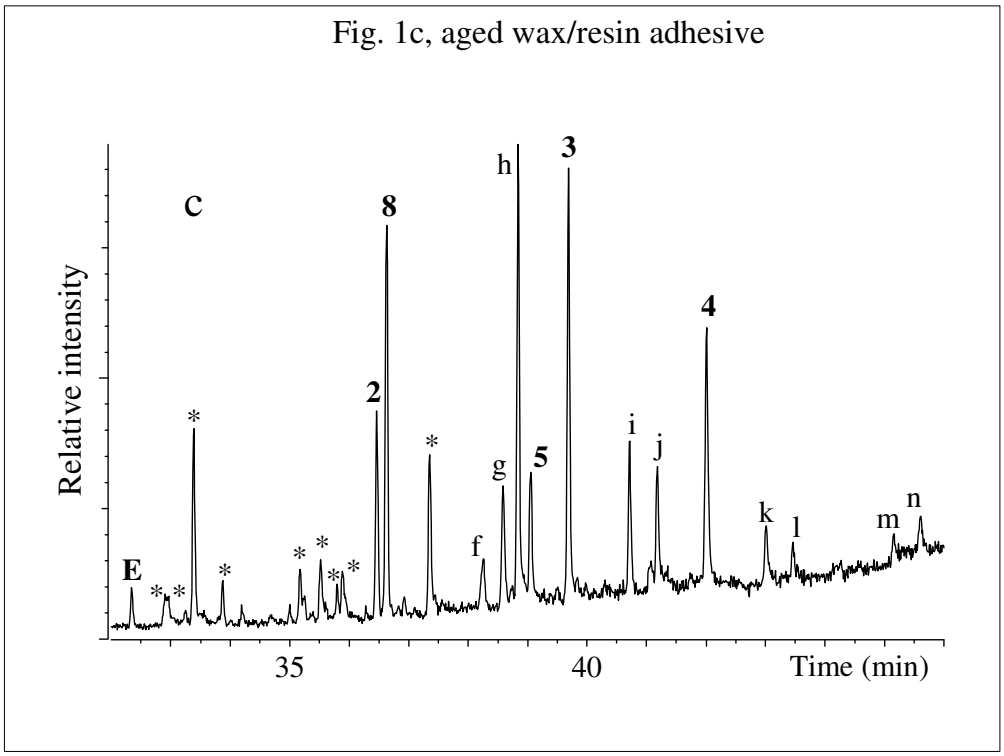


Figure 1. Partial total ion chromatograms of a) Venice turpentine, b) pine colophony, Peak labels are explained in Table 1 (below).



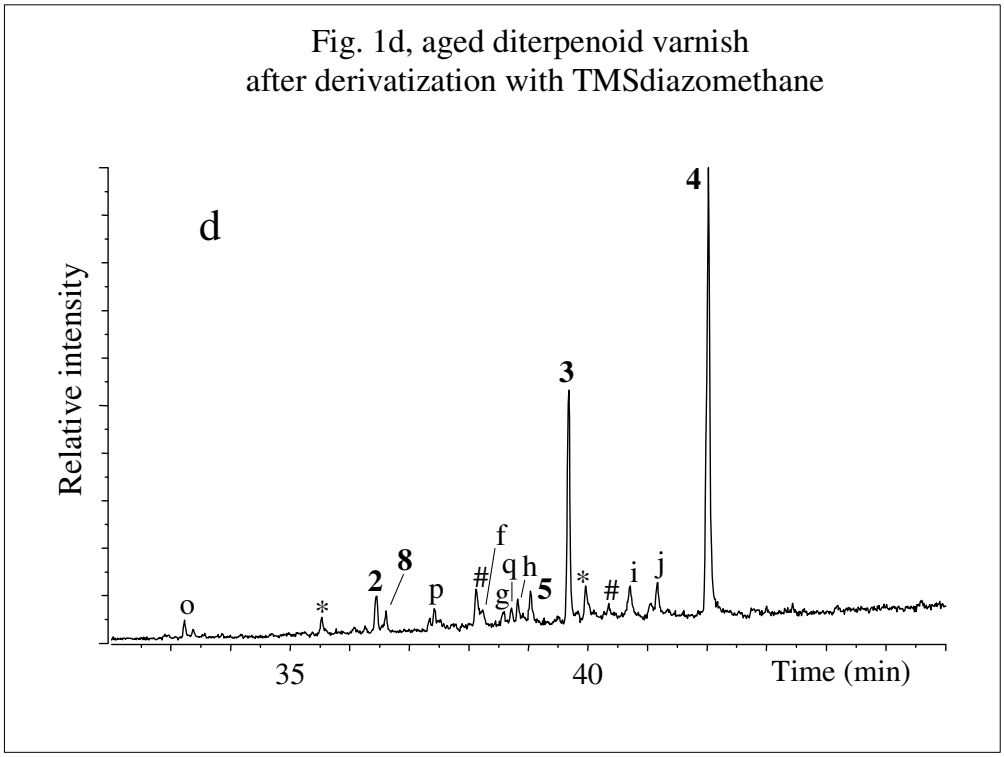


Figure 1. Partial total ion chromatograms of c) aged wax/resin adhesive, d) aged diterpenoid varnish after derivatisation with TMSdiazomethane. Peak labels are explained in Table 1 (below).

Table 1. List of precursor compounds (here depicted in their underived form, see also Scheme 1 and 3) and compound classes corresponding to the marked peaks in Fig. 1 and 2. Assignments are made on the basis of reference spectra and the interpretations explained in the text. Some mass spectra are given in the Atlas of Mass Spectra. See also'Identification and mass spectrometric fragmentation of diterpenoids'.

Compound	*label*	*atlas*	*Compound*	*label*	*atlas*
abietic acid	**1**	1	heptacosane (C 27 alkane)	i	
dehydroabietic acid	**2**	3	tetracosanoic acid (C 24 fatty acid)	j	
7-oxo-dehydroabietic acid	**3**	5	nonacosane(C 29 alkane)	k	
15-hydroxy-7-oxo-dehydroabietic acid	**4**	9	hexacosanoic acid (C 26 fatty acid)	l	
15-hydroxy-dehydroabietic acid	**5**	18	hentriacontane (C 31 alkane)	m	
7,15-di-hydroxy-dehydroabietic acid	**6**		octacosanoic acid (C 28 fatty acid)	n	
di-hydroxy-dehydroabietic acid (di-OH-DHA)	diOH		octadecanoic acid (C 18 fatty acid)	o	
larixol	**7**	25	heneicosanoic acid(C 21 fatty acid)	p	
larixyl acetate	**8**	26	docosanoic acid (C 22 fatty acid)	q	
epimanool	E		eicosanoic acid (C 20 fatty acid)	r	
pimaric acid	P	48	15-hydroxy-seco-dehydroabietic acid	s	
sandaracopimaric acid	S	49	1-tetracosanol (C 24 alcohol)	t	
isopimaric acid	I	50	1-hexacosanol (C 26 alcohol)	u	
palustric acid	Pa	30	1-octacosanol (C 28 alcohol)	v	
neoabietic acid	N	31	1-triacontanol (C 30 alcohol)	w	
seco-dehydroabietic acid	a		9,10-epoxy-octadecanoic acid (cis)	x	
dehydrogenated DHA isomer (dDHA)	b		9,10-epoxy-octadecanoic acid (trans)	y	
Non-aromatic isomer of DHA	c		9,10-dihydroxy-octadecanoic acid	z	
DHA, CH_2TMS ester	d				
AA, CH_2TMS ester	e		Unidentified compounds, presumably diterpenoids	#	
pentacosane (C 25 alkane)	f		Unidentified matrix components	*	
7-OH-DHA	g		Oxidised fatty acids	Ox FA	
di-octyl-phtalate (contamination)	h				

Results and discussion

Natural ageing of diterpenoids in pine and larch resins

In Fig. 1a and 1b, the diterpenoid region of the GCMS Total Ion Chromatograms (TIC) of the Venice Turpentine (larch balsam) and pine colophony after derivatisation with TMSdiazomethane are presented. The labels for the peaks are explained in Table 1 (see for corresponding structures also Schemes 1 and 3); the identification is based on mass spectra and relative retention times as discussed below in *Mass spectrometric fragmentation*. In both resins, varying relative amounts of isopimaric acid, **1** and isomers and **2** are present. No laevopimaric acid (Scheme 1 and 3), normally present in high amounts in the fresh balsams, is observed. This acid is known to isomerise readily to other abietic acids by exposure to light and mild heating [Mills and White, 1994]. Venice Turpentine (Fig. 1a) is distinguished from pine colophony primarily by its marker molecules **7** (larixol) and **8** (larixyl acetate, which is present in high amounts) [Mills, 1973] but also by the relatively high amount of isopimaric acid and the small fraction of abietic acids.

The pine resin shows a high ratio (>2) of pimaric and sandaracopimaric acid, which distinguishes the resin from that of other *Pinaceae species* such as spruce, larch and fir, in which sandaracopimaric acid is the more abundant [Mills and White, 1994].

In Fig. 1c and d the partial TICs of the aged wax/resin adhesive and varnish are presented. The identification of the individual compounds is discussed in *Identification and mass spectrometric fragmentation of diterpenoids* (below). The patterns are dramatically different in that no peaks corresponding to unoxidised abietic acids are present. Instead, peaks appear at higher retention times predominantly corresponding to dehydroabietic acids with oxygen incorporated, such as **5** (15-OH-DHA), **3** and **4** (Scheme 3). In all cases the integrity of the abietane skeleton is preserved.

A number of peaks appear which can be identified as matrix compounds (beeswax). In addition, high amounts of two di-OH-DHAs are observed in the lining sample, the most abundant of which is proposed to be **6** (7,15-di-OH-DHA)[*]. These compounds were found frequently in aged diterpenoid containing samples, but are predominant in wax-resin lining samples. Since linings are mostly kept in the dark, this may mean that these species are formed mainly in the dark, or, more precisely, that they are relatively unstable in daylight. The influence of daylight and associated photo-oxidation processes may also explain the higher relative amounts of **4** in the aged varnish compared to the aged wax/resin sample, which is generally observed in our analyses (See Chapter 2*, Index for the Degree of Oxidation (IDOX); a quantitative comparison*).

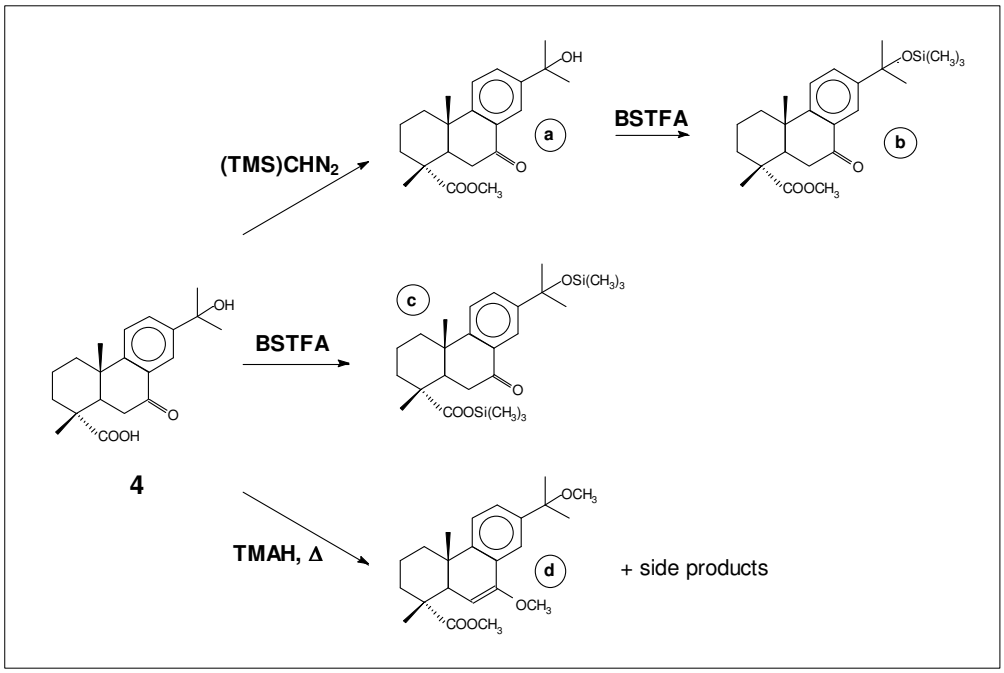
Some characteristics indicating the provenance of the resins remain in the aged samples. In the varnish, only low amounts of pimaric acid and no sandaracopimaric acid are found, suggesting that pine colophony is involved. In the wax/resin adhesive, the **7** and **8** detected demonstrate that larch resin (Venice turpentine) was used. Surprisingly, these unique markers are quite stable in the aged wax-resin lining paste. Little or no hydrolysis is observed and the original abundance ratio of **7** and **8** (<1:10)

[*] Please note: we have not observed di-OH-DHA in Figure 1c and d. Only using methods which derivatise the hydroxyl functionalies these compounds could be found (see *Identification and mass spectrometric fragmentation of diterpenoids*).

is preserved. Traces of sandaracopimaric acid are also detected. Assuming that pimaric acid is equally (un)stable as its stereoisomer sandaracopimaric acid, the lining paste must contain both larch and pine resin. This is not surprising since several well-known Dutch conservators used a mixture of beeswax, pine colophony and Venice turpentine as lining paste [Te Marvelde, 2001]. The varnish sample also contains trace amounts of larch resin and beeswax. However, these may not be original constituents of the varnish but could have been introduced in a later stage, presumably during a lining procedure.

Off-line and on-line chemical derivatisation; a qualitative comparison

The applicability of different off-line and on-line chemical derivatisation methods for the analysis of aged diterpenoid materials was studied with the wax/resin and varnish samples. An outline for these procedures is presented in Scheme 4, exemplified by the derivatisation of **4**. The TICs (diterpenoid region) of the lining wax/resin sample after methylation with TMSdiazomethane (procedure **a**), trimethyl-silylation (**c**) and after on-line methylation with TMAH (**d**) are shown in Fig 1c, 2a and 2b, respectively. Most compounds depicted in Scheme 3 are indeed observed in all TICs, with the exception of 3-OH-DHA for which no evidence could be found. Both 7-OH-DHA and **5** are identified in the aged wax/resin adhesive and the varnish, with the former invariably in relatively low concentrations.



Scheme 4. Products of derivatisation of 4 with a) TMSdiazomethane, b) TMSdiazomethane followed by BSTFA, c) BSTFA, d) TMAH.

The Kovats indices of the diterpenoids are roughly between 2300 and 2800 in case of all derivatisation methods. An important difference is the presence of more compounds in Fig. 2b due to the hydrolysis of matrix compounds and formation of

multiple products of some highly oxidised compounds (see below). Methylation with TMSdiazomethane (**a**) yields most highly oxidised compounds. As an exception, however, the di-OH-DHA compounds are not observed with this derivatisation method. This can be explained by their high polarity. In fact, slightly degraded GC columns are often found to prevent hydroxyl containing species from being analysed.

The use of TMSdiazomethane (or the more commonly used and more toxic diazomethane), which is routinely applied as derivatisation agent for the analysis of aged samples from works of art, in combination with (slightly) activated columns may explain the fact that only few examples of hydroxylated diterpenoid acids in aged painting materials have been reported in the conservation science literature. **4**, for example, the most abundant diterpenoid compound in many aged painting materials, has been recognised in these materials only relatively recently [Van den Berg, 1996].

In addition, the traditionally used procedure for the analysis of oil paints [Mills, 1966] may be of influence. In this procedure, a primary hydrolysis step using methanolic KOH is applied. Subsequently extraction of the methanolic phase with diethyl ether takes place. This extraction may not be sufficient to obtain the more polar compounds quantitatively. Experiments with this method in our hands demonstrated that hydroxylated compounds were often retained in the methanolic layer and hence lost in the subsequent analytical procedure.

Additional derivatisation of the hydroxyl compounds, *e.g.* by trimethylsilylation (**b**) can avoid this problem. However, this double derivatisation procedure is more elaborate and increases the risk of contamination and loss of material. An additional disadvantage of TMSdiazomethane is the formation of variable (albeit small) amounts of TMS-CH_2-esters in addition to methyl esters [Hashimoto *et al.*, 1981].

Direct trimethylsilylation (**c**) facilitates the distinction between free acids and methyl esters of diterpenoid acids. In this way, for example, the methyl ester of **2** could be identified in an archaeological resin-containing sample [Pastorova, 1997]. In a separate experiment, methyl esters of diterpenoid acids were found in copaiba balsam [Van der Werf, 1996]. Unfortunately, mass spectra of TMS derivatives generally contain less information than other EI spectra since the predominant fragmentation is often the loss of a methyl group.

The diterpenoid region of the TIC resulting from on-line chemical derivatisation (Fig. 2b) contains a high number of compounds compared to those of the other methods. These products are partly due to hydrolysis of ester bonds, promoted by the strongly alkaline solutions of TMAH. The alkyl methyl ethers in Fig. 2b, for example, are products of hydrolysis/transmethylation of the long-chain esters present in beeswax. With the other derivatisation procedures, these long chain esters (C40 and higher) remain intact and are not observed in the GCMS chromatogram with the present temperature program. In the derivatisation process **8** is hydrolysed to form **7** and a result no distinction between these compounds can be made anymore.

In addition, several side products are formed in the thermally assisted methylation reactions of the oxidised diterpenoid acids. These result from partial dehydration in case of the hydroxy compounds [Anderson and Winans, 1991] and partial methylation of the keto group (via the enol tautomer). See Scheme 5 for the proposed mechanism. Often even methyl substitution on a carbon atom adjacent to the keto/enol group occurs [Pastorova *et al.*, 1997].

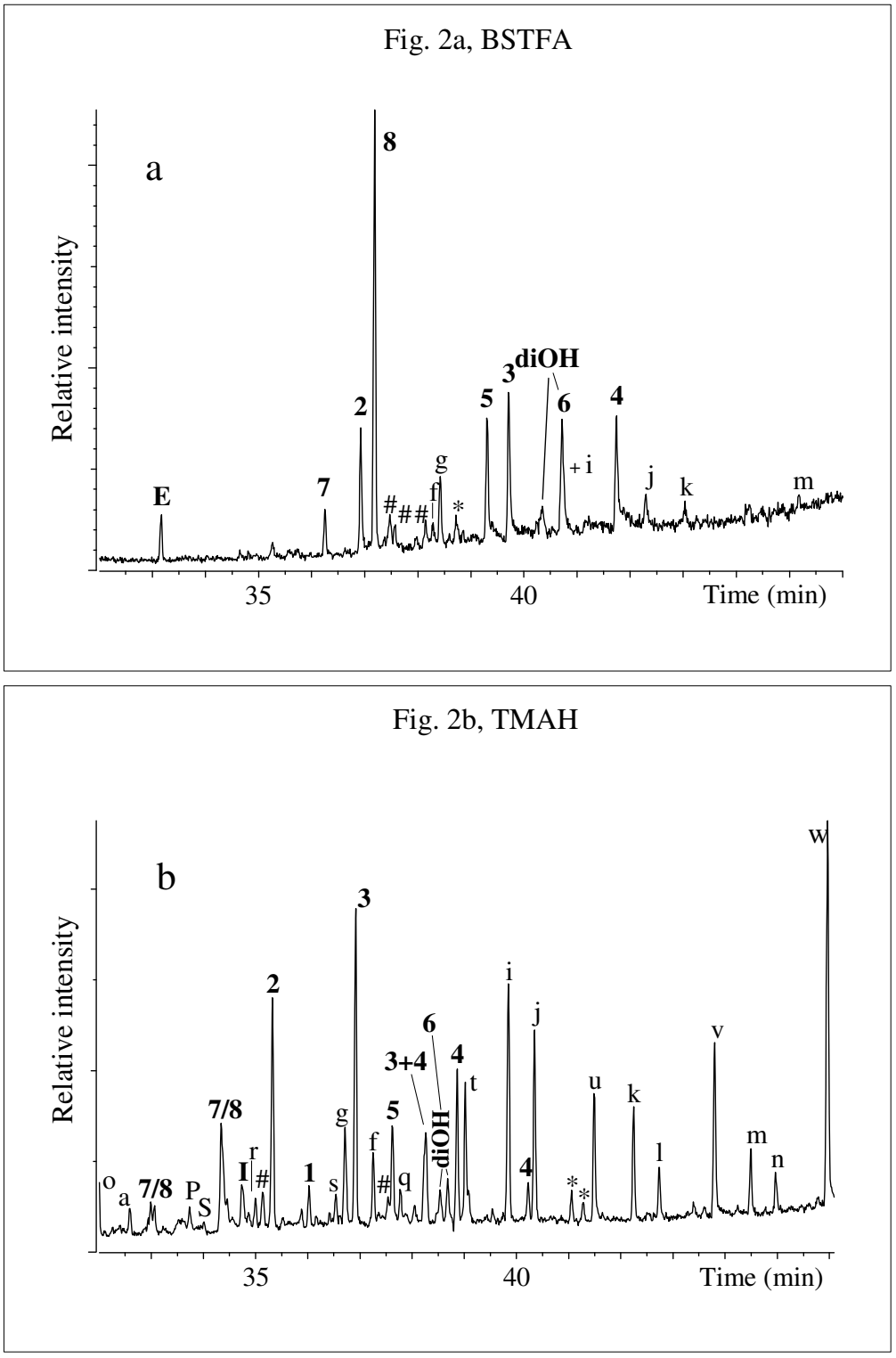
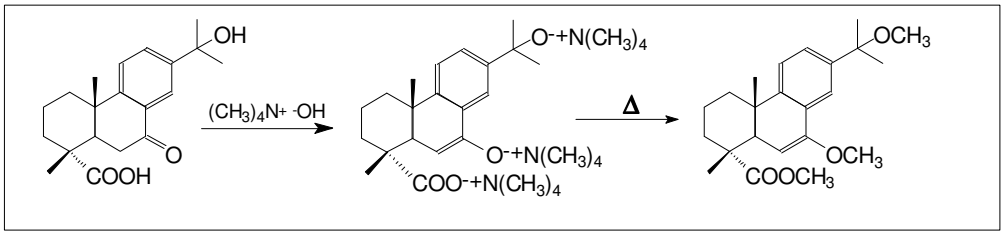
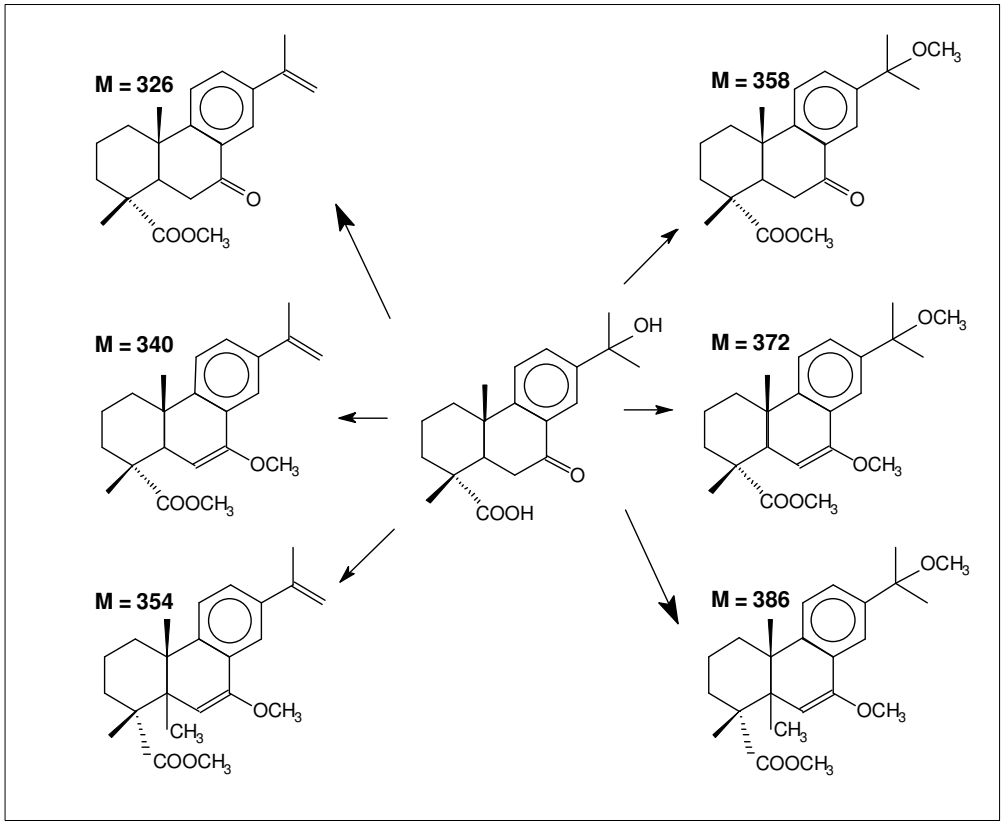


Figure 2. Partial total ion chromatograms of the aged wax/resin adhesive, derivatised with a) BSTFA and b) TMAH (on-line). Labels are explained in Table 1 and in the text.

Since these reactions are in competition, this may lead to 3 products for **3** and 6 products or more in the case of **4** (See Scheme 6). In the present experiments, several peaks of low intensity could be assigned to (partially) dehydrated **5** and other OH-DHAs (M = 312), di-OH-DHA (M = 342, 310). In case of **7** and **8**, some products deriving from partial methylation and dehydration products. We have experienced that the degree of methylation and dehydration can not be controlled and varies from experiment to experiment. However, the methyl ether products of the hydroxyl and the enol groups are always the most abundant by far.



Scheme 5. Proposed reaction mechanism for the main reaction channel of 4 and TMAH.



Scheme 6. Identified products of derivatisation of 4 with TMAH. The trimethylated compound with M = 372 is always the main product.

Multiple product formation is a drawback in the derivatisation with TMAH but this is less important if the possible products are known. 'Softer' ammonium

hydroxide reagents such as trimethyl-trifluoro-toluyl ammonium hydroxide (TMTFTH) are often presented as a good alternative since they react at lower temperatures and show less isomerisation [Kossa *et al.*, 1979; White and Pilc, 1996; Watts and De la Rie, 2002]. However, TMTFTH does not methylate hydroxy functionalities and this will again, as discussed above, complicate the detection of highly oxidised compounds such as **4**. In addition, hydrolysis can be avoided by applying a less alkaline environment. In case of tetramethylammonium acetate (TMAAc), for example, only free carboxylic acid groups are methylated and transesterification does not take place [Hardell and Nilvebrant, 1996].

In a normal off-line derivatisation procedure, only part of the sample is injected on the GC column with the off-line derivatisation methods. On-line derivatisation with TMAH has the advantage that the entire sample can be analysed. Hydrolysis and methylation is done in one step. In addition, information can be obtained from the non-hydrolysable part of the polymeric network by additional pyrolysis (See Chapter 4). These features are very important for the analysis of minute and unique samples from art or archaeology and thus an important reason to choose for the on-line derivatisation method, despite the multiple products that can be formed from highly functionalised molecules.

Identification and mass spectrometric fragmentation

The most prominent abietic acids in the different stages of oxidation given in Scheme 3 are **1-5**. The mass spectra of these acids after derivatisation (all four procedures) are presented in the *Atlas of Mass Spectra* at the end of this report, Atlas 1-21. Some spectra, or tables with relative peak intensities of the prominent peaks, have been previously presented in some form or another in the literature (mostly methyl esters, Atlas 1, 3, 5, 9 and 18 [Enzell and Wahlberg, 1969; Anderson and Winans, 1991; Krohn *et al.*, 1992]), whereas the others have been elucidated on the basis of fragmentation patterns and the relative retention times. Recent organic synthesis and analysis of **3** and **4** supports the assignment of these compounds [Pastorova *et al.*, 1997].

The mass spectra of diterpenoid abietic acids can be recognised easily by their characteristic fragmentation patterns which involve i) loss of the ester group, ii) expulsion of the C20 methyl group, iii) loss of water, trimethylsilanol or methanol from the hydroxyl moiety (Tables 2a and 2b). Expulsion of a methyl group can also occur from the TMS moiety in case of a trimethylsilylated compound. In the case of a 15-hydroxy compound, the fragment ion for the isopropyl side group, $^{+}C(CH_3)_2OR$ can be used as a marker for these side groups (m/z = 59, 131 and 73 for R = H, TMS and CH_3, respectively).

The methylated enol products of **3** and **4** with TMAH (See Scheme 6) have also been elucidated on the basis of their fragmentation patterns (Atlas 7, 8, 12-17). The discussion of their specific fragmentation behaviour is outside the scope of this chapter but can be found elsewhere [Pastorova *et al.*, 1997].

Two hitherto unknown mass spectra of additional highly oxidised diterpenoid compounds are assigned to di-hydroxy dehydroabietic acids on the basis of the respective molecular mass shifts after the respective derivatisations. The spectra of the most abundant isomer of the two compounds (with the higher retention time) are given in Atlas 22-24 (with the exception of '**6a**', from which no spectrum could be obtained, as described in the previous section). The peaks corresponding to the

$^{+}C(CH_3)_2OR$ ion indicate a hydroxy functionality on the 15-position in both cases. At this stage, we cannot be certain about the structure of the two compounds formed since the information from the mass spectra is not sufficient. However, considering the expected positions of oxidised functional groups in comparison to their potential precursors (See Scheme 3) and the mass spectra we tentatively assign the structure **6** (7,15-di-OH-DHA) to the most abundant of the two compounds. **6** has been reported in the literature as a constituent of pollen from *Cedrus deodara* Loud [Ohmoto *et al.*, 1987]. The structure was elucidated on the basis of IR and ^{1}H-NMR data of the isolated compound; no mass spectrum was presented. The other compound might be the stereoisomer of **6**, in which the 7-OH moiety could be in the other chiral positions. However, no indication for the formation of stereoisomers in similar oxidation reactions has been presented in the literature so far; 7-OH-DHA, for example, has only been observed in the α-form [Krohn *et al.*, 1992].

Table 2a. Characteristic fragment ions and corresponding m/z values of 1, 2 and 3. See Atlas of Mass Spectra and text.

Ions / Compounds	**1 a,b,d**	**1 c**	**2 a,b,d**	**2 c**	**3 a,b**	**3 c**	**3 d**
Atlas #	*1*	*2*	*3*	*4*	*5*	*6*	*7*
$M^{+\cdot}$	316	374	314	372	328	386	342
$[M-CH_3]^+$	301	359	299	357	313	371	327
$[M-COOR]^{+}$ 1)			255	255	269	269	283
$[M-HCOOR]^{+\cdot}$ 1)	256	256			268	268	
$[M-CH_3-HCOOR]^{+\cdot}$ 1)	241	241	239	239	253	253	267

1)R corresponds to the carboxylic ester; CH_3 (**a**,**b**,**d**) or $Si(CH_3)_3$ (**c**).

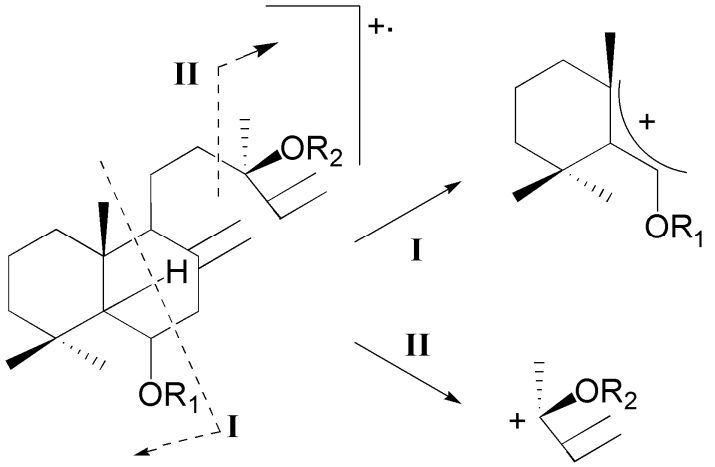
Table 2b. Characteristic fragment ions and corresponding m/z values of hydroxyabietic acids in different stages of oxidation (cont'd). See Atlas of Mass Spectra and text.

Ions / Compounds	**4 a**	**4 b**	**4 c**	**4 d**	**5 a**	**5 b**	**5 c**	**5 d**	**6 b**	**6 c**	**6 d**
Atlas #	*9*	*10*	*11*	*12*	*18*	*19*	*20*	*21*	*22*	*23*	*24*
$M^{+\cdot}$	344	(416)	474	372	330	402	460	344	490	548	374
$[M-CH_3]^+$	329	401	459	357	315	387	445	329	475	533	359
$[M-COOR]^{+}$ 1)	285	357	357	313	271	343		285	431		
$[M-CH_3-HCOOR]^{+\cdot}$ 1)	269	341	341	297	255	327	327	269			
$[M-R'OH]^{+\cdot}$ 2)		326	(384)	340	312	312	370		400	458	342
$[M-R'OH-CH_3]^{+\cdot}$ 2)			369	325	297	297	355	297	385		327
$[M-R'OH-COOR]^{+\cdot}$ 1,2)		267		281	253	253	253	253	341		283
$[M-R'OH-CH_3-HCOOR]^{+\cdot}$ 1,2)		251	251	265	237	237	237	237	325	325	267
$^{+}C(CH_3)_2OR'$ 2)	59	131	131	73	59	131	131	73	131	131	73
$[M-2\ R'OH-COOR]^{+\cdot}$ 1,2)									251		
$[M-31]^+$	313	385	443	341		371		313			343

1)R corresponds to the carboxylic ester; CH_3 (**a**,**b**,**d**) or $Si(CH_3)_3$ (**c**).
2)R' corresponds to the substitution at the hydroxylic groups; H (**a**), $Si(CH_3)_3$ (**b**,**c**) or CH_3 (**d**).

As discussed before, larixol and larixyl acetate (**7** and **8**) are typical compounds in larch resin. The mass spectra of the compounds after derivatisation are shown in Atlas 25-29. As expected, **7** and **8** are found *as such* after (**a**) and as the di-

(trimethyl)silyl ether and the 6-acetyl,13-(trimethyl)silyl ether after (**b**) and (**c**), respectively. In the reaction with TMAH (**d**) the dimethyl ether is formed as the main product from both compounds. The products are recognised easily from their characteristic fragmentation patterns (See Scheme 7 and Table 3). Cleavage of the B-ring (**I**) is predominant together with homolytic cleavage of the side chain (**II**; only predominant in case of a derivatised 13-OH-group). Other important fragmentations are loss of ROH side chains and a methyl group. The cleavage of the B-ring (**I**) in case of the acetate **8** (with formation of the ion of m/z 195) mostly concurs with the loss of ketene (CH_2CO) from **8** to form the ion of m/z 153. Since this ion is also predominant in case of **7**, the spectra of the two compounds are quite similar.



Scheme 7. Characteristic fragmentation paths of 7 and 8 after derivatisation. See Table 3, App. 7 and text.

Table 3. Important fragment ions and corresponding m/z values in the mass spectra of 7 and 8 after derivatisation. See Scheme 5, Atlas of Mass Spectra and text

	$R_{1,2}$ = *H*	R_1 = *Ac*, R_2 = *H*	$R_{1,2}$ = $Si(CH_3)_3$	R_1 = *Ac*, R_2 = $Si(CH_3)_3$	$R_{1,2}$ = CH_3
Atlas #	*25*	*26*	*27*	*28*	*29*
$M^{+\cdot}$	306[1)]	348[1)]	450[1)]	420[1)]	334[1)]
$[M - CH_3]^+$	291[1)]	333	435	405	329[1)]
$[M - ROH]^{+\cdot}$	288	288, 330[1)]	360	360, 330[1)]	302
$[M - ROH - CH_3]^+$	273	273, 315[1)]	345	345, 315[1)]	287
$[M- 2 ROH]^{+\cdot}$	270	270	270	270	270
$[M - 2 ROH - CH_3]^+$	255	255	255	255	255
I	153	195, 153[2)]	225	195, 153[2)]	167
II	71	71	143	143	85

[1)]Not observed/intensity < 0.5%. 2) After loss of CH_2CO (See text).

Py-TMAH-GCMS of a 200 year-old paint sample

A 200 year-old sample from a painting by Pieter Barbiers was studied. The fact that the paint in this work is hard and brittle in combination with the atypical UV-fluorescence of the paint raised the question what organic material could be responsible. The paint sample discussed here contained several inorganic pigments including lead white (basic lead carbonate, as studied by light microscopy).

In Fig. 3, the Py-TMAH total ion chromatogram of the paint sample is presented. The most important compounds in the spectrum are products of the drying oil fraction of the paint typically found in old oil paints: [Mills and White, 1994; Van den Berg 1998; Van den Berg, *et al.,* 2002] diacids (oxidation products from unsaturated fatty acids in the original drying oil), saturated fatty acids (predominantly palmitic acid (C16) and stearic acid, (C18) in a ratio of 1.1:1 indicating the presence of linseed oil [Mills, 1966]) and 9,10-epoxy and di-hydroxy stearic acids, oxidation products of oleic acid (Δ9-mono-unsaturated C18 fatty acid). In addition, highly oxidised abietic acids are found. No pimaric acids are observed.

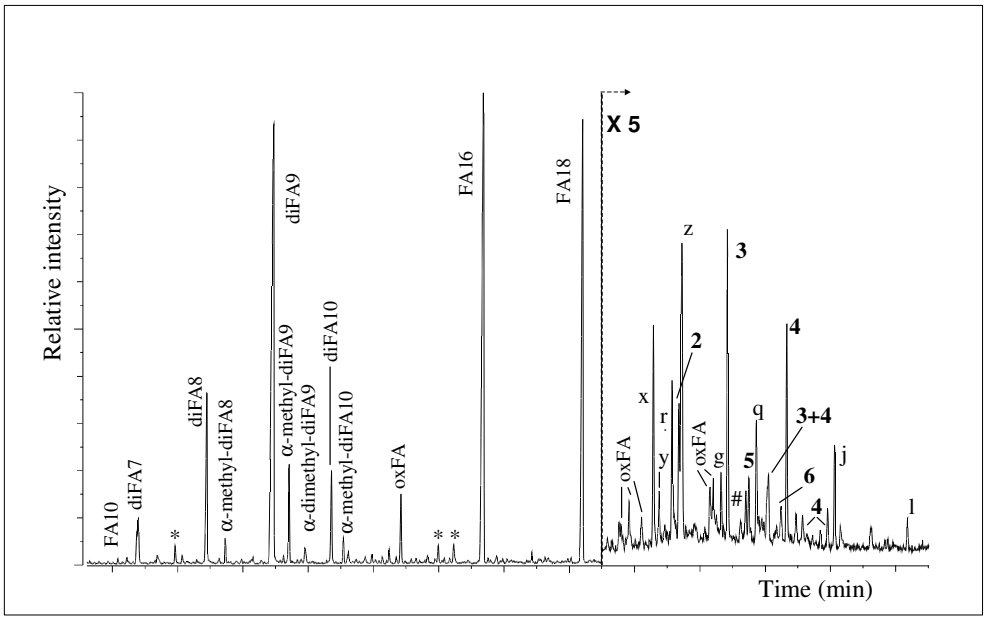


Figure 3. Total ion chromatogram of the resin/oil paint sample from Pieter Barbiers, "Jagers in de Duinen"*, derivatised with TMAH. diFA: diacid, dimethyl ester (+ number of carbon atoms); FA: fatty acid, methyl ester (+ number of carbon atoms); oxFA: oxidised FA, methyl ester. Other labels are explained in Table 1. See text.*

In conclusion, the traces of highly oxidised abietic acids in combination with drying oil in the paint sample indicate the use of a resin-oil paint. The presence of resin may explain the odd fluorescence of the paint.

The amount of resins detected in resin/oil paints is often very low. This may be explained by (co-)polymerisation with the oil in the drying process which leads to cross-linking products that cannot be analysed easily with any analytical method. It has been established that the low molecular weight fraction of *triterpenoid* resins such as dammar and mastic diminishes dramatically in the course of time when applied as varnishes [Van der Doelen *et al.*, 1998a&b; 1999]. It might also be argued that further oxidation of the abietic acids could play a role. This might involve loss of integrity of

the abietic diterpenoid acid system, for example by opening of the B-ring through oxidation [Gigante *et al.*, 1989]. From the present data it can not be concluded unequivocally that other (more highly) oxidised compounds are not formed. No evidence for these compounds was found with reversed-phase HPLC (as will be described in Chapter 2). In addition, the high relative amounts of **4**, particularly in the aged varnish, would indicate that this compound is very stable and could represent an end member.

Conclusions

Pine and larch resin can be distinguished easily using gas chromatography. After application in paintings, however, the resins lose some of their characteristic features.

The diterpenoid abietic acids from pine and larch and other members of the conifer subfamily *Pinaceae* show a natural ageing behaviour in and on paintings that is characterised by five oxidation stages. Derivatisation of the highly oxidised compounds for GCMS with BSTFA and TMAH (on-line) in combination with GCMS is most suitable. On-line derivatisation with TMAH is most suitable for complex (heterogeneous) microgram samples from *i.e.* paintings since no sample preparation is needed and no sample is lost.

Acknowledgements

The samples were taken by supervision of Manja Zeldenrust (Rijksmuseum, Amsterdam; lining adhesive), Jos van Och (Stichting Restauratie Atelier Limburg (SRAL), Maastricht; varnish) and Mireille te Marvelde (MOLART; paint sample).

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2. Mass spectrometric methodology for the analysis of highly oxidised diterpenoid acids in Old Master paintings. Part 2. LCMS and DTMS studies. Index for the Degree of Oxidation

Abstract

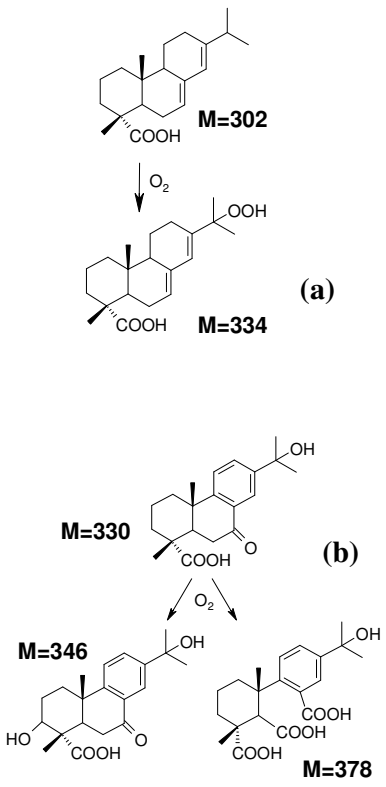
Products of diterpenoid resins present in aged works of art were studied by reverse phase HPLC-APCI-MSMS and DTMS. With both methods, a number of oxidation products of abietic acids were identified and different stages of oxidation of these acids could be distinguished in a comparable manner as with GCMS. Reverse phase HPLC does not resolve abietic acid and its isomers. A large number of oxidation products present in very low amounts in a relatively fresh pine resin (colophony) could be traced in single ion chromatograms of the HPLC-APCI-MS. No indication of products more highly oxidised than the 15-hydroxy-7-oxo-dehydroabietic acid was found. Positive ion LC-APCI-MS/MS spectra of DHA, 7-oxo-DHA and 15-OH-7-oxo-DHA show a loss of 128 a.m.u. corresponding to CID fragmentation which involves the A ring. The fragmentation reaction is complex and requires cleavage of several carbon-carbon bonds. DTMS is shown to be a fast analytical method able to analyse low amounts of diterpenoids in complex matrices.

An index for the degree of oxidation (IDOX) of the abietic acids is presented as an indicator of the degree of oxidation of the matrix in which the resin is present. The method of choice for the determination of IDOX is Py-TMAH-GCMS. The influence of age on IDOX was studied for 20 lining material samples containing beeswax and larch and/or pine resin of known age (0-140 years). The IDOX values were found to correlate reasonably well with age. IDOX rises relatively fast to 0.5 in the first 20 years and then levels off to 0.7 in the next 100 years. Besides age, light is a very important factor leading to IDOX values up to 0.93 for varnishes.

Introduction

In Chapter 1, the oxidation behaviour of diterpenoid abietic acids in painting materials was discussed (See Scheme 3 in Chapter 1). Five stages of oxidation were proposed and the molecules involved could all be identified using GCMS. Several derivatisation methods for GCMS analysis of diterpenoid resins were discussed. In this chapter, results are presented on the use of other mass spectrometric methodology, LCMS and DTMS, for the analysis of abietic acids in different stages of oxidation.

Despite the identification of the five different stages of oxidation presented in Chapter 1, the actual mechanism of degradation of diterpenoid resins, and abietic acids in particular, largely remains a mystery. Peroxide intermediates may be formed in early stages of the ageing process (see *e.g.* Fig. 1a). Diterpenoid resins may react to oligomers or to more highly oxidised species than can be elucidated with GCMS. Examples of potentially more highly oxidised species are given in Fig. 1b. GCMS requires chemical derivatisation of polar groups in the molecules. Although approaches such as Py-TMAH-GCMS are useful for direct analysis of non-purified small (less than 5 microgram) paint samples and aged varnishes in their complex matrix, it does not allow the study of transient labile oxidised species such as peroxides. It may, however, derive compounds from pyrolysed high molecular weight species.



HPLC on the other hand could give more insight in the transient oxidised species of diterpenoid resins. One of the main advantages of LCMS over GCMS is the broader range of functionalised compounds that can be studied in one analytical run without derivatisation. In the first part of this chapter, we explore the possibilities of LC-APCI-MS in positive and negative mode for the study of oxidation products of diterpenoid resins. MSMS is applied for further structural elucidation.

In the second part of this chapter, the feasibility of the analysis of these acids in complex and heterogeneous samples with Direct Temperature-resolved Mass Spectrometry (DTMS) is discussed and typical indicator masses for (oxidised) diterpenoid acids are presented. DTMS is an elegant method for fast screening of complex and small (<5 μg) samples [Boon, 1992], often used for the analysis of samples of works of art [Boon *et al.,* 1995; Van den Berg *et al.,* 1998; Van der Doelen *et al.*, 1998; Van der Doelen, 1999].

Figure 1. (a) Possible transient peroxidation product of abietic acid; (b) hypothetical oxidation producst of 15-hydroxy-7-oxo-dehydroabietic acid.

The four most important abietanes, **1** (abietic acid), **2** (dehydroabietic acid), **3** (7-oxo-dehydroabietic acid) and **4** (15-hydroxy-7-oxo-dehydroabietic acid), have been proposed and identified as potentially useful marker compounds for the assessment of the presence of oxidising environments in paintings in Chapter 1. To allow for a more quantitative measure of the degree of ageing in painting materials, an "Index for the Degree of Oxidation" (IDOX) is introduced in the last part of this chapter. This Index is calculated from Py-TMAH-GCMS analyses of a number of painting material samples, including a large set of wax/resin lining samples containing beeswax and larch and/or pine resin of known age (0-140 years).

Experimental

Materials

The samples used in this Chapter are partly from the same source as in Chapter 1. For the LCMS varnish analyses, also a extensively darkened old varnish from the painting "*Ecce homo*" (central panel, 1559) by Maarten van Heemskerk (Frans Hals museum, Haarlem) was used. This varnish was removed using a swab and isopropanol.

Another oxidised, very brown varnish was taken near the top edge of "*The Girl with the Pearl Earring*" (c. 1665-1666) by Jan Vermeer (Mauritshuis, The Hague, cat. no. 1687). It was covered by an extensive overpaint, which was applied in 1960 in a restoration treatment.

The wax-resin lining materials were taken by Mireille te Marvelde from the reverse of almost 20 paintings from several museums and collections (Frans Halsmuseum Haarlem, Mauritshuis Den Haag, Oranjezaal Huis ten Bosch, Den Haag and Rijksmuseum Amsterdam) in the framework of the MOLART wax-resin lining project [te Marvelde, 1994; 1999; 2001]. The dates of lining and therefore the age of the adhesives are accurately known and range from 1861 to 1979. The content of the materials seems to reflect the recipes that were used by the different conservators. For these traditional lining material recipes, beeswax (generally 50-65%) is mixed with (35-50%) colophony and/or Venice turpentine. The addition of copaiba balsam is mentioned in some archival sources. However, this compound has not been identified in any of our samples (see also Chapter 3). Only one sample (the 1979 sample from the Frans Hals museum) was found to contain spermaceti (wax originating from the sperm whale, with a lower melting point than beeswax) instead of beeswax. The fresh samples were made according to two traditional recipes (melting together 2/3 beeswax and 1/3 colophony or 3/6 beeswax, 2/6 Venice turpentine and 1/6 colophony, respectively) by M. te Marvelde, R. Hoppenbrouwers and K.J. van den Berg.

The chemicals used for sample preparation were commercially available and used as received.

Sample preparation

Direct Temperature-resolved Mass Spectrometry

For DTMS experiments, the sample (typically 5-10 µg) was homogenised in a mini glass mortar and made into a suspension with 20-50 µl of ethanol. Subsequently, the sample was applied on the Pt/Rh filament of a direct insertion probe.

Py-TMAH-GCMS

See Chapter 1.

Instrumentation

Reversed phase HPLC-APCI-MSMS

LCMS was performed on a VG Quattro II (Micromass Ltd., UK) tandem quadrupole mass spectrometer. Measurements were controlled and data acquired using the Masslynx 2.3 program. Sheath and drying gas was nitrogen (100 l/h and 400 l/h respectively); probe temperature 450 $^{\circ}$C; corona discharge voltage 3.15kV. The sampling cone was set typically at 25V. The MS/MS acceleration voltage was typically 22 V; collision gas was argon ($5x10^{-4}$ mbar in the gas cell).

The HPLC system is a HP1090 with a HP79881A Filter Photometric Detector (Hewlett Packard, USA). Injections were carried out using a Rheodyne 7125 injection valve (Rheodyne, USA), equipped with a 20 µl loop. The C18 reversed-phase column was a Inertsil ODS3 column (G.L.Science, USA), 5 micron beads, 1.2 cm x 0.43 cm i.d. For the analysis, water and acetonitrile (Fisher Scientific, HPLC grade) were used as eluents at a flow rate 1ml/min. The eluent (water/acetonitrile) was kept isocratic at 40/60 for 10 min. and ramped to 100 % acetonitrile in 40 min. Alternatively, the gradient was water/acetonitrile10/90 to 0/100 in 50 min.

Direct Temperature-resolved Mass Spectrometry

Data were obtained with a JEOL SX-102A double focusing (B/E) mass spectrometer using a direct insertion probe equipped with a Pt/Rh (9/1) filament (100 µm diameter). The current through the filament was ramped at a rate of 0.5 A/min for two min. to reach an end temperature of about 800 ^{o}C. Desorbed molecules were ionised (16 eV) in an ionisation chamber kept at 200 ^{o}C and accelerated to 8 keV. The mass spectrometer was scanned from m/z 20-1000, with a cycle time of 1 s. A JEOL MP-7000 data system was used for data acquisition and processing.

Gas chromatography-Mass Spectrometry

See Chapter 1.

Results and discussion

The results of HPLC-APCI-MS analysis of a "fresh resin" (colophony) are presented in Fig. 2a (positive ions) and 2b (negative ions). Reversed phase HPLC does not resolve abietic acid from pimaric acid isomers, so all these compounds are present in peak 1. DHA and various oxidation products are well separated however. A major difference with the GCMS analysis (See Chapter 1, Fig 1b) is the occurrence in relatively high abundance of at least three compounds with a molecular mass of m/z 318. These may be hydroxylated molecules of abietic acids or pimaric acids. The positive ion APCI-MS spectra (not shown here) are relatively simple compared to EI data and give only limited structural information. APCI-MS in the negative mode is about a factor of ten less sensitive than the positive mode with the exception of DHA (B, M=300) for which both modes are roughly equally sensitive. In the positive ion mode the spectra are more complex and adduct ions, pseudomolecular ions and fragment ions are not always easily distinguished. The negative mode is therefore important because reliable molecular weight information is obtained.

In Fig. 3 the positive ion chromatogram of an aged varnish is presented. The TIC is very simple, only four peaks are observed. This is also the case when another gradient is chosen. Two peaks, C and D in Fig. 1, were also identified in the Py-TMAH-GCMS analysis. The other two compounds with molecular weights of 288 and 302 have not been identified yet. The assignment of the molecular weights is tentative since these peaks are not observed in the negative ion spectrum (not shown here), which suggests other types of functionalities compared to regular diterpenoid acids.

The positive MSMS spectra of the pseudomolecular ions of the abietic acid and the most abundant oxidation products are given in Fig. 4. In the negative mode, electron detachment was the predominant process and no satisfactory MSMS spectra could be obtained.

Spectrum A is derived from various structural isomers (abietic and pimaric acids) and hence can't be related with just one known structure. However, a large number of fragment ions are observed pointing to promising structural information once a separation into pure compounds is achieved. The acid group shows by a loss of 46 a.m.u. (H_2CO_2). The spectrum of DHA is of the pure compound. The selected pseudomolecular ion at m/z 301 is fragmented to m/z 255 (loss of the acid group), m/z 173 (MH^+ -128) and m/z 133, which is interpreted as an isopropyl-tropylium ion. The formation of m/z 133 requires the cleavage of two carbon-carbon bonds in the B-ring. Ions due to a mass loss of 128 a.m.u. are also observed in the spectra of the 7-oxo-DHA (C), the15-OH-7-oxo-DHA (D) and its dehydrated isomer (D-H_2O). The constant mass loss in these compounds suggest a fragmentation process in the A ring with the charge remaining on the aromatic side. The cleavage requires a rearrangement and the resulting ion could be a two ring aromatic system (ring B and C) by transfer of the methyl group at C10 to the leaving neutral ($C_7H_{12}O_2$). An energetically favourable candidate for the structure could be an ion with a two-ring structure as given for D in Fig. 4.

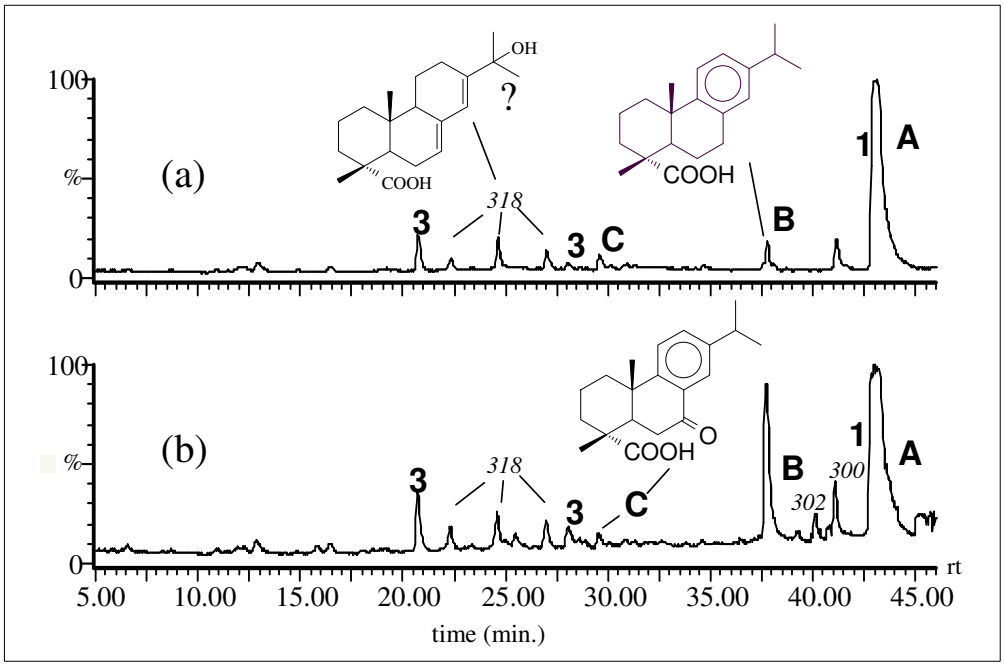


Figure 2. LC-APCI-MS analysis of colophony, in positive (a) and negative mode (b). A-C, 1,3 and 4 correspond to different oxidised abietic acids and oxidation stages of abietic acids respectively; see Scheme 3 in Chapter 1.

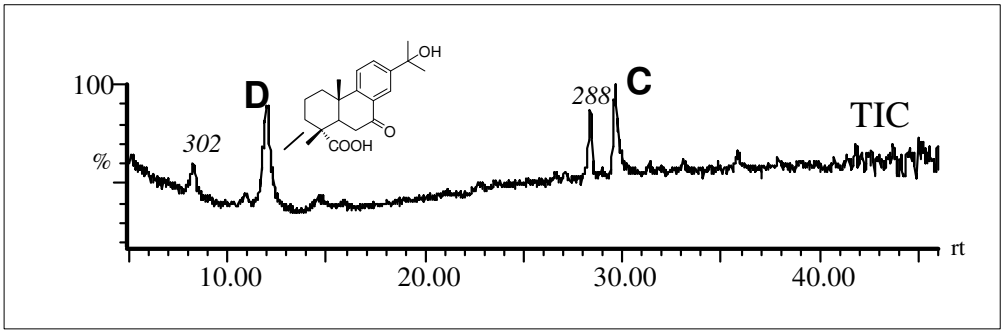


Figure 3. LC-APCI-MS analysis of an aged diterpenoid varnish. A-D, 1,3 and 4 correspond to different oxidised abietic acids and oxidation stages of abietic acids respectively; see Scheme 3 in Chapter 1.

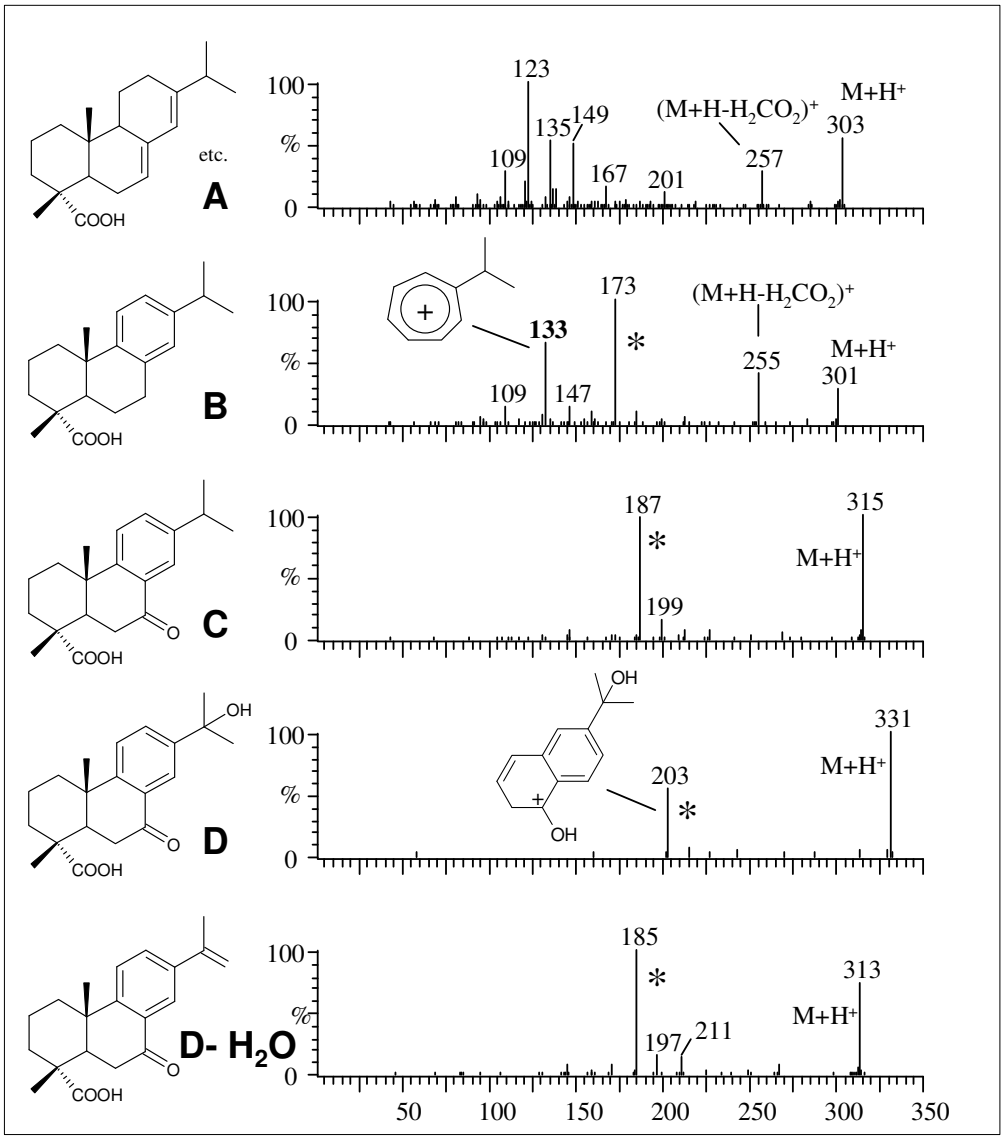


*Figure 4. MS/MS of abietic acid + isomers, dehydroabietic acid, 7-oxo-dehydroabietic acid, 15-hydroxy-7-oxo-dehydroabietic acid and its isomer after loss of water. The loss of m/z 128 (peaks marked with *) probably results in ions with a two ring structure as given for D.*

Despite the limited number of ions in the MSMS data of the spectra, the positive APCI-MSMS information provides supporting evidence of the identity of the compounds.

Single ion chromatograms (SIC) of the LCMS data under negative ion conditions of colophony (Fig. 5) show that a wealth of compounds are present once the data base is searched with mass chromatography. Compounds with two functional groups, for example a carboxylic acid group and an oxo, hydroxy or peroxy functionality, are expected to appear in the relative retention time region between 20 and 30 min. Those with three functional groups such as the di-hydroxy-DHA and 15-hydroxy-7-oxo-DHA are found approximately between 10 and 18 min (See also Fig. 3). However speculative, this might indicate that the compounds found in the SIC's of m/z 331 and 333 could be di-hydroxy acids whereas those with retention times between 20 and 30 min. are possibly peroxy diterpenoid acids. These compounds have not been found with Py-TMAH-GCMS possibly due to the severity of the

derivatisation conditions in combination with the low relative abundance of these structures. The presence of several oxidised species in this industrial colophony is an indication that the resin has oxidised easily, either in the solid resin itself or in solution (see Section *Index for the Degree of Oxidation (IDOX); a quantitative comparison* below).

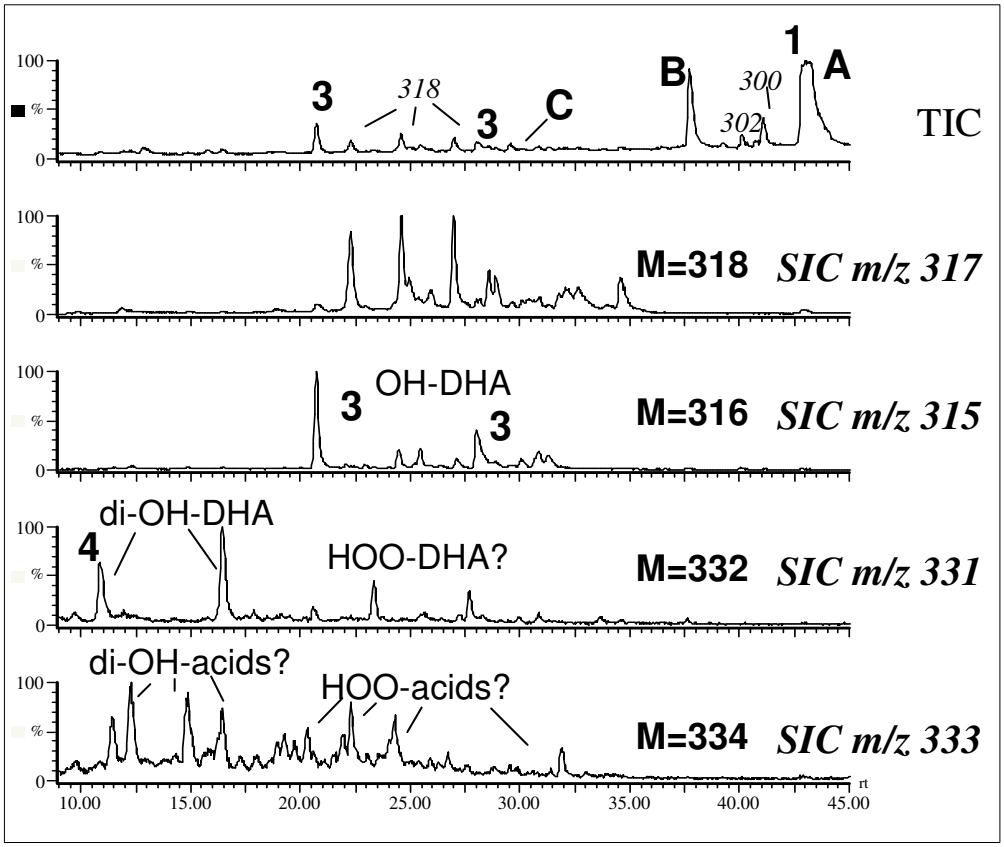


Figure 5. LC-APCI-MS negative ion mode analysis of colophony. Single ion chromatograms are used to search for traces of oxidised diterpenoid acids.

Single ion chromatograms in the negative mode of the aged varnish show peaks corresponding to M=332 in the region below 20 minutes, probably corresponding to dihydroxy-DHA isomers. Changing the solvent system to a more polar system does not improve the results. No evidence was found for unstable peroxy compounds. The relatively simple chromatogram (not shown here) suggest that only stable oxidation products are left over. We assume that potentially reactive intermediates have reacted to stable possibly larger molecular weight materials.

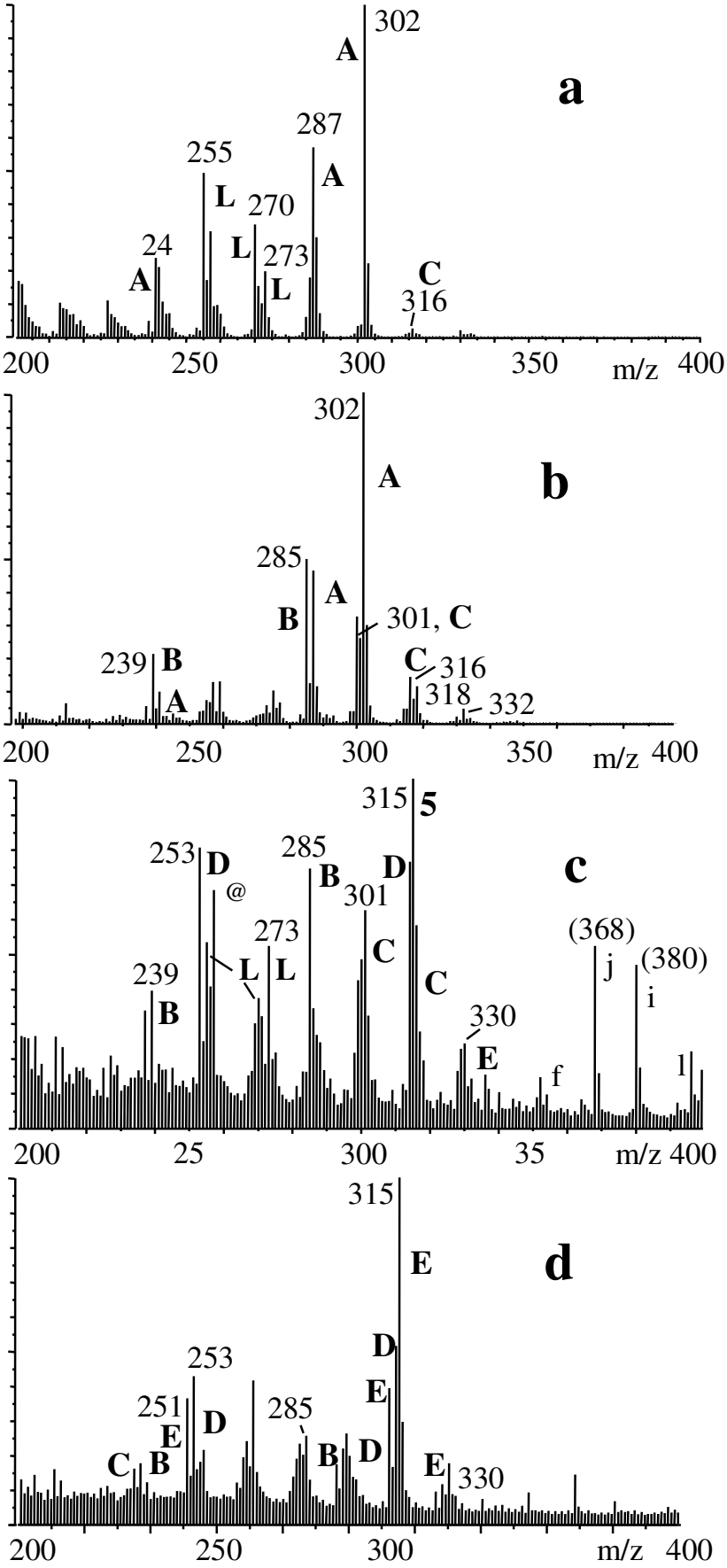


Figure 6. Summed DT mass spectra, temperature region about 100-350 °C, of (a) Venice turpentine, (b) pine colophony, (c) the wax/ resin sample and (d) the varnish. Labels are explained in Table 1 in Chapter 1; except @ which is protonated palmitic acid; fragment ion of the beeswax esters. See also Scheme 3 in Chapter 1 and Table 1 in this chapter.

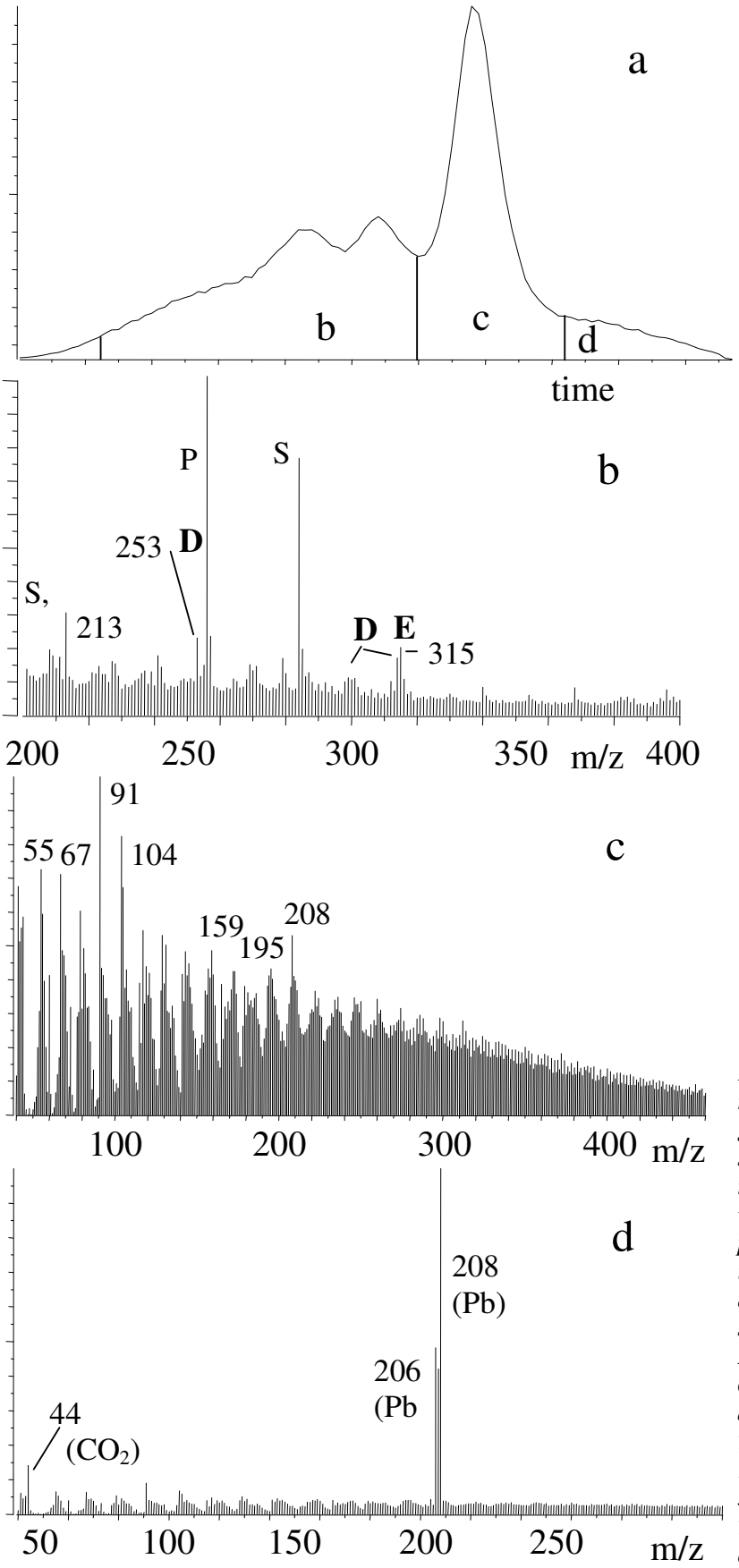


Figure 7. DTMS; total ion chromatogram (a) and summed mass spectra of (b) the volatiles, (c) the polymer and (d) the inorganic fraction of the resin/oil paint sample from Pieter Barbiers, "Jagers in de Duinen". *Labels are explained in Table 1 (Chapter 1). See also Scheme 3 in Chapter 1 and Table 1 in this chapter.*

Table 1. M/z values of characteristic molecular and fragment ions in a DTMS spectrum of abietic acids in different stages of oxidation, and larixyl acetate and larixol.

Ox. Stage	*Abietic acids*	*(Fragment) ions*[1)]
A	abietic acids, including 1[2)]	*302*, 287, 241
B	2	*300*, 285, 239
C	hydroxy-DHA's, including 5	*316*, 301, 255, 237
D	3 (+ 6 and other di-OH-DHAs')	*314*, 299, 253 (*332*, 235)
E	4	*330*, 315, 312, 251
	L = 7 or 8	288, 273, 270, 255

[1)] Molecular ion in italics; most prominent ions underlined. Fragment ions were checked with pure reference standards **1**, **2, 3** and **4**.

Table 2. Index of degree of oxidation (IDOX) of the wax/resin adhesive and the varnish and the relative amounts of the four acids (1:2:3:4) using different derivatisation methods. The degree of oxidation on the basis of DTMS data is calculated slightly differently (see text).

	TMSdiazo-methane (a)	*TMSd + BSTFA (b)*	*BSTFA (c)*	*TMAH (d)*	*DTMS (EI)*
wax/resin	0.73	0.74	0.66	0.67	0.61
	0:0.20: 0.41: 0.39	0: 0.21: 0.35: 0.44	0: 0.33: 0.38: 0.29	0.04: 0.23: 0.40: 0.33	0.08: 0.25: 0.44: 0.23
varnish	0.86	0.91	0.90	0.81	0.70
	0: 0.05: 0.32: 0.63	0: 0.04: 0.20: 0.76	0: 0.04: 0.21: 0.75	0.02: 0.09: 0.33: 0.56	0.08: 0.12: 0.42: 0.38

Microgram scale analysis with DTMS

In a typical Direct Temperature-resolved MS (DTMS) experiment, the sample (<5 μg) is homogenised in a liquid, applied toa metal wire and the liquid subsequently evaporated *in vacuo*. Then the probe is inserted directly into the ion source and heated. This results in volatilisation of compounds (roughly below 400 ^{o}C) and subsequent pyrolysis of the remaining high molecular weight compounds above 400 ^{o}C. Inorganic compounds (including metals) can be released at even higher temperatures. The compounds released from the filament are ionised at an ionisation energy of 16 eV to minimise fragmentation.

To examine the feasibility of this methodology for the analysis of diterpenoids in painting samples, the characteristic (fragment) ions of the different molecules in different stages of oxidation as well as **7/8** (larixol and larixyl acetate, see Chapter 1) were obtained from the spectra of the neat compounds **1, 2, 3** and **4** [Pastorova, 1997]. The m/z values of the fragments of other important compounds including **5, 6, 7** and **8** are educated guesses derived from corresponding 70 eV GCMS spectra. The result in Table 1 is striking because the molecular masses and predominant fragment ions are unique for all five oxidation stages. This makes identification of aged diterpenoid *Pinaceae* resin-containing samples in combination with a rough estimate of the degree of oxidation feasible (see below).

The DTMS results of pine colophony, Venice turpentine, the lining material from the Steen and the varnish from the Bol painting are given in Fig. 6. The mass spectra are summed only in the region where the resinous fraction evaporates (some 35 spectra summed, roughly from 100-300 ^{o}C), thus ignoring other information on the sample. In addition, DTMS spectra of the 200 year-old sample from a painting by Pieter Barbiers are presented in Fig. 7.

The DTMS spectrum is quite complex for the aged samples. However, with the most important compounds of (oxidised) diterpenoids elucidated, the spectra can be interpreted with confidence. Also in the case of the complex matrix of the paint sample in Fig. 7, the oxidation stages D and E can be found. In the low temperature region (Fig. 7b), in addition to traces of oxidised abietic acids, the main characteristics for drying oil are observed. These characteristics are e.g. m/z 256 and 284 (ratio about 1, characteristic for linseed oil) for palmitic and stearic acid, respectively. The higher temperature parts of the TIC give the characteristic pattern of the pyrolysis products of the oil paint network polymer (T = roughly 400-600 ^{o}C, Fig. 5c), and lead and CO_2 from the lead white pigment (T = roughly 600-800 ^{o}C, Fig. 5d).

The results show clearly that DTMS can also provide information on low amounts of resins in complex (inorganic material containing) Old Master paint samples although the results obtained with Py-TMAH-GCMS (Chapter 1) show more detail.

Index for the Degree of Oxidation (IDOX); a quantitative comparison

The degree of oxidation may be an important parameter to assess the chemical environment and the age of (a particular part of), for example, a work of art. The most important abietic acids that we have found in works of art are **1** (abietic acid, which is as a rule completely disappeared in aged samples), **2** (dehydroabietic acid), **3** (7-oxo-dehydroabietic acid) and **4** (15-hydroxy-7-oxo-dehydroabietic acid). As a semi-quantitative tool to assess and compare the degree of oxidation of the different compounds, the Index for the Degree of Oxidation (IDOX) can be calculated from the relative amounts of these four acids using the expression

$$\text{IDOX} = (1/3 * \mathbf{2} + 2/3 * \mathbf{3} + 1 * \mathbf{4})$$

In order to make this calculation, the sum of the relative intensities of the four acids (including **1** which is not present in the expression above) is set to 1. As a result, the value of IDOX ranges from 0 to 1. In our experiments, only peak heights are taken as a measure of the relative amounts of the compounds.

The IDOX of the varnish and the wax/resin sample, and the relative ratios of the four acids (derived from the Py-TMAH-GCMS analyses discussed in Chapter 1 and the DTMS analyses in Fig. 6) are presented in Table 2. The IDOX are fairly constant for the different methods and comparable for all derivatisation methods. This implies that the methods may all be used for the determination of the degree of oxidation. However, it should be kept in mind that methylation with TMSdiazomethane often underestimates the more polar compounds (See Chapter 1).

The relative intensities of the compounds in the aged samples were found to be reproducible within 5% for the various off-line derivatisation procedures (two or three

analyses). Therefore, we believe that variations in the outcomes are brought about mainly by differences in efficiency of the derivatisation methods. It must be stressed here that also the differences in ionisation cross section (relative response) of the compounds have not been accounted for. This will also result in slightly different IDOX for the different derivatisation methods; note that large molecules have a relatively high ionisation efficiency, which will result in a relatively high intensity [McDaniel, 1989].

In contrast to the aged samples, the relative intensities of the compounds in fresh, relatively unoxidised pine colophony and Venice Turpentine were found to vary quite strongly. The ratio of **1/2** differed dramatically between measurements and as a result the IDOX varied between 0.1 and 0.25. This is explained by the relatively fast oxidation of the abietane isomers in solution, both before and after derivatisation with TMSdiazomethane. This fast oxidation was found only for the non-oxidised abietic acids.

Calculation of "IDOX" values on the basis of the DTMS spectra in Fig. 6c and d, and the ions in Table 1 gave lower values than for GCMS (Table 2) due to the relatively high values for oxidation stage **A** and **C**. These values, however, are not reliable since no separation of compounds on the molecular level has taken place. Indeed, the high values for **A** and **C** can be expected since overlap with other diterpenoid compounds will occur in stage **A** (other abietic and pimaric acids) and in stage **C** (di-OH-DHAs and **3**). In addition, ^{13}C-contributions play a role and the relative amounts are calculated on the basis of only three or four (albeit prominent) peaks. Therefore, DTMS can give no more than an approximate estimate of the IDOX.

It is difficult at this stage to indicate which internal or environmental factors are most important in the oxidation of abietic acids and the value of IDOX. IDOX values for aged materials are all quite high, as we have seen above. A complicating factor is that the age is often not accurately known. Metals may play an important role in catalysing the oxidation of abietic acids in resin/oil paint, for example. However, considering the age (200 years) of the sample, the IDOX value of the Barbiers paint sample (calculated from Fig. 3 in Chapter 1) of 0.76 is, not extremely high.

Through many analyses of painting samples we found that old diterpenoid containing varnishes generally show the highest degree of oxidation. This indicates that light, possibly in combination with easy access of oxygen, is a very important factor. The highest value, 0.93 obtained so far was from an old, possibly original, varnish from Vermeer (Fig. 8) [Groen *et al.*, 1998].

In order to obtain some idea of the influence of age and the usefulness of IDOX as an estimate of the oxidising environment, a large amount of diterpenoid resin containing lining adhesive samples was analysed with Py-TMAH-GCMS. All samples contained beeswax (except for one containing spermaceti, see *Materials and methods*) in addition to colophony and/or Venice turpentine, of lining material samples containing beeswax and larch and/or pine. Despite the variations in formulation, application and the exact origin of the resin components, the history of the samples is relatively similar. The photochemical degradation factor can be ruled out since all samples were all naturally aged in the dark (more or less on the surface of the back of the painting) Therefore, the most obvious remaining variable is time; the samples were all from linings of known age (0-140 years after application).

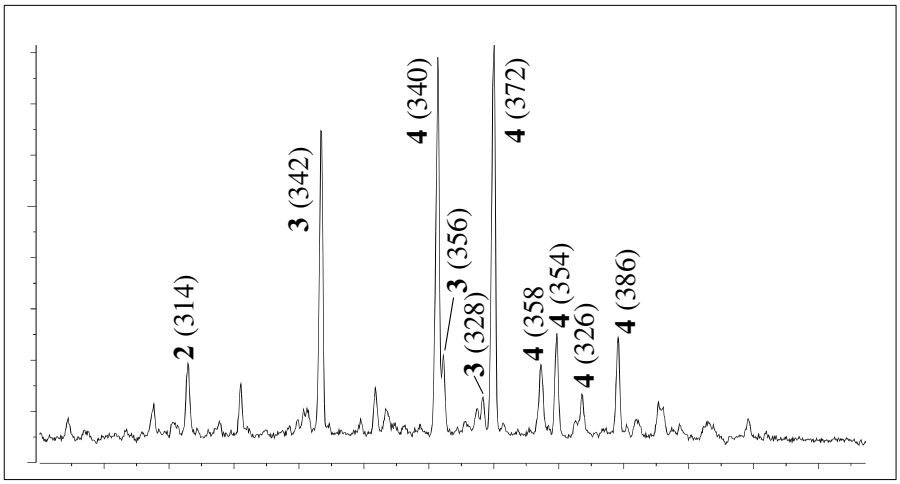


Fig 8. Py-TMAH-GCMS spectrum of an old varnish from "The Girl with the Pearl Earring" *by Vermeer. The peaks associated to the four IDOX compounds have been marked (as well as their molecular masses, see Chapter 1). The IDOX has been calculated as 0.93.*

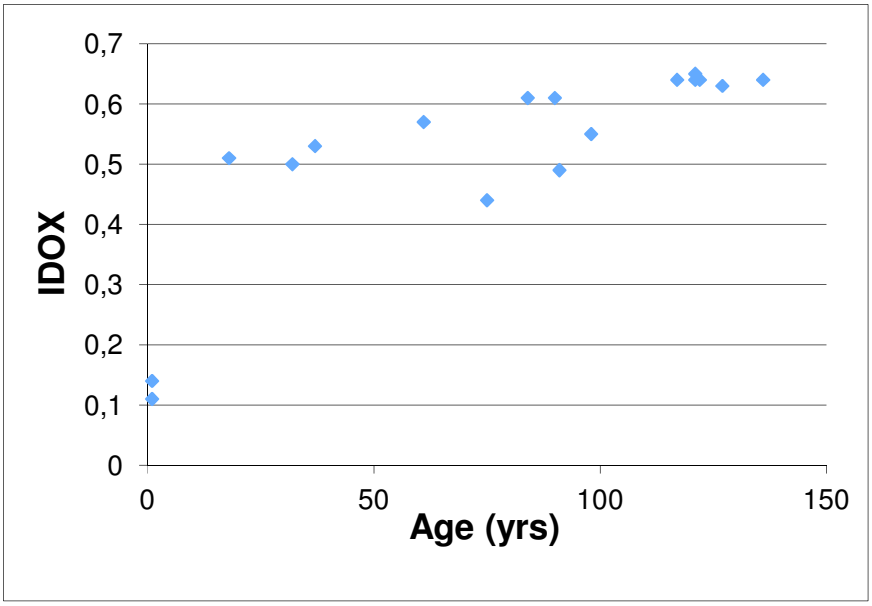


Fig 9. IDOX of different wax/resin lining samples as measured with Py-TMAH-GCMS.

The calculated IDOX of the 17 wax/resin samples are given in Fig. 9. The results show that, despite some clearly deviating results, the degree of oxidation measured with Py-TMAH-GCMS seems to be fairly dependent on age. An additional sample which was not included because of its slightly uncertain age (some 15-20 years) would fit in very nicely (IDOX=0.41). The IDOX is found to rise from a low degree (0.15) after application to 0.5 in 20-30 years. It then levels off to 0.65-0.70 in samples of more than 100 years old.

Two samples that deviate from the curve are the 76 year-old Mauritshuis sample (IDOX 0.44) and the 92 year-old Rijksmuseum sample (IDOX 0.49), which both show relatively low IDOX values. It is difficult to explain these relatively large deviations. The low oxidation states may be related to a relatively high content of

resin for both samples; it is known, for example, that pure lumps of colophony can remain in a very low degree of oxidation for a very long time. Only after mixing with other compounds such as wax or oil, oxygen uptake is facilitated, starting more pronounced oxidation.

DTMS analyses were carried out on the same samples. As expected (see the discussion earlier in this section), the IDOX values were found to be generally lower than those obtained from Py-TMAH-GCMS analysis. Moreover, no relation with age was found. This means that DTMS is indeed not reliable for the examination of IDOX.

The clear dependence of the IDOX on age and less on the relative amount of wax and resin and other (unknown) variations from Py-TMAH-GCMS is encouraging. However, there still is some variation in the results which will make it still impossible to assign a definite age to a sample of unknown age. In addition, the apparent asymptotic behaviour at ages >100 years is difficult to study since there are no wax/resin linings known of an earlier date than 1861.

An interesting application of IDOX, however, is one particular dating problem. A painting in the Oranjezaal ("*The Education of Frederick Henry*" (1648) by T. van Thulden) had a lining which could only have been carried out, according to the relatively well-kept restoration records, either 50 or 190 years ago [te Marvelde, 1999]. The IDOX of this lining was measured to be 0.64. Although no definitive answer could be given, it appears from the curve in Fig. 9 that the date of 1810 is more likely, meaning that this case is the earliest known use of wax-resin in a lining process.

Conclusions

Both reversed-phase HPLC-APCI-MS and DTMS are methods well suited to analyse abietic acid containing resins in different stages of oxidation.

Reversed-phase HPLC analysis of colophony separates oxidised abietane diterpenoid acids and DHA from a non-resolved mixture of abietic and pimaric acids. APCI-MS positive ion data of compounds in colophony and aged varnish confirm earlier data obtained by thermally assisted methylation GCMS (Py-TMAH-GCMS). Negative ion APCI-MS detects a number of unidentified oxidised species, which are thought to be hydroxy- and peroxy- DHA derivatives. These compounds were not detected in aged varnish samples. Two non-identified main compounds in aged varnish (molecular weight unknown, possibly M = 288 and 302) can be observed in the positive but not in the negative APCI-MS mode. Positive APCI-MSMS data on DHA and its oxidised isomers reveal the presence of a characteristic fragment ion (loss of 128 a.m.u.), which is proposed to have a protonated dicyclic structure.

In addition, the high relative amounts of **4**, particularly in the aged varnish, would indicate that this compound is very stable and could represent an end member.

DTMS is a fast and elegant method to obtain a fingerprint of very small and complex samples from (for example) Old Master paintings. The method can detect relatively low amounts in *Pinaceae* diterpenoid resins.

The degree of ageing is quantified by the Index for the Degree of Oxidation (IDOX). The IDOX of a resinous fraction is determined from the relative amounts of the four most prominent molecules in oxidation stages **A**, **B**, **D** and **E**. Derivatisation of the highly oxidised compounds for GCMS with BSTFA and TMAH (on-line) in combination with GCMS is most suitable. On-line derivatisation with TMAH is most

suitable for complex (heterogeneous) microgram samples from *i.e.* paintings since no sample preparation is needed and no sample is lost.

Typical values for wax-resin lining adhesive, which were aged in the dark, are up to 0.7 for >100 year-old samples. Varnishes are generally more oxidised with values found up to 0.93. Light and time are the major driving forces in the development of IDOX.

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3. Molecular characterisation of copaiba balsam as used in painting techniques and restoration procedures*

Abstract

An overview of the use of copaiba balsams in paint media and in restoration practice is given. Twenty-eight samples of copaiba balsam, which were obtained from pharmacies, artist materials suppliers, various museums and archives, were analysed by Gas Chromatography Mass Spectrometry (GCMS) in order to identify their sesquiterpene and diterpenoid constituents. Two types of copaiba balsam could be distinguished, which probably originate from Copaifera langsdorfii *L. and* Copaifera multijuga *Hayne. In particular, the "historical" samples, which were taken from old remnants of copaiba balsam provided by museums and archives, have been produced by* C. langsdorfii *L., whereas the samples, which are still commercially available, probably originate from* C. multijuga *Hayne. Moreover, the analyses of the twenty-eight samples of copaiba balsam indicated that almost all samples had been adulterated with a Pinus balsam.*

Copaiba balsam is not stable and changes by evaporation, oxidation and polymerisation. Natural ageing tests showed the complete evaporation of the sesquiterpenes and the oxidation of some diterpenoid acids but also revealed various stable diterpenoid acids, which were used as marker molecules for the presence of copaiba balsam. This is illustrated by the analytical results of the samples of three paintings: "The Girl with the Pearl Earring" *by J. Vermeer (Mauritshuis, The Hague),* "Unknown Lady with a Carnation" *by F. Bol (Staatlichen Museen, Kassel) and* "Farming Village at Twilight" *by V. van Gogh (Rijksmuseum, Amsterdam).*

* This chapter was mainly written by dr. Inez van der Werf.

Introduction

Copaiba balsam is a natural resin produced by trees of the genus *Copaifera* [Mills and White, 1994]. Since the seventeenth century the balsam has been imported from South America mostly for medical purposes in Europe. In the nineteenth century copaiba balsam has been utilised as an additive to oil paint and for restoration, such as the "Pettenkofer regeneration treatment". More recently copaiba balsam is also used in cosmetics and for the production of biofuel [Calvin, 1983]. Since the balsam is considered to have negative effects on paintings [Schmitt, 1990a], a reliable and sensitive analysis method is required to assess the presence of the balsam and to investigate its possible influence.

Botanical sources of copaiba balsam

Trees of the *Leguminosae* are noted for copious resin production. In particular, within the subfamily of *Caesalpinioideae*, the genera belonging to the *Detarieae* tribe produce various resins. Copaiba balsam, a mixture of diterpenoids and sesquiterpenes, is secreted by various species of the genus *Copaifera*. Thirty species of *Copaifera* are recognised to occur in South America, especially in Brazil, Venezuela and Colombia, and four in Africa [Langenheim, 1973]. A relatively recent amber (fossil resin) from north-eastern Angola has been attributed to *Copaifera braedii* [Langenheim, 1969].

In other than botanical and chemical literature the different types of copaiba balsam are indicated by names that refer to the place of origin. Parabalsam, from the state of Pará in Brazil, and Maracaibo balsam, collected in Venezuela, are mentioned most frequently [von Eibner, 1908; Jantsch, 1939; Dieterich, 1930; Doerner, 1949]. For artistic and restoration purposes the fluidity, which corresponds to the content of essential oils, is an important factor. Parabalsam is the less viscous variety, while Maracaibo balsam and Angostura balsam are much thicker [Doerner, 1949].

Copaiba balsam as a constituent of paint

By addition of copaiba balsam to their paint media artists intended to achieve different purposes. The inhibitory effect of the balsam on the drying of oil paint, which permitted a longer working process, was already known at the beginning of the nineteenth century [Jantsch, 1939]. Painters like Van Gogh added the balsam to obtain deep and saturated colours [Hummelen and Peres, 1993]. Furthermore, the presence of copaiba balsam in the paint was meant to allow high impasto painting without the formation of tears, wrinkles and premature cracks [von Eibner, 1928].

In an outline on terpenoid varnishes and balsams by White, the earliest evidence of the use of copaiba balsam for paint media and varnishes is reported [White, 1986]. The British painter Sir Joshua Reynolds (1723-1792), for example, used the balsam in different medium and varnish compositions, especially for glazes. In Germany the addition of copaiba balsam to paint media goes back to the beginning of the nineteenth century [Jantsch, 1939, Schiessl, 1987]. In literature recipes for mixtures of wax and copaiba balsam [Knirim, 1839] as well as the recommendation of using the more fluid Parabalsam on an absorbent glue/chalk ground can be found [Urban, 1939].

In his "Nuenen" period (1884-1885) Van Gogh used to add copaiba balsam to his paint to prevent the sinking in of dark colours. This has been documented in a letter written by Kersemakers, a pupil and friend of Van Gogh [Kersemakers, 1912]. According to this letter Van Gogh had asked the local pharmacist, supplier of the balsam, if copaiba balsam could be diluted in turpentine.

Summarising, the balsam must have been mixed with oil paint by several painters themselves, but there are accounts of the use of copaiba balsam in the production of commercial paint as well, especially in Germany [von Eibner, 1908; Schiessl, 1987; Schmidt, 1936]. In the Roberson archive a recipe from 1928 reports "Zinc white in balsam of Copaiba 3 Tubes" [HKI MS. 797-1993 folio 113v]. addition, the Dutch firm Talens seems to have been producing a medium consisting of linseed oil and copaiba balsam, according to a catalogue of 1933 and an article on painting materials of 1928 [Talens, 1928]. Moreover, the balsam was available separately as a drying-retardant [Schiessl, 1987].

Copaiba balsam in restoration practice

The use of copaiba balsam in restoration practice is primarily confined to the removal of old varnishes. For example, conservators intended to mitigate the solvent action of alcohol by adding some balsam. Other "mild" cleaning mixtures were made by forming a soap of copaiba balsam and ammonia [von Pettenkofer, 1888; Martin, 1921]. Furthermore the balsam was applied to the surface of oil paintings, eventually followed or preceded by the exposure to alcohol vapours. This procedure is known as one of the variants of the "Pettenkofer treatment". It was used in order to regenerate blanched varnishes and/or saturate turbid oil paint films. An extensive study of the "Pettenkofer method", its use, history and its effects on paintings has been carried out by Schmitt [Schmitt, 1990b]. Max von Pettenkofer patented the additional use of copaiba balsam in 1867 but, during the last century, copaiba balsam had already been in use for some decades in Germany, where it became of common practice until at least 1940. The "Pettenkofer method" has been applied in other European countries as well, especially at the end of the nineteenth and in the beginning of this century.

Another restoration method involving the balsam, is the impregnation of the reverse of canvas or panel paintings with the intention to create a barrier against humidity [Doerner, 1949]. In lining procedures copaiba balsam has occasionally been added to the lining adhesive in order to improve the flexibility and adhesion. The "Dutch Method", for example, was reported by Tudor-Hart to consist of the use of a lining mixture composed of resin and wax dissolved in Canada balsam or copaiba balsam [Tudor-Hart, 1931]. These mixtures, however, were not recommended because "… the presence of gum resins and balsams in any quantity is regarded as being potentially deleterious to the paint on account of the possible solvent action of their essential oils" [Plenderleith and Cursiter, 1934]. Furthermore, the balsam has been applied for the consolidation of paint flakes and blisters since it was considered to soften the paint film and sticks it back to the surface [Kainzlbauer, 1910], and as a medium for retouching.

The extent of the use of copaiba balsam in current restoration practice is not very clear. Restoration treatments involving the balsam, such as the "Pettenkofer method", may still be in use [Seddon, 1982] and in some commercially available picture cleaners, for example, the balsam was found to be present (see below).

Possible effects of copaiba balsam on paintings

The negative effects of copaiba balsam on paintings were already known when the balsam came into wider use at the turn of this century [Schiessl, 1987]. Already in 1845 Fernbach is very conscious of the inhibitory effect of copaiba balsam on the drying process of oil paint [according to Jantsch, 1939]. Doerner [Doerner, 1949] warns against the addition of copaiba to paint media, because of darkening and softening effects, but recommends the use of the balsam for several restoration purposes. In 1939 the German restorer Jantsch writes [Jantsch, 1939]:

> *[Regarding] the process of softening the "linoxynfilm" [i.e. fully dried oil paint], [I wonder] if copaivabalsam will act somehow chemically with the swollen "linoxyn", it is outside my knowledge. I do doubt it. I suppose it [the product of copaiva and linoxyn] to be a (purely physical) mixture. But it would be of highest interest, if chemists would like to take up this problem.* [*]

When used in restoration procedures, copaiba balsam is considered to cause softening, swelling and darkening of some paint as well as the migration of compounds. The latter process causes intermingling of the different layers, as has been shown by careful studies of cross-sections [Schmitt, 1990b; Brammer, 1987]. Of main concern to restorers, however, is the extraordinary reaction of the regenerated paint layers to various solvents [Brammer, 1987]. High sensitivity of copaiba balsam-containing paint films against humidity and heat have been recognized already in 1908 by Eibner [von Eibner, 1908] and in the 1930's by Schmidt [Schmidt, 1936]. The use of commonly used solvents, like ethanol, isopropanol, white spirit and others, for cleaning and varnish removal is often not possible in paintings where copaiba balsam is present. For these reasons the determination of the quantity and location of the balsam can play an important role in the preliminary phase of a restoration. In addition, information regarding painting techniques and previous restoration treatments can be obtained.

Analytical studies of copaiba balsam

In the beginning of this century the exact composition of the various types of copaiba balsam was not known but simple chemical tests were performed trying to distinguish true copaiba balsam from falsifications like Gurjun- and Segura balsam [von Eibner, 1908, p.348-352]. In 1930, Dieterich [Dieterich, 1930, p. 47] reports "the officinal balsams were falsified with Gurjun balsam, fatty oils (castor oil, olive oil), styrax, colophony, turpentine and paraffin oil, while the other balsams were exchanged or adulterated. Maracaibo balsam was most frequently falsified with the fluid Parabalsam." While earlier Gurjun balsam was often used, at that time African balsam, Hardwickia balsam and Segura balsam were preferred for adulteration [Dieterich, 1930].

Later studies revealed that copaiba balsam consists of a mixture of terpenes, in which the sesquiterpenes act as the solvent for the diterpenoids. The sesquiterpene hydrocarbons of different types of copaiba balsam have been identified by gas

[*] "Ob Kopaivabalsam beim Erweichen der Linoxynschicht mit dem gequollenen Linoxyn eine chemische Verbindung irgendwelcher Art eingeht, entzieht sich meiner Kenntnis, es will mir nicht einleuchten, ich halte es eher für ein Gemenge. Aber es wäre von großem Werte, wenn die Chemiker sich dieses Problems annehmen wollten" (translation by the authors from [Jantsch, 1939])

chromatography and infrared (IR) spectroscopy [Nigam and Levi, 1962 & 1966; Wenninger *et al.,* 1967; Langenheim, 1981; Ferrari *et al.,* 1971]. Several diterpenoid constituents of two types of copaiba balsam, produced by *Copaifera multijuga* Hayne and *Copaifera langsdorfii* L., have been isolated. The identification was performed with nuclear magnetic resonance (NMR) and infra red (IR) spectroscopy and chemical tests [Ferrari *et al.,* 1971; Delle Monache *et al.*, 1969 & 1970; Mahajan and Ferreira, 1971].

Two previous studies report the identification of copaiba balsam in paintings. In both cases the use of the balsam is related to the painting technique. In the first study a sample of a glaze from Reynolds' "*Self-portrait*" (Royal Collection, London) has been analysed by Gas Chromatography Mass Spectrometry (GCMS) [White, 1986]. In addition to the methyl esters of fatty acids from linseed oil and oxidised abietanes, some labdanes were found, though not identified. The abietanes and labdanes represent two different kinds of diterpenoid skeletons. The labdanes were attributed to a *Leguminosae* resin, probably copaiba balsam. A second study concerns "*The Potato Eaters*" (1885) by V. van Gogh (Van Gogh Museum, Amsterdam) in which the presence of copaiba balsam was indicated with infrared spectroscopy and thin layer chromatography [Hummelen and Peres, 1993]. In both studies, however, the assignments are not based on the identification of characteristic molecular constituents of copaiba balsam.

Aim of this study

In order to be able to characterise copaiba balsam as it was used both in the past and at present, twenty-eight samples were analysed by GCMS and by on-line thermally assisted methylation GCMS (Py-TMAH-GCMS). In this paper the discussion is focused on the diterpenoid fraction because the diterpenoid acids are less volatile than the sesquiterpenes. They will be present in larger amounts and are more indicative for the presence of copaiba balsam in paintings. A large number of mass spectra are presented in the Atlas. A more detailed discussion on the identification of the diterpenoid constituents and their mass spectra is given elsewhere [Van der Werf, 1996; Van der Werf *et al*., 1997].

Relatively fresh, viscous samples of copaiba balsam, stored in glass vials, were studied. In addition, some of these samples were exposed to natural ageing conditions for three years in order to determine the stable diterpenoid acids which may act as marker molecules. These marker molecules can indicate the presence of copaiba balsam, as will be illustrated by the analytical results of various samples taken from three paintings: "*The Girl with the Pearl Earring*" by J. Vermeer, "*Unknown Lady with a Carnation*" by F. Bol and "*Farming Village at Twilight*", by V. van Gogh.

Table 1 Samples of commercially available fresh copaiba balsam. Most reference materials were purchased from suppliers of artist materials. Some were obtained from pharmacies.

Label	*Provenance*	*Year purch-ased*	*Type of balsam*	*Purity*[1]
Bals. Copaivae	Pharmacy 'Hedwig'-Markt Ebrach (Germany)	1990	C. langsdorfii L.	+++
Balsamum Copaivae Maracaibo	Pharmacy 'Riemerschmid' - Munich (Germany)	1990	Pinus	- [2]
Balsamo Copaive (Type sample A)	Bizarri - Florence (Italy)	1991	C. multijuga Hayne	+++
Copaivabalsam (Type sample B)	Schachinger - Munich (Germany)	1990	C. langsdorfii L.	pure
Copaivabalsam	Schachinger - Munich (Germany)	1990	C. multijuga Hayne	+
Copaivabalsam dick	Kremer - Aichstetten (Germany)	1990	C. multijuga Hayne	+
Nr. 6210 Copaivabalsam mitteldick	Kremer - Aichstetten (Germany)	1990	C. multijuga Hayne	++
Copaiva Balsam, Ein altbewährtes Mittel für alte Ölgemälde, Reizend. Enthält Terpentinöl	C. Kreul - Frankfurt am Main (Germany)	1991	C. multijuga Hayne	+
Copaiva Balsam	Signum - Gauting (Germany)	1992	C. multijuga Hayne	+
Balsamo di Copaive	Zecchi - Florence (Italy)	1992	C. multijuga Hayne	+
Copaiva balsem	Verfmolen de Kat - Zaanstad (The Netherlands)	1995	C. multijuga Hayne	+++
Copaiv-Balsam 50003, dickflüssiges Weichharz für Restauratoren	Schmincke - Erkrath (Germany) [3]	?	C. multijuga Hayne	+
Copaive Balsam	Schmincke - Erkrath (Germany) [3]	?	C. multijuga Hayne [4]	+

[1] Degree of purity: >75% copaiba balsam (+++), ±50% copaiba balsam (++), <25% copaiba balsam (+).
[2] This balsam has probably been prepared by dissolving colophony in the distillate of copaiba balsam from *C. multijuga* Hayne (see text).
[3] Sample provided by the Netherlands Institute for Cultural Heritage (Amsterdam).
[4] The determination of the type of balsam is uncertain because some of the characteristic compounds (agathic acid **L9**, 3-hydroxy-copaiferic acid **L10**, 3-acetoxy-copaiferic acid **L11**, hydroxy-Hardwickiic acid **C2** and acetoxy-Hardwickiic acid **C3**) were not found.

Table 2 'Historical' samples of copaiba balsam, provided by museums and archives.

Label	*Provenance*	*Est. age*	*Type of balsam*	*Purity*[1]
	Bay. Nat. Museum - Munich (Germany)		C. langsdorfii L.	++
	Staatlichen Museen - Kassel (Germany)		C. multijuga Hayne	++
Copaiba - David G. Jones Pharmacist	Antiquities - Montevideo (Uruguay)		C. multijuga Hayne [2]	+
Balsamum Copaivae Ph. N. V	Rijksmuseum - Amsterdam (The Netherlands)	± 1930	C. langsdorfii L.	++
Resins Apothek Theodor Martius	Doerner Institute - Munich (Germany) [3]	± 1880	C. langsdorfii L.	pure
Balsam Copaivae Maracaibo Ph.G.VI	Dr. Bender & Dr. Hobein - Munich (Germany)		C. langsdorfii L.	+
Lechertier Barbe Ltd Copaiba Balsam	Roberson Archive - Cambridge (Great Britain)		C. langsdorfii L.	pure
Copaiva	Luitwieler - Rotterdam (The Netherlands)	pre 1940	C. langsdorfii L.	++
Copaiva balsem	Hesterman Archive - Amsterdam (The Netherlands)	pre 1940	C. multijuga Hayne	+
	pharmacy - Dresden (Germany)		C. langsdorfii L.	+++
	pharmacy - Dresden (Germany)		C. langsdorfii L.	++
	Studio gallery - Dresden (Germany)		C. langsdorfii L.[4]	+
	Studio gallery - Dresden (Germany)		C. langsdorfii L.[4]	+
	HBK[5] - Dresden (Germany)		C. langsdorfii L.	+++
	HBK[5] - Dresden (Germany)		C. langsdorfii L.	+

[1] Degree of purity: >75% copaiba balsam (+++), ±50% copaiba balsam (++), <25% copaiba balsam (+).
[2] The determination of the type of balsam is uncertain because some characteristic compounds (agathic acid **L9**, 3-hydroxy-copaiferic acid **L10**, 3-acetoxy-copaiferic acid **L11** and hydroxy-Hardwickiic acid **C2**) were not found.
[3] Sample provided by the Netherlands Institute for Cultural Heritage (Amsterdam).
[4] The determination of the type of balsam is uncertain because no polyalthic acid **L6** and few pinifolic acid **L8** were found.
[5] Hochschule für Bildende Künste

Materials and methods

Samples[*]

Two groups of samples of copaiba balsam were studied in this paper. The first group of samples is commercially available and was obtained from pharmacies and suppliers of artist materials (Table 1). The second group consists of samples of 'historical' copaiba balsam, provided by various museums and archives (Table 2). The age of these samples goes back to approximately hundred years ago (sample from the Doerner Institute).

The 'Gemäldegalerie Dahlem' in Berlin provided two samples of picture cleaners (one fresh and one 'old') that are/have been produced both by Winsor & Newton. On the label of the fresh sample the following constituents are reported: copaiba balsam, dipentene (monoterpene), 'pine oil' and ammonia.

The paint samples were taken from "*The Girl with the Pearl Earring*" (Johannes Vermeer, 1665, MH 1687 Mauritshuis, The Hague), "*Unknown Lady with Necklace*" (Ferdinand Bol, 1642 or 1644, GK 238 Staatlichen Museen, Kassel) and "*Farming Village at Twilight*" (Vincent van Gogh, 1884, A3307 Rijksmuseum, Amsterdam).

Sample preparation

Copaiba balsam samples of 0.5-3 mg were methylated with trimethylsilyl-(TMS) diazomethane according to the following procedure [Hashimoto *et al.*, 1981]: the sample was dissolved in benzene (40µl/mg sample), and subsequently methanol (10µl/mg sample) and TMS-diazomethane 2.0 M in hexane (5µl/mg sample) were added. The solution was left at room temperature for 15 minutes. Then, the solvents were evaporated under nitrogen and the sample was dissolved in (200 µl/mg sample) dichloromethane (DCM) with an internal standard (n-hexadecane, 6.5 µl/50 ml). A volume of 1 µl was injected into the Gas Chromatograph (see below).

For the Py-TMAH-GCMS analysis the sample of copaiba balsam (0.5-3 mg) was dissolved in approximately 1 ml of tetrahydrofuran (THF). About 5 µl of this solution were applied to a ferromagnetic probe (Curie point 610°C). The solvent was evaporated *in vacuo*. Subsequently, one or two drops of a 2.5% w/w solution of tetramethyl ammonium hydroxide (TMAH) in water were added and the sample probe was dried *in vacuo*. Then, the ferromagnetic probe was inserted into a glass liner.

Paint samples of typically 5-10µg were homogenised in a mini glass mortar and made into a suspension with a few µl of a 2.5% w/w solution of TMAH in water. An aliquot of the suspension was applied to the analytical filament (Curie point

[*] Samples of copaiba balsam, picture cleaners and/or paint were kindly provided by: Hans Brammer (Staatlichen Museen, Kassel), Karin Groen and Wilma Roelofs (Instituut Collectie Nederland, Amsterdam), Gisela Helmkampf (Gemäldegalerie Dahlem, Berlin), Hélène Kat (Rijksmuseum, Amsterdam), Mireille te Marvelde, Jørgen Wadum (Mauritshuis, The Hague), Annetje Boersma (private conservator, Rotterdam), Christoph Schölzel (Zwinger, Gemäldegalerie, Dresden), Prof. Schramm and Ms. Schramm (Labor des Fachbereichs Restaurierung, Hochschule für Bildene Künste, Dresden), Dr. J. Koller (Doerner Institut, München) and Sally Woodcock (Hamilton Kerr Institute, University of Cambridge) who also retrieved recipes mentioning copaiba balsam in the Roberson Archive.

610°C) and dried *in vacuo*. Subsequently, the sample probe was inserted into a glass liner. The subsequent analytical procedures are described under *Py-GCMS*.

Gas Chromatography Mass Spectrometry (GCMS)

GCMS data were obtained using a fused silica SGE BPX5 column (25 m, 0.32 mm i.d., 0.25 mm film thickness) in a Carlo Erba gas chromatograph (series 8565 HRGC MEGA 2) coupled directly to the ion source of a JEOL DX-303 double focusing (E/B) mass spectrometer. Helium was used as the carrier gas at an initial flow rate of approximately 2 ml/min. The initial temperature of the gas chromatograph was 50° C for 5 minutes. In case of the samples of copaiba balsam the oven temperature was programmed with a ramp of 6° C/min up to 206° C or alternatively with 2° or 4° C/min to 270° C or 290° C. For the paint samples a temperature program with a ramp of 6° C/min to the final temperature of 350° C was used.

Ions were generated by electron ionisation (70 eV) in the ionisation chamber, accelerated to 3 keV, mass separated and postaccelerated to 10 keV before detection. The mass spectrometer was scanned from m/z 40-700, with a cycle time of 1 s. A JEOL MP-7000 data system was used for data acquisition and processing.

Py-GCMS

The sample probe, inserted into a glass liner, was flushed with argon and placed in the pyrolysis chamber (190 ° C) of the FOM-4LX pyrolysis unit. At this temperature the tetramethylammonium salts are decomposed to methylated compounds, which evaporate [Van den Berg *et al.*, 1996] and are cold-trapped in the first part of the column. For additional pyrolysis of the remaining material inductive heating (610 ° C) with an RF field was used during 6 seconds in the Curie-point pyrolyser (FOM 4-LX system) [Van Loon and Boon, 1994]. The pyrolysate was transferred by the carrier gas into the GC column and focused at a temperature of approximately 50° C. GCMS data were obtained as described above.

Identification

The identification of most compounds was performed by comparison of the mass spectra with reference spectra from literature and from the Wiley library of MassLib version 7.3 (Chemical Concepts, Weinheim). Others were assigned on the basis of the fragmentation patterns observed in the mass spectra of various derivatives [Van der Werf, 1996].

Results and discussion

Identification of the constituents of copaiba balsam by GCMS

The GCMS analyses of the twenty-eight samples of copaiba balsam reveal two distinct groups with similar compositions. In this paper the two types of balsam are represented by the typical sample A, labelled "Balsamo Copaive" from Bizarri (Florence) and by sample B, labelled "Copaivabalsam" from Schachinger (Munich).

The main sesquiterpenes in sample A are α-copaene **S3**, trans-caryophyllene **S6**, α-bergamotene **S7**, α-humulene **S8**, β-bisabolene **S11** and δ-cadinene **S12**, while in sample B β-elemene **S4**, α-bergamotene **S7** and β-bisabolene **S11** were most prominent (Figure 1 and Table 3). In previous studies [Nigam and Levi, 1962 & 1966; Wenninger *et al*., 1967; Ferrari *et al*., 1971] of various samples of copaiba balsam, of which the botanical origin is not always specified, the same sesquiterpenes have been isolated in addition to some others.

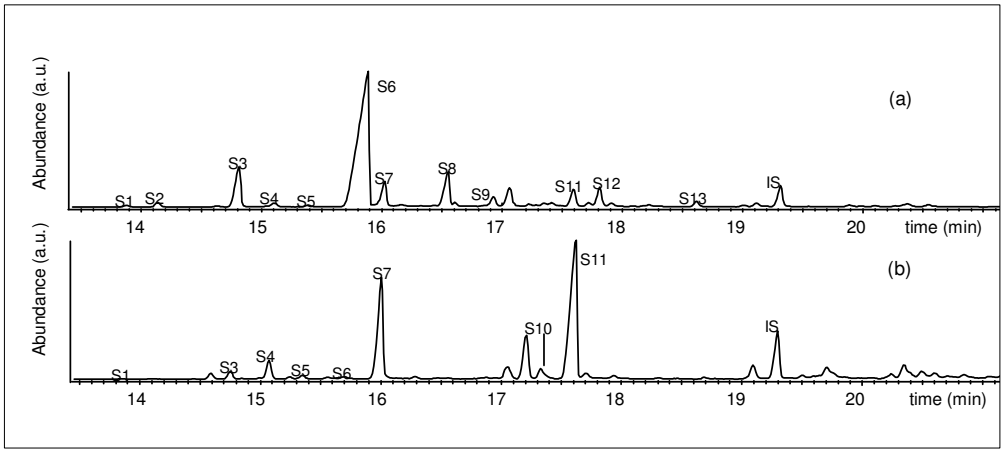

Figure 1 Total Ion Current Chromatograms of the Gas Chromatography Mass Spectrometry analyses of the sesquiterpenoid fraction of (a) sample A and (b) sample B. The compounds indicated by labels are listed in Table 3. IS refers to the internal standard (n-hexadecane).

The GCMS analyses of sample A and B (Figure 2a and 3a, Table 4) reveal that the diterpenoid fractions consist of dicyclic molecules with labdane and clerodane skeletons. Sample B also contains tetracyclic compounds with a kaurane structure (Figure 4). The principal diterpenoid acids are mono- or dicarboxylic acids with additional hydroxyl groups, furanoic rings, extra double bonds and some of their oxidation products. 3-Acetoxy-copaiferic acid **L11**, hydroxy-Hardwickiic acid **C2** and acetoxy-Hardwickiic acid **C3** (Figure 4) have been assigned by interpretation of the mass spectra of various derivatives. The position of the hydroxyl and acetoxy group can not be established in the compounds hydroxy-Hardwickiic acid **C2** and acetoxy-Hardwickiic acid **C3** respectively, but is thought to be at C_7 in both cases [Van der Werf *et al.*, 1996]. In a previous study [Delle Monache *et al.*, 1969] 7-hydroxy-Hardwickiic acid has been isolated and identified in a balsam from *Copaifera multijuga* Hayne.

Table 3 Results of the GCMS analysis of the samples A and B, relative to the sesquiterpenoid fraction[1]. The Total Ion Current Chromatograms are reported in Figure 1. Mass spectra are presented in the Atlas

Component label	*Identification*	*Atlas*
S1	δ-elemene	51
S2	α-cubebene	52
S3	α-copaene	53
S4	β-elemene	54
S5	cyperene	55
S6	trans-caryophyllene	56
S7	α-bergamotene	57
S8	α-humulene	58
S9	γ-cadinene	59
S10	α-selinene	60
S11	β-bisabolene	61
S12	δ-cadinene	62
S13	γ-elemene	63
IS	n-hexadecane (internal standard)	

[1] The identification of all compounds was performed by comparison of the mass spectra with reference spectra from the Wiley library of MassLib version 7.3.

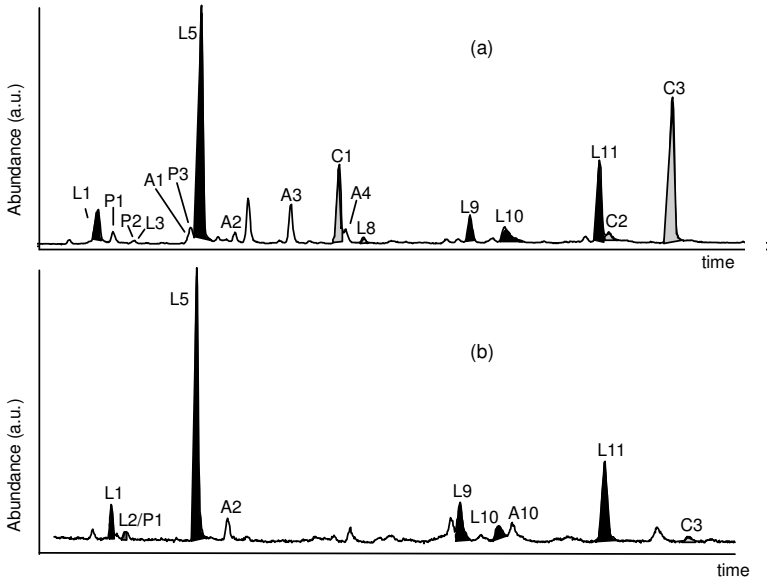

Figure 2 TIC Chromatograms of the GCMS analyses of the diterpenoid fraction of sample A (a) unaged and (b) naturally aged for three years. The compounds indicated by labels are listed in Table 4. The light gray peaks represent the unstable compounds, while the black peaks in-dicate stable marker molecules and ageing products. Trimethylsilyl-diazomethane was used for derivatisation.

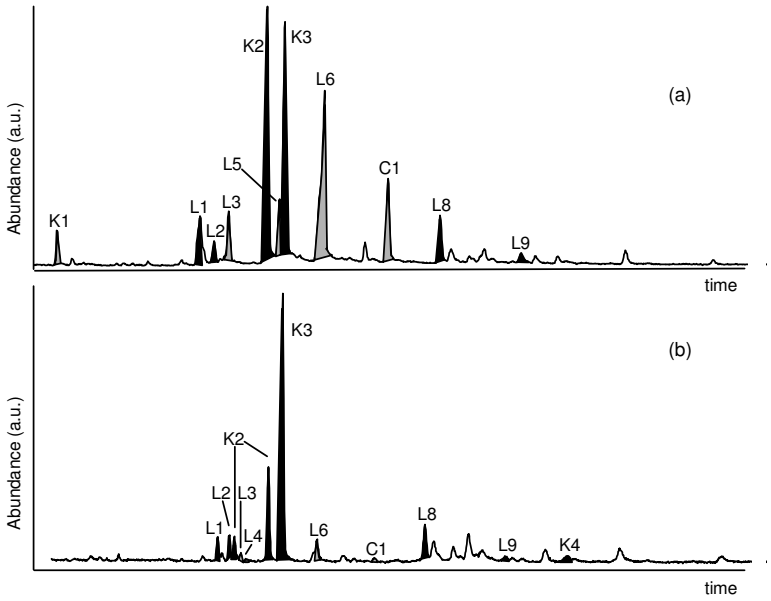

Figure 3 Total Ion Current Chromatograms of the GCMS analyses of the diterpenoid fraction of sample B (a) unaged and (b) naturally aged for three years. The compounds indicated by labels are listed in Table 4. The light gray peaks represent the unstable compounds, while the black peaks indicate stable marker molecules and ageing products. Trimethyl-silyl-diazomethane was used for derivatisation.

Table 4 Identification of the diterpenoids in sample A and B indicated in the Total Ion Current Chromatograms of Figure 2 and 3[1]. Mass spectra are presented in the Atlas

Compound label[2]	*M*	*Identification*	*Atlas*	*Reference spectra*
L1	320	methyl ester of eperuic acid	39	[Enzell and Ryhage, 1965]
L2	322	methyl ester of labdanic acid	40	[Enzell and Ryhage, 1965]
L3	320	methyl ester of cativic acid	41	[Enzell and Ryhage, 1965]
L4	332	unidentified labdane		
L5	318	methyl ester of copalic acid	42	[Sandermann *et al.*, 1967]
L6	330	methyl ester of polyalthic acid	43	[Enzell and Ryhage, 1965]
L8	364	dimethyl ester of pinifolic acid	44	[Enzell and Ryhage, 1965]
L9	362	dimethyl ester of agathic acid	45	[Enzell and Ryhage, 1965]
L10	334	methyl ester of 3-hydroxy-copaiferic acid	46	[Braun and Breitenbach, 1977]
L11	376	methyl ester of 3-acetoxy-copaiferic acid	47	[Van der Werf, 1996].
C1	330	methyl ester of Hardwickiic acid	32	[Misra *et al*. 1979]
C2	346	methyl ester of hydroxy-Hardwickiic acid	33	[Van der Werf, 1996].
C3	388	methyl ester of acetoxy-Hardwickiic acid	34	[Van der Werf, 1996].
K1	272	(-)-16-kaurene	35	[Kalinovskii *et al.*, 1971]
K2	316	methyl ester of kaur-16-en-19-oic acid	36	[Kalinovskii *et al.*, 1971]
K3	318	methyl ester of 16β-kauran-19-oic acid	37	Wiley Library of MassLib version 7.3
K4	360	unidentified kaurane	38	
P1 #	316	methyl ester of pimaric acid	48	[Anderson and Winans, 1991; Challinor, 1993]
P2 #	316	methyl ester of sandaracopimaric acid	49	[Anderson and Winans, 1991; Challinor, 1993]
P3 #	316	methyl ester of isopimaric acid	50	[Anderson and Winans, 1991; Challinor, 1993]
A1 #	316	methyl ester of palustric acid	30	[Anderson and Winans, 1991; Challinor, 1993]
A2 #	314	methyl ester of dehydroabietic acid	3	[Anderson and Winans, 1991; Challinor, 1993]
A3 #	316	methyl ester of abietic acid	1	[Anderson and Winans, 1991; Challinor, 1993]
A4 #	316	methyl ester of neoabietic acid	31	[Challinor, 1993]

[1] The carboxylic groups were methylated with trimethylsilyl-diazomethane.

[2] The compounds supposedly due to adulteration are marked with #.

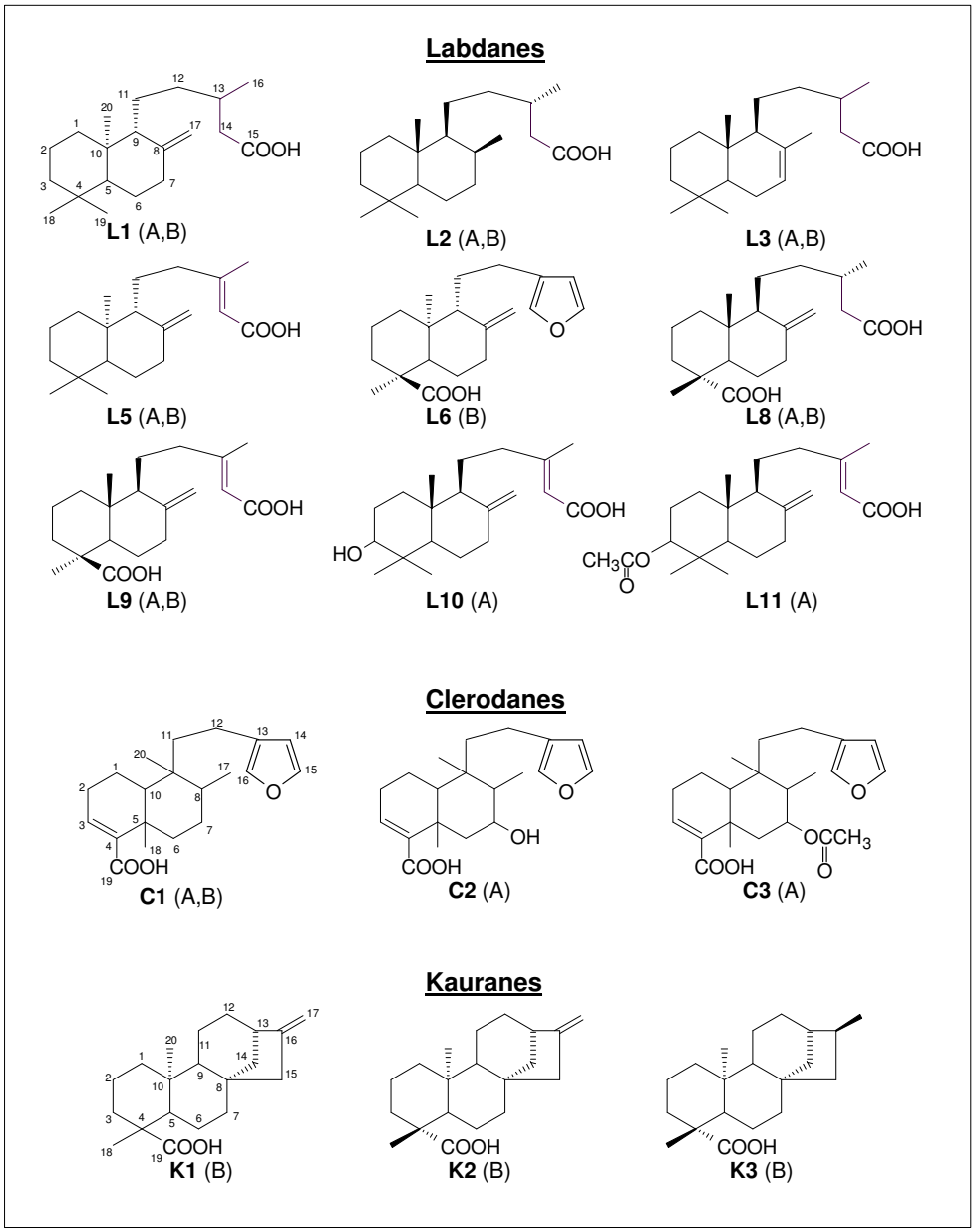

Figure 4 Structures of the diterpenoids identified in sample A and sample B. The compounds are listed in Table 4.

Almost all diterpenoid acids present in copaiba balsam have been isolated from resins or balsams produced by other trees than *Copaifera*, such as for example *Hymenaea*. Nevertheless, the combination of certain diterpenoid acids seems to be characteristic for the *Copaifera* resins. Type sample A contains many diterpenoid acids similar to those found in a balsam produced by *C. multijuga* Hayne. Diterpenoids like eperuic acid **L1**, pinifolic acid **L8**, agathic acid **L9**, 3-acetoxy-copaiferic acid **L11** and acetoxy-Hardwickiic acid **C3**, could be identified [Delle Monache *et al*., 1970]. However, copaiferolic acid and 11-hydroxy-labd-8(20),13-dien-15-oic acid, also reported to be constituents of the balsam from *C. multijuga* Hayne [Delle Monache *et al*., 1970], are not found in sample A.

The compounds found in type sample B are identical to those isolated from a sample of balsam produced by *C. langsdorfii* L. [Ferrari *et al*., 1971]. In addition, however, the following diterpenoid acids were identified: (-)-16-kaurene **K1**, eperuic

acid **L1**, labdanic acid **L2**, cativic acid **L3**, Hardwickiic acid **C1** and agathic acid **L9**. The latter compounds are present in small amounts.

Adulteration

With exception of sample B, the samples from the Roberson Archive and from the Doerner Institute, all samples were found to be adulterated with other balsams (Table 1 and 2). Type sample A contains only a small amount of added balsam. The compounds related to the adulteration are marked in Table 4. A pimaric/sandaracopimaric acid ratio larger than two indicates that a *Pinus* balsam is involved rather than e.g. an *Abies*, *Picea* or *Larix* balsam [Mills and White, 1994; Van den Berg *et al*., 1996]. *Pinus* balsam is composed of a very volatile fraction, the monoterpene hydrocarbons, as well as of colophony, which consists of acids with abietane and pimarane skeletons. Due to the sample preparation methodology, the major part of the monoterpenes was lost and could not be detected. A rough estimation of the amount of *Pinus* balsam added is therefore based on the diterpenoids alone. The degree of adulteration varies consistently between the samples, as indicated in Table 1 and 2. The apparent viscosity of the samples is not influenced by the degree of adulteration but might be related to the quantity of monoterpenes present. Some visual correlation, however, was found between the sample colour, varying from light yellow to dark brown, and the amount of added *Pinus* balsam. The yellow samples all contain about 25% or less copaiba balsam, while in the brown ones 70% or more copaiba balsam was found. The most likely reason for the adulteration is that *Pinus* balsam is much cheaper than copaiba balsam.

One sample, labelled "Balsamum Copaivae Maracaibo" from a pharmacy in Munich (Table 2), only contains diterpenoid acids with pimarane and abietane skeletons. However, large amounts of α-copaene **S3**, trans-caryophyllene **S6** and α-bergamotene **S7**, which represent the major constituents of the sesquiterpenoid fraction of sample A, are also present. The sample involved might have been prepared by mixing oil of copaiba, that is the distillate of copaiba balsam, with colophony. Dieterich [Dieterich, 1930, p. 57], has described the use of colophony to increase the viscosity.

Analysis of copaiba balsam by microscale Py-TMAH-GCMS

The analytical procedure for fresh and aged balsam used above is too elaborate for the analysis of painting samples suspected of copaiba balsam additions. Painting samples are analysed in our laboratory by an online chemical derivatisation method which involves direct desorption from a heatable sample probe (Py-TMAH-GCMS). Py-TMAH-GCMS enables the direct analysis and identification of oil, wax and resin compounds in microgram samples, thus avoiding extraction and isolation steps. The sensitivity is high since the whole sample is placed onto the ferromagnetic filament and will be analysed, in contrast to many off-line methods in which only part of the sample can be studied. Comparative studies were undertaken to examine the chemical stability of the various diterpenoid acids in copaiba balsam under these analytical conditions. Although the presence of certain diterpenoid acid methyl esters can easily be confirmed by Py-TMAH-GCMS, the data and the chromatograms may become somewhat more complex compared to those obtained after off-line chemical derivatisation methods. The increased number of peaks is due to the fact that some

heat- and alkali-sensitive compounds can isomerise or degrade under conditions of high alkalinity (due to the reagent) and the temperatures that may be employed [Kossa *et al*., 1979; Ding *et al*., 1997]. It is known that some oxidised diterpenoid acids may convert into more than one derivative as a result of the thermally assisted methylation with TMAH [Anderson and Winans, 1991]. Dehydration may take place [Pastorova *et al*., 1997] and in particular, several methylation products of the oxidised abietanes have been found; the enol tautomer of methyl 7-oxo-dehydroabietate is methylated by TMAH and additional methyl groups can be incorporated at C_5 [Van den Berg *et al*., 1996; Pastorova *et al*., 1997].

The Py-TMAH-GCMS analysis of samples of copaiba balsam revealed the isomerisation of kaur-16-en-19-oic acid **K2**, copalic acid **L5**, 3-hydroxy-copaiferic acid **L10**, 3-acetoxy-copaiferic acid **L11,** agathic acid **L9**, Hardwickiic acid **C1** and hydroxy-Hardwickiic acid **C2** [Van der Werf *et al*., unpublished results]. Thus, diterpenoid acids with double bonds conjugated with the carboxylic group tend to isomerise upon treatment with TMAH.

In spite of these complications, Py-TMAH-GCMS is a very sensitive microscale technique for the simultaneous analysis of a wide range of substances and is very useful for the identification of copaiba balsam in paint samples.

Picture cleaners

Picture cleaners have been used and are still in use for ‘cleaning’ the varnishes instead of removing them. They are mixed either individually by hand or produced commercially and most of them contain an additive of copaiba balsam. In the sample vials of both the ‘old’ and fresh commercial Winsor & Newton picture cleaners studied for this paper a phase separation takes place and two layers are formed with time. The bottom layer consists of a yellow liquid while the top layer is brown and more viscous. All layers were analysed by Py-TMAH-GCMS.

The composition of the diterpenoid fraction of the top and bottom layer of the ‘new’ sample is comparable. In addition to some unidentified diterpenoids, the methyl esters of the compounds eperuic acid **L1**, labdanic acid **L2**, cativic acid **L3**, copalic acid **L5**, pinifolic acid **L8**, agathic acid **L9**, Hardwickiic acid **C1** and acetoxy-Hardwickiic acid **C3** were found, pointing to a copaiba balsam similar to type sample A. Furthermore, the methyl esters of 3-methoxy-copaiferic acid and methoxy-Hardwickiic acid were identified. Since both hydroxy and acetoxy groups react with TMAH to form a methoxy group, no distinction can be made between 3-hydroxy-copaiferic acid **L10** and 3-acetoxy-copaiferic acid **L11** and between hydroxy-Hardwickiic acid **C2** and acetoxy-Hardwickiic acid **C3** by performing a Py-TMAH-GCMS analysis. In the top layer various sesquiterpenes are present, whereas in the bottom layer only some of them were found. The sesquiterpene hydrocarbons that were identified in the top layer are α-cubebene **S2**, α-copaene **S3**, δ-selinene, calamenene and δ-cadinene **S12**. It is rather puzzling that these sesquiterpenes do not correspond qualitatively nor quantitatively to those found in sample A.

The top layer of the ‘old’ sample is composed of *Pinus* diterpenoids, the labdanes eperuic acid **L1**, copalic acid **L5** and pinifolic acid **L8** and several sesquiterpenes of which only α-bergamotene **S7** and curcumene could be identified. In the bottom layer the same diterpenoids as in the top layer were found, in addition to large amounts of unidentified polar monoterpenes. In this picture cleaner the layer separation is probably due to the incompatibility between a polar phase, mainly

consisting of functionalised monoterpenes, and an apolar phase of sesquiterpene hydrocarbons. The diterpenoid acids are soluble in both phases. Considering the small amount of labdanes and characteristic sesquiterpenes it was not possible to assign the botanical origin of the copaiba balsam present in this picture cleaner.

Ageing of copaiba balsam

Regarding the presence of copaiba balsam in paint samples one must take into account that ageing often results in the loss of some characteristic compounds by processes of evaporation, oxidation and polymerisation during the lifetime of the work. These processes may also occur inside the tree and once the balsam is collected and stored. For example, diterpenoid acids with a diene-containing side chain are known to polymerise [Anderson, 1995; Clifford and Hatcher, 1995]. Other diterpenoids and their oxidation products will remain unchanged and may serve as taxonomic markers [Langenheim, 1981]. Here only the non-polymeric fraction is taken into account.

In order to study the effect of natural ageing on both types of copaiba balsam, a thin layer of balsam was applied on a glass plate and aged by exposure to moderate light and ambient air for three years. Most sesquiterpenes were found to have disappeared in the aged samples, probably by evaporation or polymerisation. In the Figures 2 and 3 the diterpenoid fractions of the fresh samples are compared with those of the samples after natural ageing.

The more stable compounds of sample A are eperuic acid **L1**, copalic acid **L5**, agathic acid **L9** and 3-acetoxy-copaiferic acid **L11**. The clerodanes Hardwickiic acid **C1** and acetoxy-Hardwickiic acid **C3**, which are present in large amounts in the fresh sample, are found to be unstable (Figure 2). From literature it is known that oxidation of clerodanes with a Δ^3 double bond can produce an epoxy at C_3 -C_4 [Anthonsen and Bergland, 1971; Henderson and Murray, 1973; Kusumoto *et al*., 1969], a keto group at C_2 [Anthonsen, *et al.*, 1973] or an extra double bond at C_1 -C_2 [Pandey *et al.*, 1984]. When a furan ring is present in the side chain, as in Hardwickiic acid **C1**, hydroxy-Hardwickiic acid **C2** and acetoxy-Hardwickiic acid **C3**, this can also be oxidised to a lactone [Anthonsen and Bergland, 1971; Zdero *et al*., 1991; Zhao *et al*., 1991]. However, in the Total Ion Current (TIC) Chromatogram of the aged sample A (Figure 2b) no peaks that may be related to clerodanes with extra oxygen atoms or extra double bonds appear. The ageing of the *Pinus* constituents of the balsam was found to result in the oxidation of the abietanes to dehydroabietic acid and 7-oxo-dehydroabietic acid [Mills and White, 1994; Van den Berg *et al*., 1996].

In sample B the compounds labdanic acid **L2**, 16β-kauran-19-oic acid **K3** and pinifolic acid **L8** are relatively stable upon natural ageing (Figure 3). The inert character of these compounds becomes evident from their structures. No double bonds or other reactive functional groups are present. Kaur-16-en-19-oic acid **K2** is still found after three years of ageing, but seems to have partially been transformed into an isomer. The ageing product indicated by **K4** (Figure 3b) has a Molecular Weight (MW) of 360 and has not been identified so far. The mass spectrum points to a kaurane structure. The compound labelled **L4** was not found in the fresh sample B, but might have been 'hidden' under the peak of cativic acid. The mass spectrum suggests a labdane structure, a MW of 322 and a carboxylic group at C_4.

"The Girl with The Pearl Earring", J. Vermeer (1665)

This 17th-century Dutch canvas painting has been restored several times in the past. It was documented in the archives of the Mauritshuis, The Hague that restorer D. de Wild regenerated the varnish in 1915 and 1922 [Wadum *et al*., 1995]. No copaiba balsam was mentioned, but the balsam, eventually in combination with alcohol vapours, was often used for the regeneration of blanched varnishes.

Several paint and varnish samples were analysed by Py-TMAH-GCMS and copaiba balsam was found in most of them [Groen *et al.,* 1998]. In Figure 5a the partial TIC of the analysis of a sample of the dark background, including the ground- and paint layers and some varnish, is reported. The diterpenoid acids present are oxidised abietanes (as a result of the thermally assisted methylation with TMAH several methylation products of the oxidised abietanes were found) and relatively large amounts of the methyl esters of labdanic acid **L2**, two isomers of kaur-16-en-19-oic acid **K2**, 16β-kauran-19-oic acid **K3** and pinifolic acid **L8** (Table 5) in addition to **K4**. The latter compound, possibly a kaurane, has already been mentioned as a typical ageing product in sample B. In a sample of the wax-resin lining mixture no copaiba balsam was found. Therefore, copaiba balsam of a similar type of balsam as sample B has probably been used for the regeneration of the varnish.

"Unknown Lady with a Carnation", F. Bol (1642 or 1644)

This painting belongs to the collection of the Gallery of Old Masters of the Staatlichen Museen in Kassel (Germany). From the archives of the museum it is known that a large number of paintings of its collection were submitted to regeneration treatments several times between 1880 to 1950. In 1929 Doerner visited the Gallery and recommended the application of copaiba balsam for regeneration treatments. The different layers of the painting "*Unknown Lady with Necklace*" have been studied by microscopy [Brammer, 1987]. Cross sections clearly show intermingling and swelling of the layers; phenomena that point to regeneration treatment, most probably alternating application of copaiba balsam and alcohol vapours.

Some samples of this painting were analysed by Py-TMAH-GCMS. A partial TIC of a sample containing the various varnish layers is reported in Figure 5b. The TIC clearly shows peaks corresponding to methyl esters of kaur-16-en-19-oic acid **K2**, 16β-kauran-19-oic acid **K3** and pinifolic acid **L8** in addition to several oxidised abietanes (Table 5). Various side reaction products of the thermally assisted methylation of the oxidised abietanes were identified.

The amount of copaiba balsam is very small and relates again to the ageing pattern of sample B. In the samples of the paint- and ground layer the presence of copaiba balsam was not confirmed so far but investigations are continued.

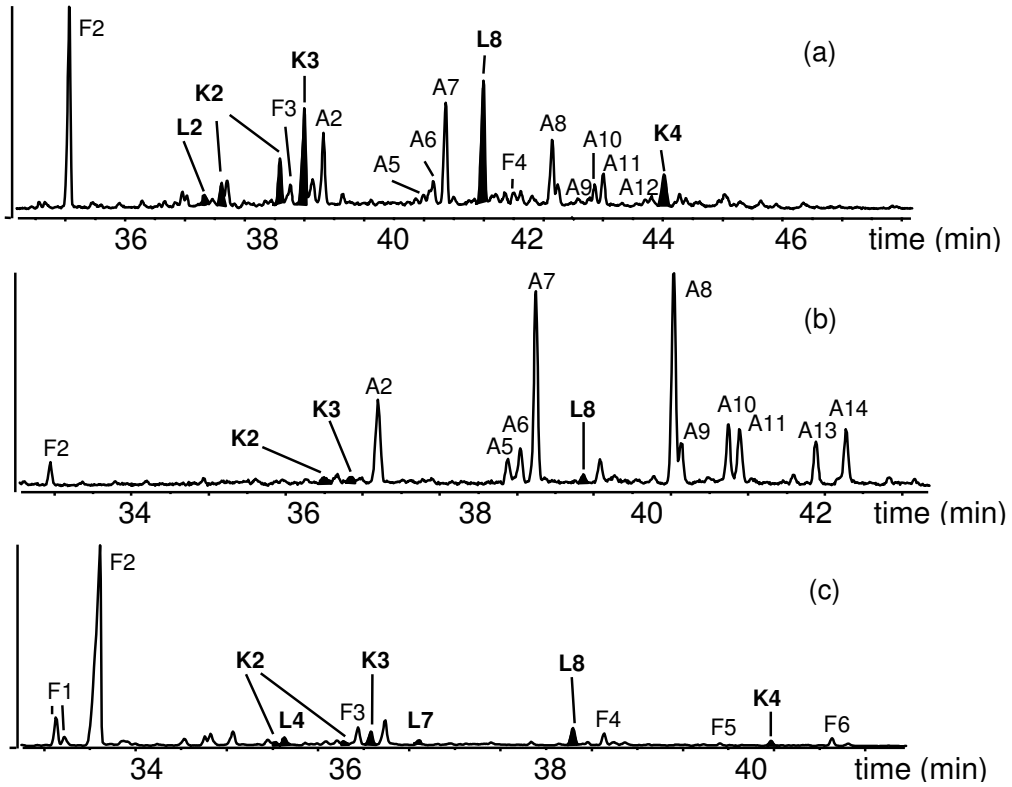

Figure 5 Total Ion Current Chromatograms of the in situ thermally assisted methylation Gas Chromatography Mass Spectrometry (Py-TMAH-GCMS) analysis of (a) a paint sample taken from the background of "The Girl with The Pearl Earring", by J. Vermeer (Mauritshuis, The Hague), including ground and paint layers with some varnish, (b) a varnish sample from the left edge of "Unknown Lady with Necklace", by F. Bol (Staatlichen Museen, Kassel) and (c) a paint sample taken from the left edge of "Farming Village at Twilight", by V. van Gogh (Rijksmuseum, Amsterdam). The compounds indicated are listed in Table 5.

"Farming Village at Twilight", V. van Gogh (1884)

This painting belongs to Vincent van Gogh's so called "Nuenen period" (1884-1885). At that time Van Gogh worked in Nuenen in The Netherlands, where he also painted "*The Potato Eaters*". "*Farming Village at Twilight*" (Rijksmuseum, Amsterdam) is a complex painting because the actual picture has been executed on top of another one, which represents a basket of potatoes. This 'potato' study has probably been painted in the same period.

The results of the Py-TMAH-GCMS analysis of the paint layers from the left edge of the painting are reported in Figure 5c and Table 5. The diterpenoids present, that is the methyl esters of the two isomers of kaur-16-en-19-oic acid **K2**, pinifolic acid **L8** and the compounds labelled **K4** and **L4**, coincide with those of the naturally aged sample B. In addition another unidentified labdane, indicated as **L7** (MW 336), was found. The mass spectrum of this compound is very similar to that of compound **L4** and points to a carboxylic group at C_4. In the aged sample B only a small peak for **L4** was found, while in the sample from the Doerner Institute, which is about a hundred years old, both labdanes **L4** and **L7** are present.

The amount of characteristic diterpenoids is small compared with that of the methyl ester of stearic acid. This fatty acid belongs to the binding medium, most likely consisting of linseed oil, as concluded from the P/S ratio of 1.7 [Mills and White 1994]. In addition small amounts of other fatty acids were found (Table 5). There might be several reasons for the presence of such a small amount of copaiba balsam in the paint layers.

In the first place the painter might have added just a small quantity of balsam to his paint. This is, however, in contrast with an observation of his pupil Kersemakers that "At that time, in Copahu balsam, he believed he had found a way of preventing the paint sinking in, which he so hated, but because he was rather lavish in the use of this substance, as well as his paints, he used too much of it with the result that the sky of the painting came floating down (...)". The painting Kersemakers refers to is "*Watermill at Gennep*" (Thyssen collection) [Hummelen and Peres, 1993].

Another explanation might be that, since the balsam was added to fresh oil paint, some diterpenoids might have reacted with the unsaturated fatty acids present. In fresh paint the fatty acids have not formed a cross-linked network yet and reactive double bonds are still available.

Conclusions

For this paper twenty-eight samples of copaiba balsam were analysed and two groups with different chemical compositions, represented by sample A and B, were distinguished. The first group of samples is probably related to C*opaifera multijuga* Hayne, while the samples of the second group might have been produced by *Copaifera langsdorfii* L. This assumption is based on the analytical results of previous studies relative to the diterpenoid fractions of balsams produced by *C. multijuga* Hayne and *C. langsdorfii* L. [Ferrari *et al*., 1971; Delle Monache *et al*., 1969 & 1970; Mahajan and Ferreira, 1971]. Nonetheless, this has still to be confirmed by the GCMS analysis of reference samples of both botanical species obtained directly from the corresponding trees.

The majority of the samples recently purchased from pharmacies and artist materials suppliers was found to have a composition similar to sample A (i.e. *C. multijuga* Hayne). This is also the case for the copaiba balsam present in one of the Winsor & Newton picture cleaners. On the other hand, in the group of 'historical' samples of copaiba balsam, old remnants from museum workshops and archives, there is a preference for a balsam resembling sample B (i.e. *C. langsdorfii* L.). Furthermore, the analytical results of the samples taken from three paintings show the same combination of characteristic diterpenoids, corresponding to those found after three years of natural ageing of sample B.

Summarising, it is likely that balsam B, probably produced by *C. langsdorfii L.*, was more frequently used in the past. This observation seems to be in accordance with an article, entitled "Kopaïvabalsam", from 1893 [Keim, 1893, p.223], where *Copaifera langsdorfii* Desf. is mentioned.

Table 5 Identification of the compounds indicated in the Total Ion Current Chromatograms of Figure 5.7.3.[1] Mass spectra are presented in the Atlas.

Compound label	*M*	*Identification*	*Atlas*
L2	322	methyl ester of labdanic acid	40
L4	322	unidentified labdane	
L7	336	unidentified labdane	
L8	364	dimethyl ester of pinifolic acid	44
K2	316	methyl ester of kaur-16-en-19-oic acid	36
K3	318	methyl ester of 16β-kauran-19-oic acid	37
K4	360	unidentified kaurane	38
A2	314	methyl ester of dehydroabietic acid[2]	3
A5	310	methyl ester of 2,8,11,13,15-abietapentenoic acid	
A6	312	methyl ester of 8,11,13,15-abietatetraenoic acid[2]	
A7	342	methyl ester of 7-oxo-dehydroabietic acid (enol)[2]	7
A8	340	methyl ester of 7-oxo-8,11,13,15-abietatetraenoic acid (enol)[2]	16
A9	356	methyl ester of 5-methyl-7-oxo-dehydroabietic acid (enol)[2]	8
A10	328	methyl ester of 7-oxo-dehydroabietic acid[2]	5
A11	372	methyl ester of 15-methoxy-7-oxo-dehydroabietic acid (enol)[2]	12
A12	358	methyl ester of 15-hydroxy-7-oxo-dehydroabietic acid (enol)[2]	
A13	354	methyl ester of 5-methyl-7-oxo-8,11,13,15-abietatetraenoic acid (enol)[2]	17
A14	326	methyl ester of 7-oxo-8,11,13,15-abietatetraenoic acid[2]	
F1a	296	methyl ester of cis-oleic acid (C18:1)	
F1b	296	methyl ester of trans-oleic acid (C18:1)	
F2	298	methyl ester of stearic acid (C18)	
F3	326	methyl ester of eicosanoic acid (C20)	
F4	354	methyl ester of docosanoic acid (C22)	
F5	368	methyl ester of tricosanoic acid (C23)	
F6	382	methyl ester of tetracosanoic acid (C24)	

[1] The reference mass spectrum was found in the Wiley Library of MassLib version 7.3 (Chemical Concepts, Weinheim)
[2] See Chapter 1.

Natural ageing tests showed that pure films of copaiba balsam change substantially within three years. The sesquiterpenes have completely evaporated or polymerised, but some diterpenoid acids are relatively stable and may indicate the presence of two different types of aged copaiba balsam. Eperuic acid **L1**, labdanic acid **L2**, copalic acid **L5**, agathic acid **L9**, 3-hydroxy-copaiferic acid **L10** and 3-acetoxy-copaiferic acid **L11** can act as marker molecules for type A of copaiba balsam (probably produced by *C. multijuga* Hayne). For type B (probably originating from *C. langsdorfii* L.), eperuic acid **L1**, labdanic acid **L2**, kaur-16-en-19-oic acid **K2**, 16β-kauran-19-oic acid **K3**, pinifolic acid **L8** and the unidentified kaurane **K4** are valuable markers.

The copaiba balsam found in the three paintings under study has also changed with time. In all cases the sesquiterpenes have completely evaporated or polymerised and only some diterpenoid acids are still present in relatively small amounts. The

presence of these marker molecules enabled the identification of the balsam and the assignment of its botanical origin.

Nevertheless, in some paintings, which have been treated with copaiba balsam in the past, no copaiba balsam can be retrieved. This may have several reasons. Firstly, we found that manufacturers have adulterated the majority of the samples of copaiba balsam with a *Pinus* balsam. In addition, copaiba balsam was often diluted in turpentine before application or only the oil of copaiba balsam was used which evaporates almost completely. Furthermore, we should take into account the detection limit of the analytical technique involved and the possible irregular distribution of the balsam between the different layers and parts of the painting.

Some final considerations concern the negative effects of copaiba balsam on paintings. In view of the substantial changes between fresh and aged copaiba balsam (only small amounts of some diterpenoid acids could be detected), how should we explain the sensitivity to solvents, heat and humidity and the phenomena observed in cross-sections and by microscopic examination of paintings, which have probably been treated with copaiba balsam? The existence and nature of any chemical interaction between diterpenoid acids and oil paint film compounds has not been investigated yet. However, chemical reactions might occur between the reactive sites of the oil network and those of the diterpenoid acids (double bonds, carboxyl and hydroxyl groups). Moreover, the carboxylic acid groups of the diterpenoid acids might interact with the positive ions of the pigment particles in competition with the negative sites (poly-anions) of the oil network [Boon *et al*., 1996], reducing the chemical stability of the paint film.

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4. Recognition of copals in aged resin/oil paints and varnishes; identification of copal in paintings of the Pre-Raphaelite Brotherhood*

Abstract

The polymer fractions of three copals and sandarac were examined with Direct Temperature-resolved Mass Spectrometry and pyrolysis-GCMS with thermally assisted methylation with TMAH. The polymers were found to consist mostly of polymers of communic acid and communol. Congo copal predominantly contained ozic acid and some enantio-*biformene and ozol.*

A new analytical approach for the identification of polymer fractions in small and insoluble paint samples is introduced. The procedure involves a two-step pyrolysis-GCMS technique with on-line derivatisation with TMAH in the first step. In the second step, traces of polymer fractions in small complex samples are analysed.

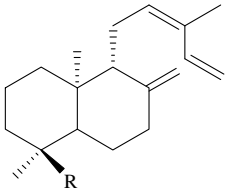
A copal/oil varnish was studied. The characteristic copal components had disappeared to a large extent, in the manufacturing process and especially during ageing. However, some characteristic marker compounds could still be found.

Using the new methodology, copal resins could be detected in several resin/oil varnises. In addition, they were found in paint media in several paintings from artists from the Pre-Raphaelite Brotherhood.

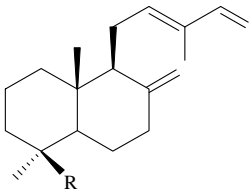
* This chapter is based on the publication: Van den Berg, K.J., J. van der Horst and J.J. Boon, (1999), "Recognition of copals in aged resin/oil paints and varnishes", in *Preprints ICOM Committee for Conservation 12th Triennial Meeting, Lyon, France, 29 Aug.-3 September 1999*, Vol. II, p. 855-861. James & James, London, and previously unpublished results in collaboration with Joyce Townsend, Tate and Henk van Keulen, RCE.

Introduction

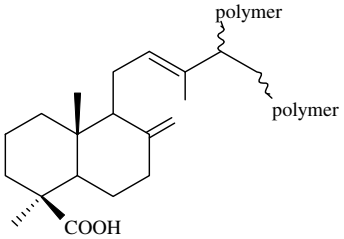
Polymerisation of low-molecular weight compounds is an important process in the ageing of natural resins for example in triterpenoid varnishes [Feller *et al.* 1985, de la Rie, 1988; Van der Doelen 1999]. Resins added to drying oil in resin/oil paints or varnishes (megilps, copal oils, etc.) may be incorporated into the polymer structure already in the manufacturing and curing process. The fate of polymer fractions of resins and resin-containing varnishes and paint media is not very well understood and has often been neglected. This is mostly due to the difficulties that arise for the analysis of these high-molecular-weight materials, that are inaccessible for *e.g.* most GCMS techniques. However, a better understanding of the nature of these fractions is very important since they determine the mechanical properties of varnishes and paints, such as brittleness and swelling sensitivity during solvent cleaning, to a large extent.



ozic acid (R=COOH)
ozol (R=CH_2OH)
enantio -biformene (R=CH_3)



communic acid (R=COOH)
communol (R=CH_2OH)
biformene (R=CH_3)



polycommunic acid

Some resins are known to form polymers already upon exudation from the tree by polymerisation of low molecular weight terpenoids containing dienes [Lagercrantz and Yhland, 1962]. Natural rubber is a well-known polymer of the smallest terpene, isoprene [Kishore and Pandey, 1986]. The polymer fraction of diterpenoid resins has been reported as polycommunic acid for Manila copal (*Agathis dammara*) [Mills and White, 1977] and sandarac (*Tetraclinis articulata*) [Gough, 1964]. A copolymer of communic acid and communol was suggested for Kauri copal (*Agathis australis*) [Thomas, 1966] and polyozic acid for Congo copal (*Daniellia*) [Bevan *et al.* 1968] (See Fig. 1). Structural identification of these polymer fractions has often been based solely on the analysis of 'polymerisable' monomers in the resins. Polymer fractions have been studied with Nuclear Magnetic Resonance (NMR) and Pyrolysis-Gas Chromatography MS (Py-GCMS) in fossil resins [Van Aarssen, 1992; Clifford and Hatcher 1995; Anderson, 1995] and fresh dammar [Van Aarssen *et al.* 1994]. The polymer fraction of gum mastic (from *Pistacia lentiscus* L.) has long remained a mystery but was recently identified as a poly-myrcene, the first reported naturally occurring monoterpene polymer (reported in Chapter 5).

The use of sandarac in spirit varnishes has been reported in painting treatises from early times until the 19th century. Copals have been used extensively in 18th/19th century varnishes and resin/oil media [Carlyle, 1991].

Fig. 1. Labdane diterpenes and a communic acid polymer (see text).

Though sometimes used as spirit varnishes, they were mostly manufactured with oil. In this process, high temperatures were applied (often >300 ^{o}C) to melt the hard copal resin before adding the oil. Due to these extreme circumstances, chemical changes may be expected in both the resin and the oil fraction. This may be the main reason why little or no analytical evidence so far has been presented for the use of copal [Mills and White, 1994]. A solution to this problem may be the analysis of the polymer fraction.

In this report we focus on the polymer fractions of resins and their fate in the manufacturing and ageing processes. Polymer fractions of three different copals and sandarac were studied by Direct Temperature-resolved Mass Spectrometry (DTMS) and Pyrolysis-Gas Chromatography/Mass spectrometry (Py-GCMS) to identify characteristic structural elements. A novel two-step Py-GCMS technique is introduced and used for selective analysis of remnants of polymer structures in aged varnishes and paint media. With this technique, the fate of these structures after heat treatment and ageing is investigated.

Materials and methods

Materials

Kauri copal: A.F. Suter. Congo copal, Manila copal, sandarac: Verfmolen de Kat, Zaanstad, The Netherlands. The varnish was manufactured by Dr. L. Carlyle after an original recipe from Manila copal and linseed oil (1/2 w/v) in turpentine (19% resin content). This included heating and melting of the resin (350 ^{o}C), adding hot linseed oil and fusing of the two components for a total time of 3 hours at about 300 ^{o}C [Carlyle and Bourdeau, 1994]. The varnish was applied on a tile and artificially light-aged at the CCI for 28 months (fluorescent tubes, 2300 lux, 15µW/l) [Carlyle *et al*., 1999] and stored in the dark for 1.5 years until analysis.

All chemical reagents were commercially available and used as supplied.

Instrumentation

Most of the methods have been referred to the literature, with the exception of:

Size exclusion chromatography

Solvent delivery Applied Biosystems 400 system at 1 ml/min (THF, unstabilised) on a Polymer Laboratories (UK) 10^3 Å column (300 x 7.5 mm) using an LDC/Milton Roy SM4000 programmable wavelength detector at 240 nm. Injection volume was 100 µl; polystyrene standards were used for calibration.

Two-step Curie-point Pyrolysis setup

This technique involves a modified Cu-pyrolysis setup [J.J. Boon, unpublished results] in which the sample wire can be heated to any temperature below the Curie-point temperature of the wire by electronic manipulation of the electromagnetic field.

The analytical filament (Curie point 610 ^{o}C) with the sample is inserted into a glass liner, flushed with argon and placed in the pyrolysis chamber (180 ^{o}C). In the first step, the temperature is raised to 250 ^{o}C to evaporate low MW compounds and inject them on the capillary GC column. In the second step the wire is reinserted in the pyrolysis unit and heated to the Cu-point temperature to pyrolyse the residual fraction.

Polymer fractions of copals and sandarac

The polymer fractions of sandarac, Kauri and Manila copal were isolated by dissolution in dichloromethane and precipitation with methanol [Van Aarssen, 1992]. The composition of the precipitate was monitored using Size Exclusion Chromatography. The insolubility of the polymer fraction of Congo copal was such that it could only be isolated by extraction of the finely ground material with tetrahydrofuran and dichloromethane. The molecular weight distribution of the resins was found to extend to about 100-150 kDa for Kauri and Manila. The upper limit for sandarac was about 75 kDa and for Congo copal at least 150 kDa. Due to the low solubility of the resin the maximum molecular weight could not be determined. Fractions >20 kDa of all resins were collected using preparative SEC.

Part of the polymer fractions of the diterpenoid resins was derivatised with BSTFA to convert free hydroxyl and carboxylic acid groups into trimethyl-silyl ethers and esters, respectively [Blau and Halket, 1993]. Alternatively, fractions were derivatised *on-line* by mixing the sample with an aqueous solution of tetramethylammonium hydroxide (2.5% TMAH) prior to Direct Temperature-resolved MS (DTMS) analysis (See also Chapter 2). By heating the mixture, the hydroxyl and carboxylic acid moieties are methylated at 150-200 ^{o}C.

Isobutane chemical ionisation (CI) was applied to ionise the molecules that evaporated from the filament in the DTMS experiment [Van Aarssen *et al*., 1994]. Pyrolysis occurred in all instances at temperatures above 400 ^{o}C. The corresponding CI mass spectra predominantly showed $[M+H]^+$ ions of m/z 375 with an increment of 374 Da after trimethylsilylation, or of m/z 317 with an increment of 316 Da after *on-line* derivatisation with TMAH. Oligomer ions up to the pentamer (m/z 1872) were observed after trimethylsilylation (Fig. 2). This indicates that the polymer predominantly consists of monomer units of 302 Da (before derivatisation). The only exception is Kauri copal, which failed to show ions from oligomers. Only dimer structures were observed after methylation with TMAH (m/z 605, 619 and 633). This points to a copolymer consisting of monomer units with molecular weights (MW) of 302 and 316 (after derivatisation).

The polymers were then subjected to Py-TMAH-GCMS (See Chapter 2 and [Van den Berg *et al*., 1998]. The GCMS traces (not presented here, see Table 1) show a large number of pyrolysis products, the by far most abundant compounds being diterpenoids. The mass spectra of the derivatised diterpenoid pyrolysis products are given in the Atlas of Mass Spectra (Communic acid, 64; Communol, 65; Ozic acid, 66; ozol, 67 and *enantio*-biformene, 68). The mass spectra of communol, ozol (methyl ethers) and ozic acid (methyl ester) have (to our knowledge) not been reported before but are assigned based on the analogy with the known mass spectra of free ozic acid and methylated communol and communic acid.

At least two isomers of communic acid and communol were detected. This is due to the isomerisation of the diene side chain in the monomers which is known to occur upon heating [Atkinson and Crow, 1970]. Surprisingly, this was not the case for ozic acid; here only one isomer was observed. No indication for biformene and related compounds were found in the resins other than Congo copal. All structures were confirmed by the results of ^{1}H and ^{13}C-NMR (not shown). Succinic acid has been reported to occur in ambers [Mills *et al*., 1984/85; Boon *et al*., 1990] but was not detected in our studies.

The GCMS results generally confirm those of DTMS and the structures proposed in the literature. However, the presence of traces of communol monomer

units in Manila copal and sandarac polymer fractions has not been reported previously. The same holds for *enantio*-biformene and ozol in Congo copal.

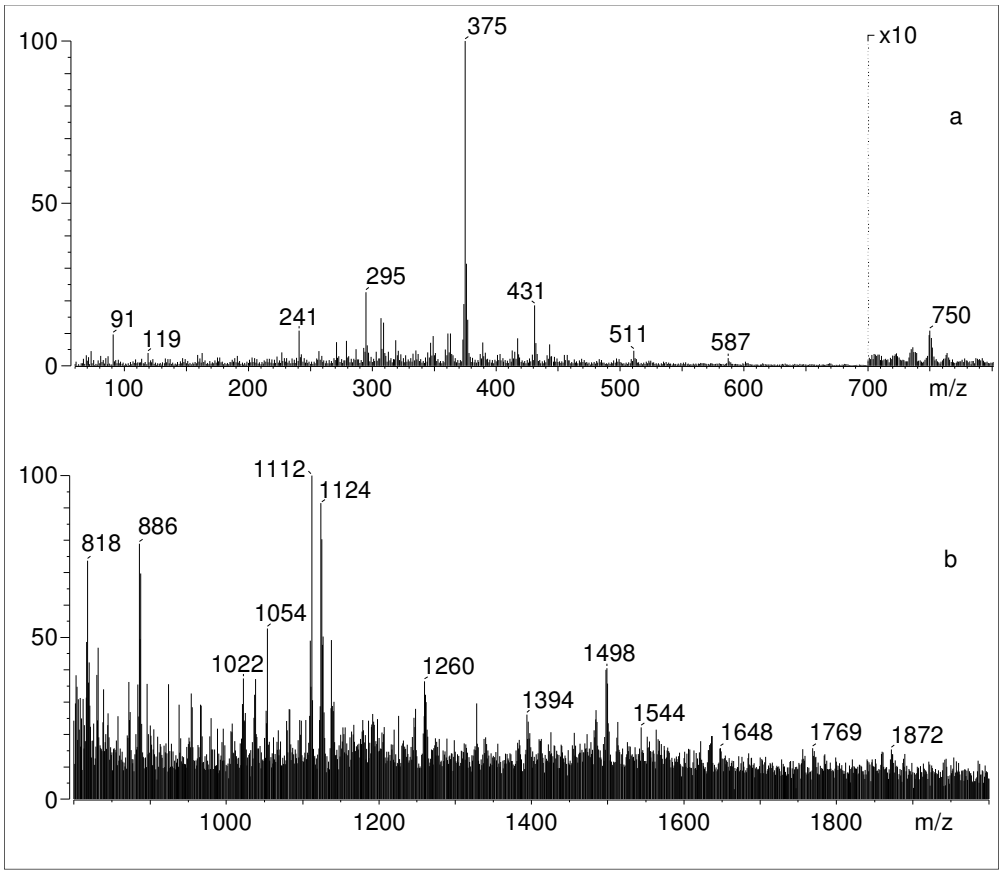


Fig. 2. DTMS-CI spectrum of the polymer fraction of Manila copal after trimethylsilylation; a) m/z 60-800, b) 800 to 2000. The ion of m/z 1112 is an artefact.

Table 1. Identity and relative abundance (peak heights measured) of diterpenoids as observed in the Py-TMAH-GCMS experiments for the polymer fractions of the resins studied. See text.

Resin	*Diterpenoid monomers observed*
Manila copal	communic acid, methyl ester; communol, methyl ether (trace)
Kauri copal	communic acid, methyl ester (60%) communol, methyl ether (40%)
Sandarac	communic acid, methyl ester; communol, methyl ether (trace)
Congo copal	enantio-biformene (16%), ozic acid, methyl ester (79%) ozol, methyl ether (5%)*

* also eperuic acid present, probably a left over from the monomer fraction

An important additional structural element observed in GCMS spectra was a molecule with MW 236 (Atlas of Mass Spectra, 69). This constituent was also found in DTMS spectra (m/z 237, after trimethylsilylation m/z 295). The spectrum probably corresponds to the ring structure in Atlas 69 in which the side chain has been lost in the pyrolysis process. In addition, similar structures are formed with molecular masses of 248 and 250. The structures of these compounds were found previously as products

from fossil resins containing polycommunic acids [Van Aarssen, 1992; Anderson, 1995]. Mass spectra and chemical structures are given in Atlas 70 and 71. Additionally, the expected corresponding spectra for the products of the communol entities in the kauri resin (MW = 222, 234 and 236) were also observed [Anderson, 1995].

The polymers were found to be relatively unstable to air after isolation. Upon atmospheric exposure, a decrease in solubility of the fractions was observed which is interpreted as an indication that oxidative cross-linking was taking place.

Analysis of a copal varnish; a new two-step approach

A (Manila) copal varnish made in reconstruction experiments by Leslie Carlyle was studied using a newly developed two-step Cu-pyrolysis technique which is capable of separating and analysing the low MW and polymer fractions on-line requiring little preparation (see *Materials and Methods*). In our experiments, the sample was mixed with TMAH and heated to 250 ^{o}C to (simultaneously hydrolyse and) methylate the sample (see below) and analyse all low MW compounds with GCMS. In the second step, the remaining high MW fraction was pyrolysed at 610 ^{o}C and the products analysed.

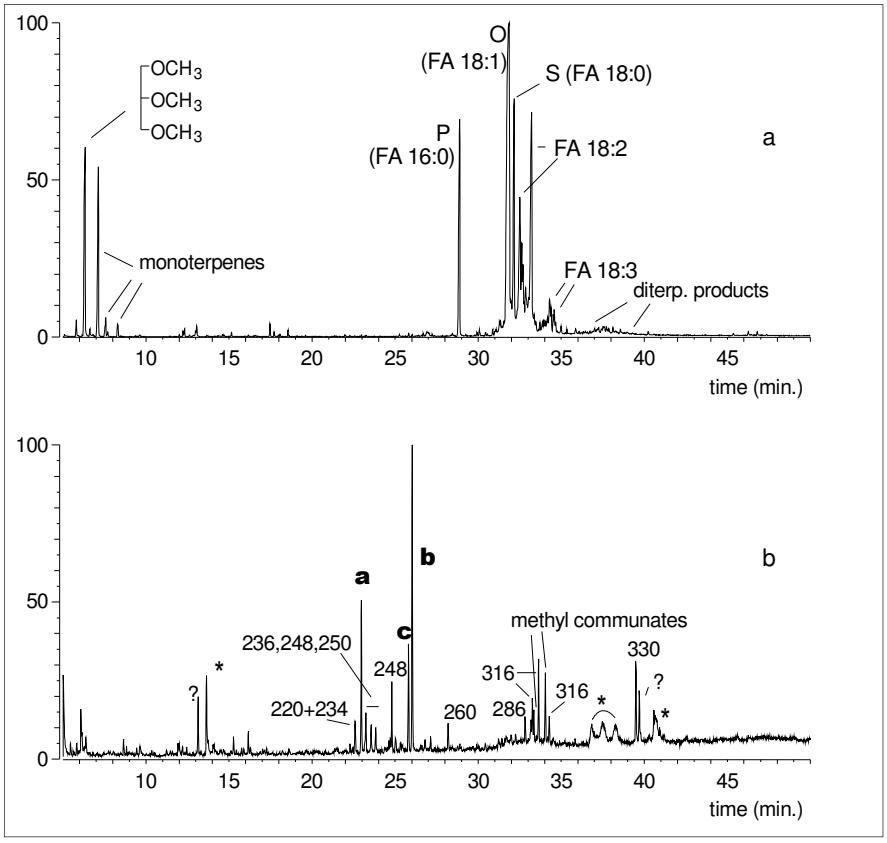


Fig. 3. Py-TMAH-GCMS total ion chromatogram of varnish 'Co2', stock solution; a) step 1 at 250 ^{o}C, b) step 2 at 600 ^{o}C. a,b,c refer to Atlas *69, 70, 71. FA = fatty acid, methyl ester; diFA = fatty diacid, dimethyl ester; numbers refer to chain length and number of unsaturations. Other peak labels refer to molecular masses of the compounds; * = contamination.*

The Py-TMAH-GCMS traces of the varnish are presented in Fig. 3. In the first step, residual amounts of monoterpenes (from the oil of turpentine), glycerol and various saturated and singly and doubly unsaturated fatty acids are predominant. The double bonds in the fatty acids have partly migrated as a result of the heat applied in the manufacturing, resulting in a large number of partly unresolved isomeric structures.

This especially holds for isomers of the most reactive fatty acid moiety, linolenic (all-*cis* triply unsaturated) acid which is most abundant in fresh linseed oil. However, most linolenic acid moieties have reacted away as a result of cross-linking with other oil or copal compounds by e.g. Diels-Alder reactions. These reactions are responsible for the increase in viscosity of the oil. Cyclopentenyl- and cyclohexenyl-containing products formed by internal cyclisation may also have taken place [Dobson *et al*., 1996] but have not been identified in the varnish.

The diterpenoids present initially (predominantly agathic acid, but also communic acid, sandaracopimaric acid, etc.; see e.g. [Mills and White, 1994, Van den Berg *et al.*, 2002a]) mostly reacted away although a large number of unresolved small peaks (isomers and reaction products) were still present. The molecular masses of these products are in the range of 318-380. No diterpenoid-like structures of lower MW were found, indicating that decarboxylation is probably not an important process during the manufacturing of the varnish, as was assumed by Mills and White (1994).

In the second analytical step, only pyrolysis products of the copal polymer fraction have been formed, predominantly consisting of the communate monomeric units and the small bicyclic compounds with molecular mass of 236, 248 and 250. The latter compounds are now predominant (MW = 248 being most abundant). Interestingly, more isomers of methyl communate are present than in the spectrum of the polymer isolated from the fresh resin. The mass spectra of these isomers all show a characteristic fragmentation pattern in which m/z 235 (probably through loss of the side chain) and 175 (additional elimination of the methyl carboxylate side chain) are dominant peaks.

In the heating and fusion process with the oil, extensive rearrangements in the polymer fraction of the copal has occurred. Both isomerisation of double bonds and condensation reactions with double bonds in fatty acid moieties by Diels-Alder reactions may be responsible. This has resulted in a decrease of the characteristic products from pyrolysis compared to that of the 'fresh' resin polymer. However, the integrity of the two-ring alicyclic structures remains intact to a large extent and decarboxylation is limited (see below).

The spectra of the copal/oil varnish after drying and artificial ageing are given in Fig. 4. The low MW spectrum in the first step (Fig. 4a) shows only the characteristic fatty acids from cured oil. The varnish has dried completely as can be reasoned from the fact that unsaturated fatty acid moieties have reacted away completely, including the less-reactive oleic acid. The relatively large amount of diacids other than azeleic acid (C9 diacid; C8/C9 = 1 : 3.8, peak areas measured) is a reflection of the severe heat-bodying treatment the oil/resin mixture has undergone. In addition, all characteristic monomers of copal resin have now disappeared and no distinction can be made with regular heat-bodied oil paint.

The chromatogram corresponding to the high temperature step (Fig. 4b), shows very little chemical information. Apart from some remaining diacids, peak identification is extremely difficult. However, after careful searching of characteristic peaks from the small bicyclic compounds (m/z 161, 175 and 189, respectively) traces of corresponding compounds could be found (Fig. 4c and d).

Obviously, the polymer fraction has altered dramatically in the drying and ageing process and it is difficult to trace the polymer. It can be argued that these processes affect the polymer even more than the extreme heat (> 300 ^{o}C) involved in the manufacture of the varnish, by further oxidation and polymerisation. Eventually, degradation to lower molecular weight compounds may take place.

It has to be borne in mind that dissociation products from e.g. polycommunic acids can not distinguish between e.g. Manila copal, sandarac and many ambers (all used frequently in the past). Nevertheless, the small bicyclic compounds are very useful as characteristic products of polycommunates and polyozates.

Conclusions and outlook

Polymer fractions of sandarac and most copals (excluding Congo copal) can be isolated easily by dissolution/precipitation and by monitoring the fractionation process with SEC. DTMS of the derivatised polymers with chemical ionisation provides information on the polymers up to m/z values well over 1000, indicative of the masses of the predominant monomeric units. Pyrolysis-GCMS of the polymers with *on-line* derivatisation with TMAH can then release the most important monomers in the polymer. The polymer fractions of Manila copal and sandarac are polycommunic acids with traces of communol incorporated. Kauri copal contains relatively large amounts of communol and the polymer of Congo copal consists of polyozic acid with low amounts of *enantio*-biformene and ozol.

The ageing process dramatically changes the relative amount of compounds characteristic for copals, especially when the resins have been subjected to severe heat treatments in combination with drying oil. This especially holds for diterpenoid, low MW compounds that are analysed in traditional GCMS procedures. The characteristic diterpenoid compounds in copal-oil varnishes completely disappear in the drying and ageing process. However, using a two-step pyrolysis GCMS method with on-line thermally assisted methylation, tracers of characteristic compounds released from the polymer fraction of copal (oil) resins could be identified.

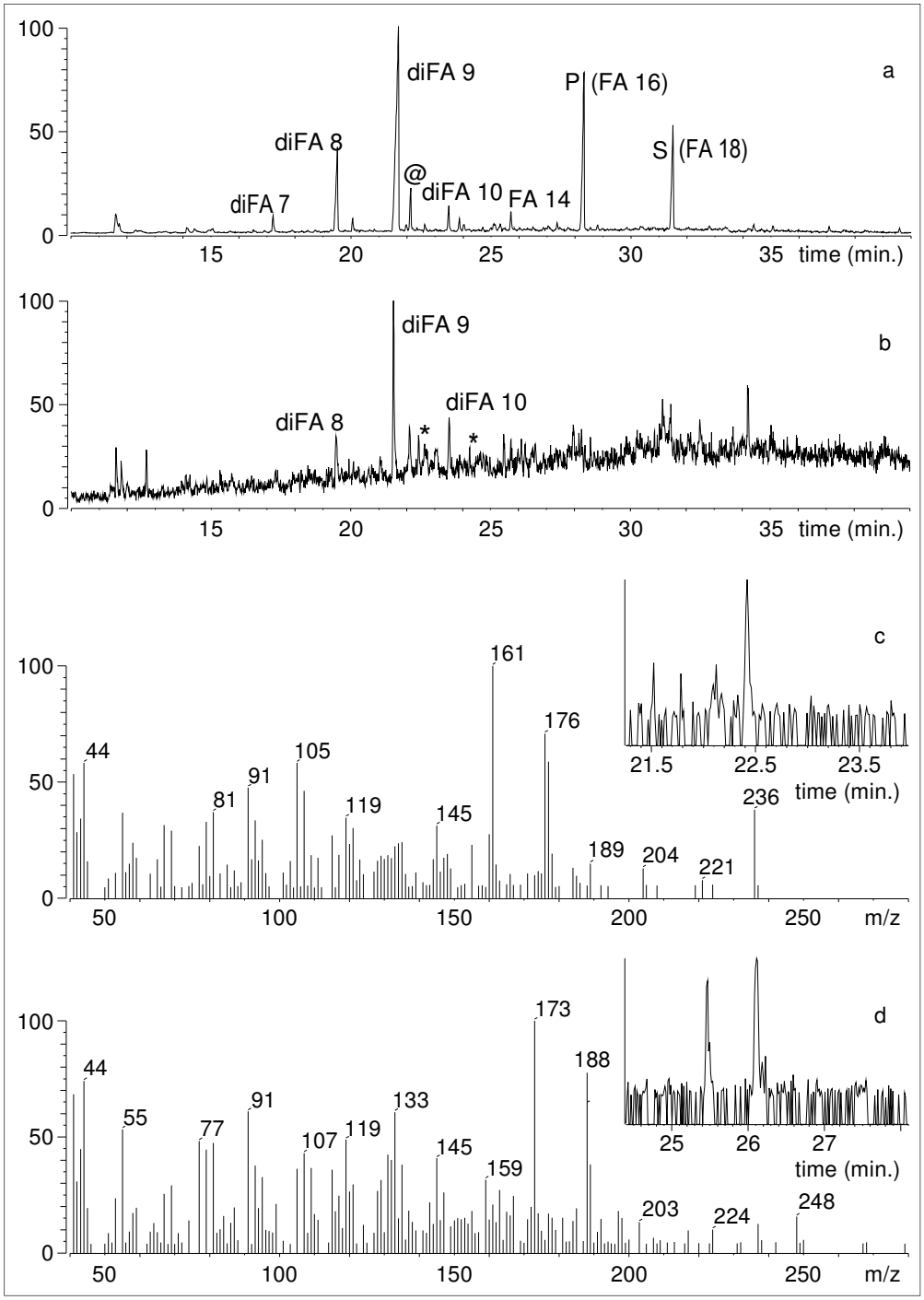


Fig. 4. Py-TMAH-GCMS total ion chromatogram of varnish 'Co2' after drying and light-ageing; a) step 1 at 250 °C, b) step 2 at 600 °C (refers to c) and d), respectively), c and d) partial single ion chromatograms with mass spectra of compounds with M= 236 and 248 (See also Fig. 3). FA = fatty acid, methyl ester; diFA = fatty diacid, dimethyl ester; numbers refer to chain length. @ = α-methyl-diFA 9. Other peak labels refer to molecular masses of the compounds.*

Occurrences of copal in paintings

Since this chapter was first drafted in early 1999, we have found evidence for copal using the two-step method in a number of cases. In several cases, copal containing 19th Century varnishes were analysed in varnish samples from Dutch paintings from for example the Hague school [Van den Berg *et al*., 2002a, 2002b]. These were all from the polycommunic acid type. A rare example of an African copal (ozic acid type) was found on a painting by T. Eakins in the Philadelphia Museum of Art [Van den Berg *et al*., 2002a].

In addition, polycommunic acids indicative of copal resin in oil paint were detected in several paintings from the Pre-Raphaelite Brotherhood, a group of artists who used copal/oil resin media to obtain brighter and warmer colours [Hackney et al., 2001, 2002]. This use was well-documented; see also the cover of this specialist report]. These paintings are from Millais: The Vale of Rest, Tate Gallery Cat. # N01507, Mariana in the Moated Grange, # L01871, The Order of Release, # N01657, possibly Ophelia, # N01507, and Hunt, The Light of the World, Keble college. In 10 paint samples from other works by Pre-Raphaelite artists these traces were not found, although evidence for heat-bodied oil was found in most of these cases. See for an example the total ion chromatogram from the analysis of a green paint sample Fig. 5.

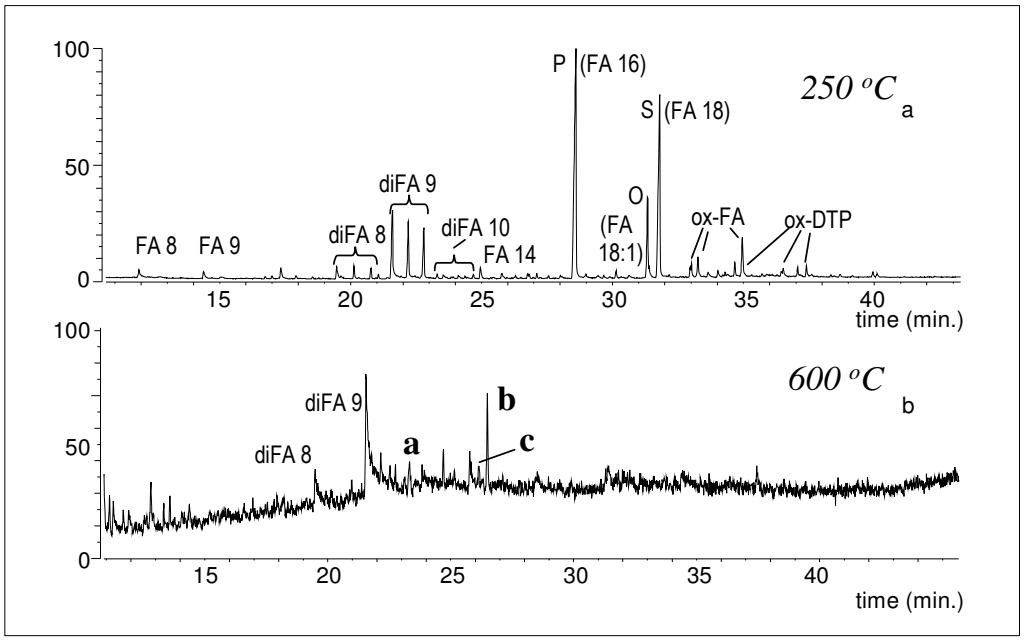


Fig. 5. Py-TMAH-GCMS total ion chromatogram of green paint sample from tree foliage (containing emerald green) in John Everett Millais, The Vale of Rest, *Cat. # N01507, Tate Gallery; a) step 1 at 250 °C, b) step 2 at 600 °C.*

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5. C*is*-1,4-poly-β-myrcene; the structure of the polymeric fraction of mastic resin (*Pistacia lentiscus* L.) elucidated

Abstract

The polymer fraction of mastic resin from Pistacia lentiscus *L. was isolated and purified by extraction and Size Exclusion Chromatography. The molecular weight distribution of the polymer was broad and stretched up to 100,000 Da. The structure was characterised as 1,4-poly-β-myrcene, predominantly present in the* cis-*conformation. The compound is the first known example of a naturally occurring polymer of a monoterpene.*

Introduction

Mastic, or gum mastic, is a bleed resin formed in teardrops from the *Pistacia lentiscus* L. tree from the *Anacardiaceae* family. It has been known and was extensively traded in antiquity around the Mediterranean as adhesive, coating, medicine etc. [Mills and White, 1977 & 1989; Koller *et al.,* 1997]. The best known international application in the last couple of centuries is the use as a spirit varnish for furniture and paintings [Koller *et al.,* 1997; Mills and White, 1994]. Other species of the *Pistacia* genus which produce resin are *P. terebinthus* L. which produces a turpentine and *P. atlantica* Desf., which brings forth a mastic of inferior quality [Koller *et al.,* 1997]. Gum mastic consists of large amounts of triterpenoid molecules which have been studied in varying detail by a number of research groups [Mills and White, 1989; Barton and Seoane, 1956; Seoane, 1956; Monaco *et al.*, 1973; Caputo *et al.,* 1978; Boar *et al.*, 1984; Marner *et al.*, 1991; Van der Doelen *et al.,* 1998a & 1998b, Van Aarssen, 1992 & 1994; Scheijen *et al*., 1989]. The resin also contains a considerable amount of polymeric material of which the structure was not known before. In this chapter, we describe the experiments for the elucidation of this polymeric fraction and present its chemical structure.

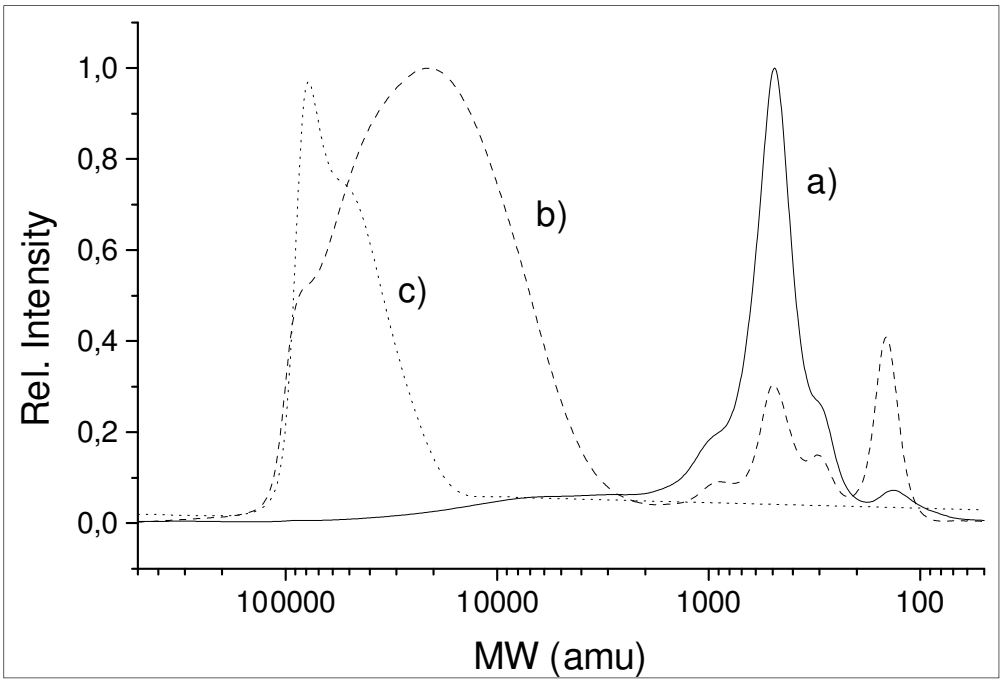


Figure 1. Size exclusion chromatograms of a) mastic, b) the polymer fraction after several dissolution/precipitation steps, c) the isolated polymer with SEC.

Results and discussion

The polymer fraction of gum mastic teardrops (Schmincke, Erkrath, Germany) was isolated by dissolution with dichloromethane and precipitation with methanol [Van Aarssen, 1992] which was monitored using Size Exclusion Chromatography

(SEC, absorption at 240 nm). The polymer fraction was found to have a molecular weight distribution up to about 100,000 Da (Fig 1). Polymeric material of 20,000-100,000 Da was collected using preparative SEC for further study.

Direct Temperature-resolved MS (DTMS) after isobutane chemical ionisation (CI) [Van Aarssen *et al.*, 1994] showed a low amount of monomeric material (triterpenoid molecules) still present, which desorbed at temperatures below 350 °C. Pyrolysis occurs at temperatures above 400 °C. The corresponding CI mass spectrum predominantly shows [M+H]+ ions with an increment of 136 Da, indicating that the polymer consists of monomer units of 136 Da (Fig. 2). Oligomer fractions up to the 14-mer, with a nominal mass of 1904 Da (m/z 1906 due to ^{13}C contribution), are visible in the spectrum.

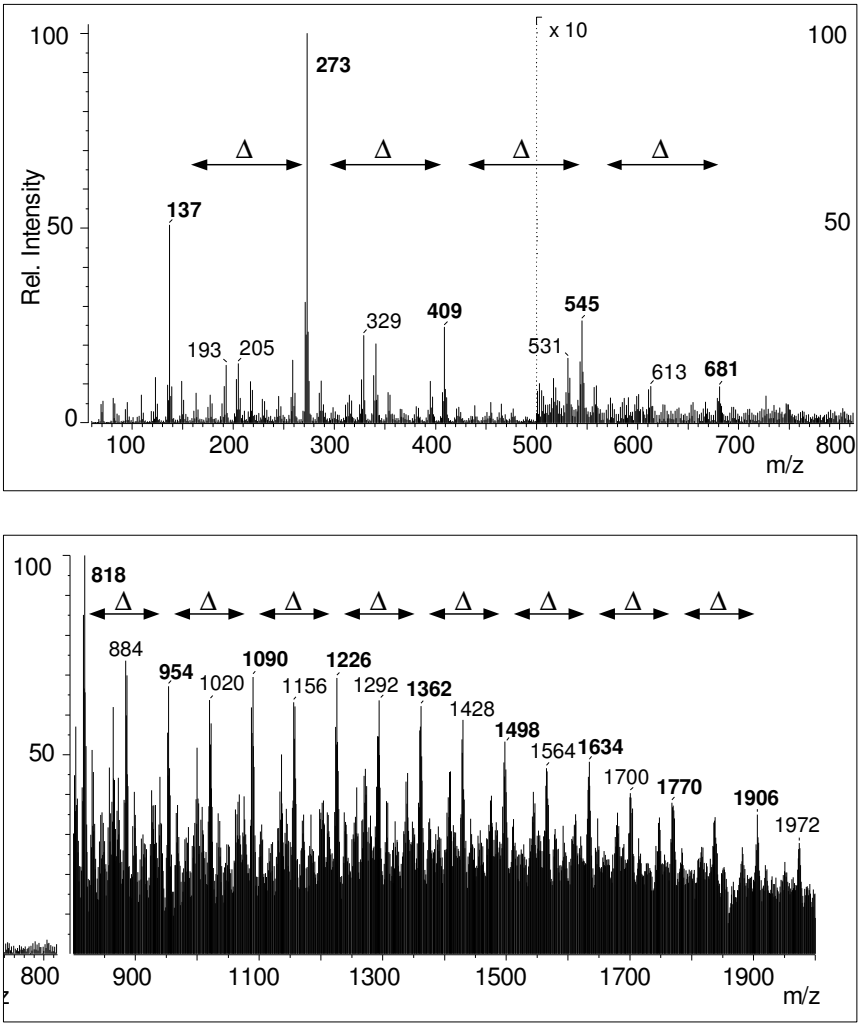


Figure 2. Mass spectra of the pyrolysate (>400 oC) of the isolated polymer of gum mastic, m/z 60-800 (a) and m/z 800-2000 (b). Δ = 136 Da (see text).

Curie-point pyrolysis GCMS (Cu-Py-GCMS, Cu-point 610 oC) [Scheijen et al., 1989] showed a large number of products, the most abundant of which are hydrocarbons CxHy (Fig. 3). The most abundant species was identified as β-myrcene ($C_{10}H_{16}$, 136 Da) which points towards β-myrcene as the monomeric base unit of the polymer. Several dimeric structures ($C_{20}H_{32}$) were found as well but the structure of these compounds could not be identified unequivocally. In addition, also some oxygen-containing molecules are present in the pyrolysate which may have been formed from low molecular weight impurities as well (see below).

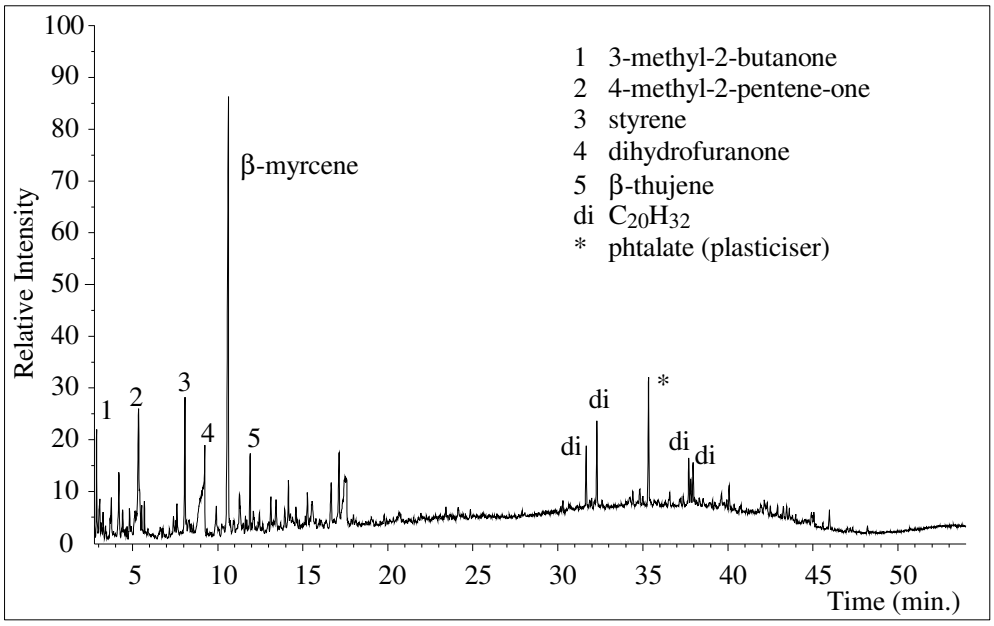


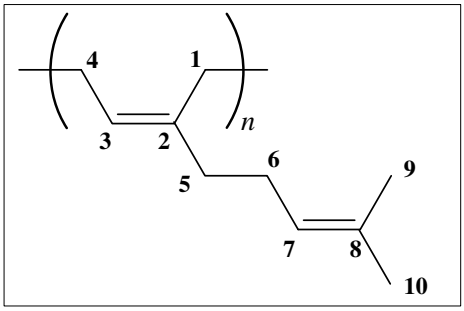
Figure 3. Py-GCMS total ion chromatogram of the polymer fraction of gum mastic.

FT-IR, ^{1}H-NMR (not shown) and ^{13}C-NMR data (Fig. 4) point to a hydrocarbon such as latex and gutta percha, natural rubbers that consist of 1,4-polyisoprenes [Kishore and Pandey, 1986]. The NMR data (supported by DEPT) show signals which relate both to sp^2 and sp^3 carbon atoms involved in C-H and C-C bonds. Isoprene, however, is a C_5H_8 molecule which is exactly half of that of β-myrcene. DTMS and Py-GCMS indicated that the best candidate is a poly-monoterpene. The chemical shifts and intensities of the peaks in the ^{13}C-NMR spectrum show a remarkable similarity to that of cis-1,4-poly-β-myrcene (Table, Scheme 1) that has been synthesised and characterised by Newmark and Majumdar and others [Newmark. and Majumdar, 1988].

Table. Chemical shifts and intensities of the relevant peaks in the 13C-NMR spectra of mastic polymer fraction and synthetic cis-1,4-poly-β-myrcene. [Newmark and Majumdar 1988]

	Polymer of gum mastic		**Synthetic polymer**	
Assignment	*Chemical Shift*	*Intensity (integral)*	*Chemical Shift*	*Intensity (peak heights)*
C_1	31.5	80	30.56	148
C_2	139.5	92	138.86	99
C_3	125.0	100	124.52	102
C_4	28.1	80	26.74	147
C_5	38.1	98	36.85	193
C_6	27.8	84	26.92	300
C_7	125.0	100	124.36	225
C_8	131.8	118	131.01	22
C_9	18.8	130	17.64	182
C_{10}	26.7	118	25.62	400

The NMR spectra show some 20 additional peaks (Fig. 4), mostly corresponding to aliphatic carbons of which the abundance is too high to originate from low molecular weight compounds. They may be explained by the presence of additional (yet unknown) monomeric units or by products of intramolecular cyclisation produced in the polymerisation process [Quack and Fetters, 1978]. Small signals at 109.5 and 152.0 ppm suggest that some 3,4-polymerisation product (Scheme 1) of β-myrcene is present. In addition, several (broadened) peaks as well as information from 2D and DETP NMR suggest that the β-myrcene units partly have a trans-configuration. For example, the broadened peaks at 30.5 and 29.2 may be explained by C_1 and C_4 in the cis/trans mode, respectively. The chemical shifts of the other carbon atoms are anticipated to be similar and to cause some peak broadening at the most. The ratio of cis- and trans-poly-β-myrcene is estimated as 3/1 based on the integrated signals at for C_1 at 31.5 and 30.5 ppm.



Scheme 1. Cis-1,4-poly-β-myrcene.

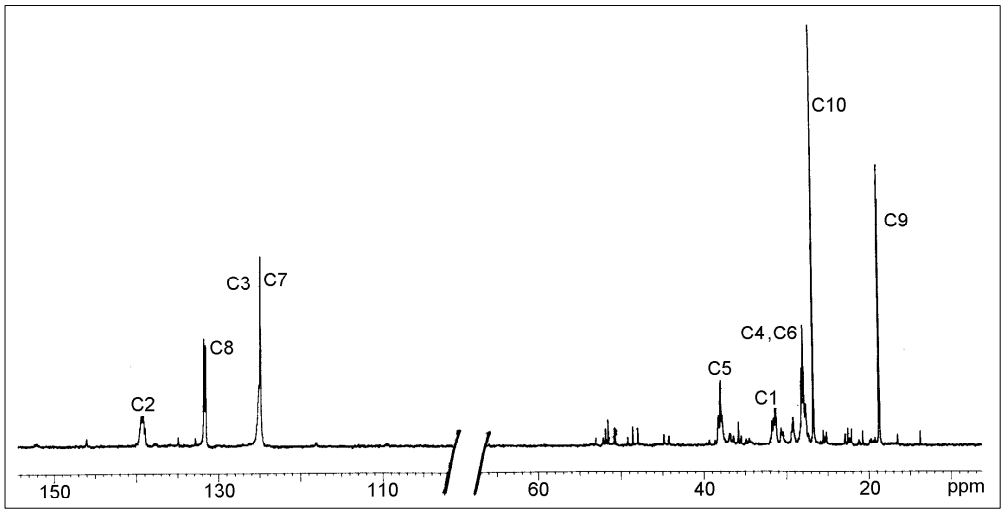


Figure 4. 13C-NMR spectrum ($C_2D_2Cl_4$) of the polymer fraction of gum mastic.

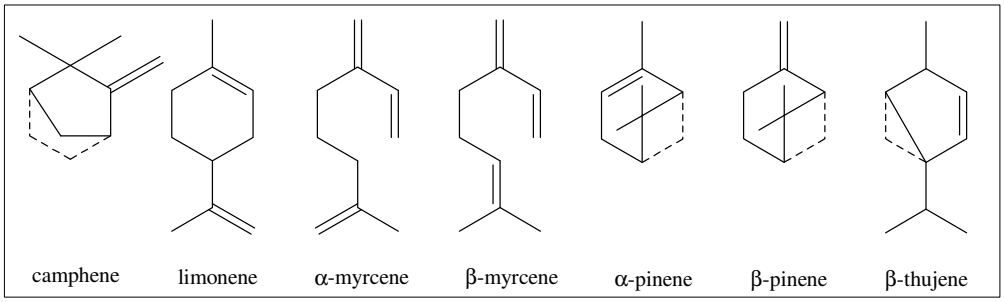
Little or no evidence for products of oxidation or oxidative cross-linking is found. Chemical shifts of 60 ppm or more are expected to correspond to sp^3 C-O functionalities which are not present in any manifest abundance. Therefore the oxygen-containing species analysed by Py-GCMS and FT-IR analysis (C=O bend vibration at 1700 cm^{-1}) may well be explained by the triterpenoid impurities and possibly some phtalate (Fig. 3).

The polymer proved to be relatively unstable after isolation when no precautions to avoid degradation were taken which was indicated by the rapid decrease of solubility of the polymer after isolation. This suggests that, due to the large number of unsaturations, oxidation and/or cross-linking takes place rapidly. In addition, the cis/trans isomerisation may have taken place. The instability of the poly-β-myrcene may hamper the positive identification of this polymer in, for example, aged varnish samples from paintings. The degraded polymer was not investigated at this stage; this will be done in future research.

Conclusion

The polymeric fraction of mastic from *Pistacia lentiscus* L. is 1,4-poly-β-myrcene which has the cis-conformation for about 75%. Analysis of the oil of turpentine content of mastic teardrops (which makes up for only 2%) showed that α-pinene is the most abundant compound [Papageorgiou, 1981]. In addition, also β-myrcene and low amounts of limonene, camphene and β-pinene were identified. Of these compounds, β-myrcene is the only compound with conjugated double bonds which are relatively prone to polymerisation (Scheme 2). We infer that the tree produces relatively large amounts of β-myrcene which is polymerised once the resin exudes from the tree. The reason why *P. terebinthus* L. produces a turpentine rather than a solid resin might then be explained by relatively low amounts of myrcene and relatively high amounts of non-polymerisable essential oil compounds. Monoterpenes such as β-myrcene are relatively common components of the volatile fraction of tree balsams [Mills and White, 1994] and it is also known to be secreted by butterflies [Honda, 1990]. Polymers of isoprenes (natural rubber) [Kishore K. and Pandey, 1986], polysesquiterpenoids (polycadinene) and polyditerpenoids (e.g. polycommunic acid) [Mills and White, 1977; Van Aarssen, 1992; Langenheim, 1995] have been

known for a long time. This new discovery is important since it is the first reported naturally occurring polymer of a monoterpene.



Scheme 2. Monoterpenes

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About the author

The author received his PhD in Chemistry at the University of Amsterdam in 1994 for the thesis *Gas-phase Organometallic Negative Ion/Molecule Chemistry*. Then he spent a short period of time at the Free University/Institute for Environmental Studies.

From 1995 to 1999 he was a project leader in MOLART at the FOM Institute for Atomic and Molecular Physics, in charge of development of strategies for (organic) chemical analysis and molecular ageing studies of painting materials. Since 2000, he has been working at the Cultural Heritage Agency of the Netherlands (RCE) studying material aspects and colour changes in 19th and 20th Century painted art.

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Other publications

MOLART publications, not included in this report:

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9. G.A. van der Doelen, K.J. van den Berg and J.J. Boon, (2000), "A comparison of Weatherometer-aged dammar varnishes and varnishes aged naturally on paintings", in *Art et chimie, la couleur. Actes du congrès*, J. Goupy and J.-P. Mohen (Eds), CNRS Editions, Paris, p. 146-149.
10. J.D.J. van den Berg, K.J. van den Berg and J.J. Boon, (2001), "Determination of the degree of hydrolysis of oil paint samples using a two-step derivatisation method and on-column GC/MS". *J. Progr. Org. Coatings*, 1074: 1-13.
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Other *pre*- and *post*-MOLART publications by or involving the author can be found on the RCE website, via www.cultureelerfgoed.nl.

Atlas of Mass Spectra

In this Atlas are presented the mass spectra of compounds that have been discussed in the previous chapters. Identification of these compounds was done through synthesis of model compounds, comparison of the fragmentation patterns observed in the mass spectra of several derivatives, and comparison of the mass spectra with reference spectra from literature and from the Wiley library of MassLib version 7.3 (see previous chapters and literature cited therein).

The mass spectra were obtained with the JEOL-DX303 GCMS instrument at FOM Institute AMOLF (see *Materials and methods* sections in the previous Chapters) under electron ionisation conditions (70 eV). Spectra were transferred from the JEOL data system, and processed in TopDraw 3.0. Structures were drawn with ACD Chemsketch 2.0.

Index of spectra by Molecular Weight

MW	*Atlas*	MW	*Atlas*	MW	*Atlas*
204	*51*	316	*31*	348	*26*
204	*52*	316	*36*	354	*17*
204	*53*	316	*48*	356	*8*
204	*54*	316	*49*	358	*13*
204	*55*	316	*50*	360	*38*
204	*56*	316	*64*	362	*45*
204	*57*	316	*66*	364	*44*
204	*58*	318	*37*	372	*4*
204	*59*	318	*42*	372	*12*
204	*60*	320	*39*	374	*2*
204	*61*	320	*41*	374	*24*
204	*62*	322	*40*	376	*47*
204	*63*	326	*15*	386	*6*
236	*69*	328	*5*	386	*14*
248	*70*	330	*18*	388	*34*
250	*71*	330	*32*	402	*19*
272	*35*	330	*43*	416	*10*
272	*68*	334	*29*	420	*28*
302	*65*	334	*46*	450	*27*
302	*67*	340	*16*	460	*20*
306	*25*	342	*7*	474	*11*
314	*3*	344	*9*	490	*22*
316	*1*	344	*21*	548	*23*
316	*30*	346	*33*		

Alphabetical index of spectra

Cativic acid, methyl ester (L3)	41
Communic acid, methyl ester	64
Communol, methyl ether	65
Copalic acid, methyl ester (L5)	42
Cyperene (S5)	55
Dehydroabietic acid (DHA), methyl ester	3
Dehydroabietic acid (DHA), TMS ester	4
enantio-biformene	68
Eperuic acid, methyl ester (L1)	39
Hardwickiic acid, methyl ester (c1)	32
Hydroxy-Hardwickiic acid, methyl ester (c2)	33
Isopimaric acid, methyl ester (P3)	50
Kaur-16-en-19-oic acid, methyl ester (k2)	36
Labdanic acid, methyl ester (L2)	40
Larixol	25
Larixol, dimethyl ether	29
Larixol, di-TMS ether	27
Larixyl acetate	26
Larixyl acetate, TMS ether	28
Neoabietic acid, methyl ester (a4)	31
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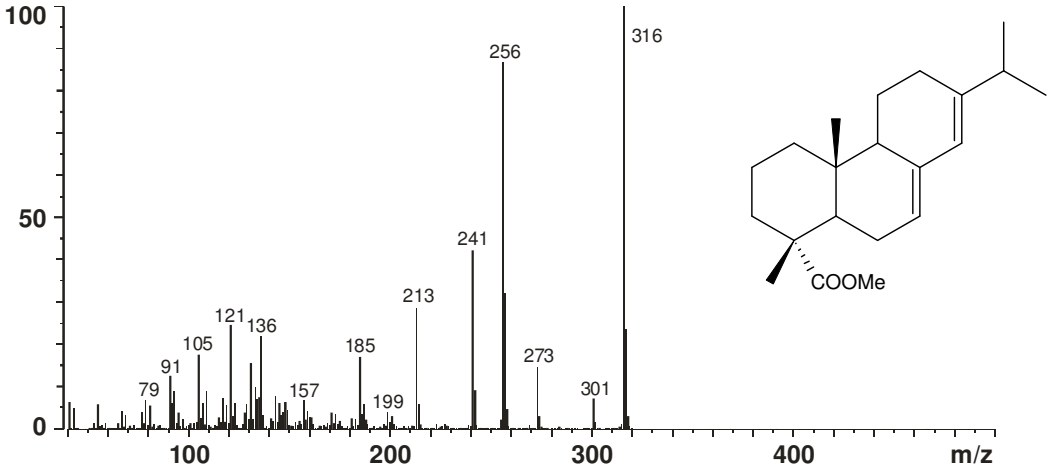
Pyrolysis product of polycommunic acid, methyl ester	70
Pyrolysis product of polycommunic acid, methyl ester	71
Sandaracopimaric acid, methyl ester (P2)	49
Trans-caryphyllene (S6)	56
Unidentified kaurane (k4)	38



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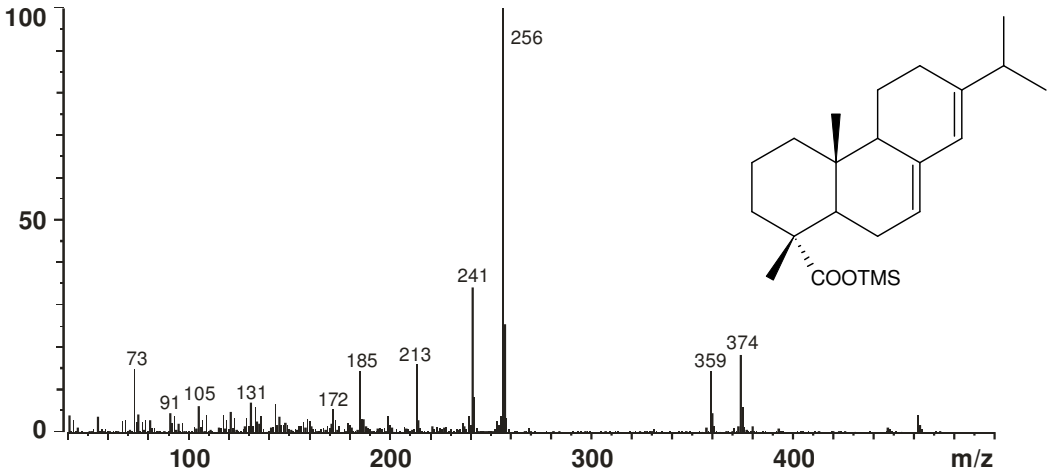
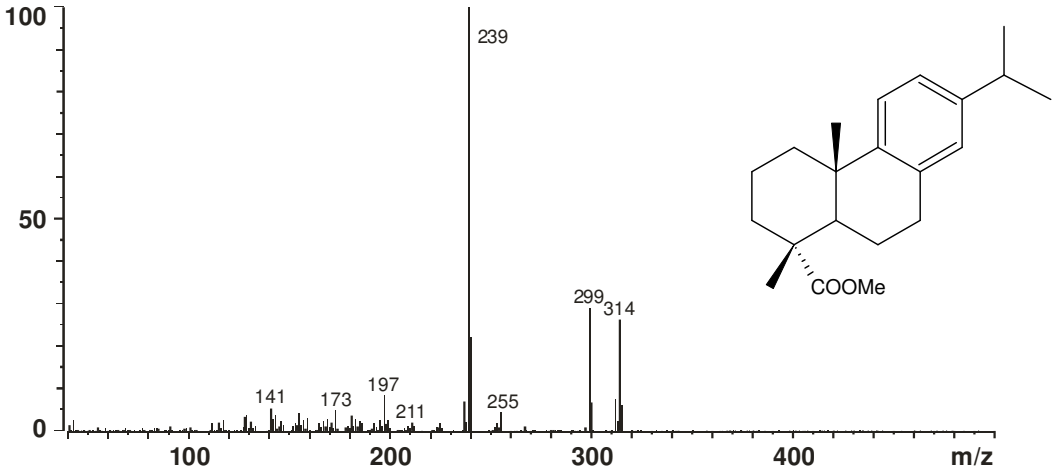
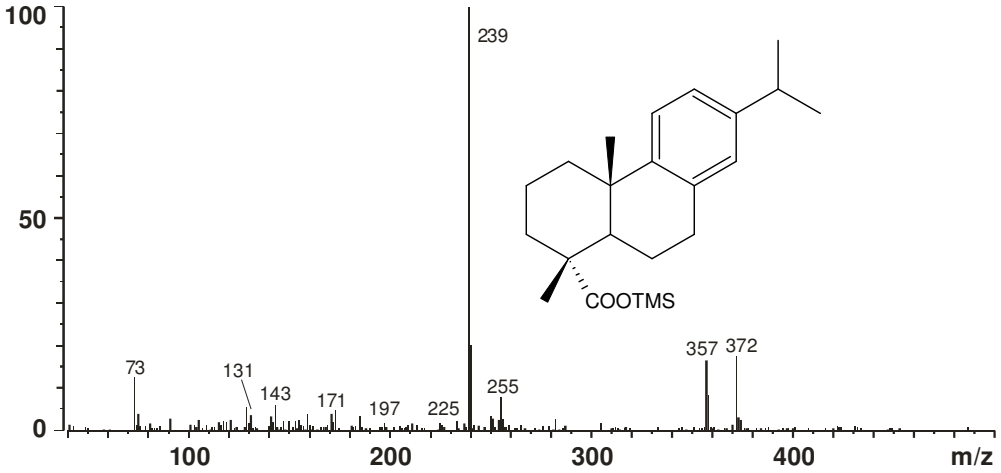


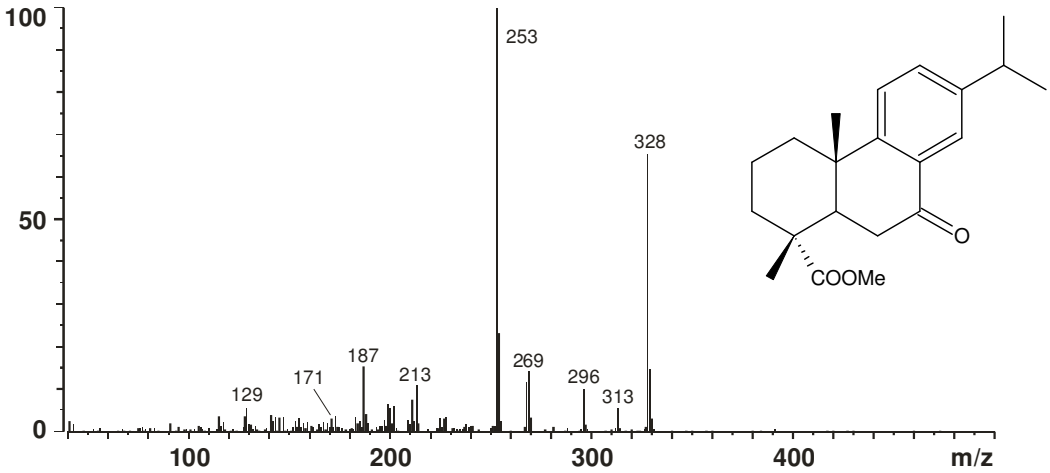
2 MW=374 Abietic acid, TMS ester



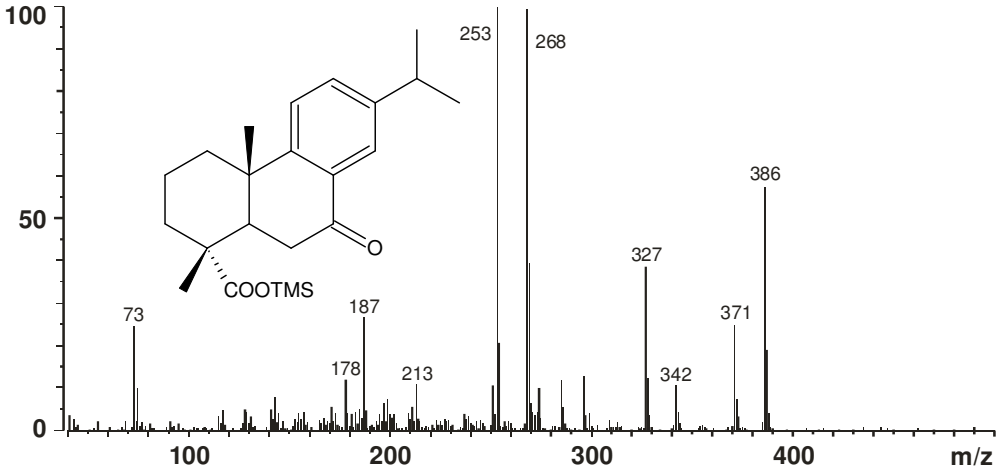
3 MW=314 Dehydroabietic acid (DHA), methyl ester



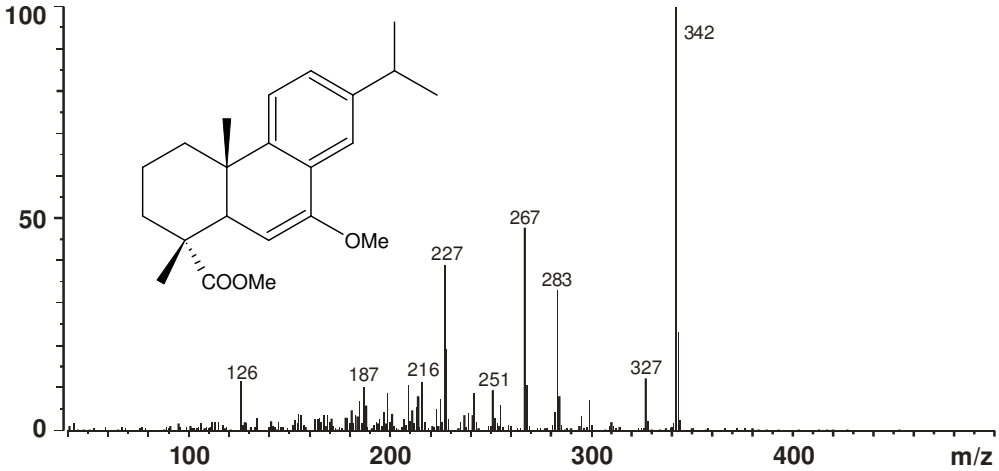
4 MW=372 Dehydroabietic acid (DHA), TMS ester



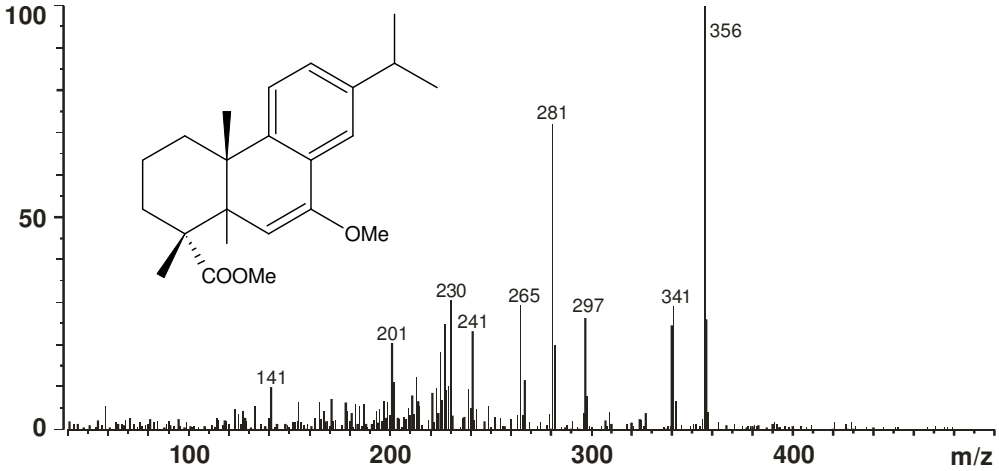
5 MW=328 7-oxo-DHA, methyl ester



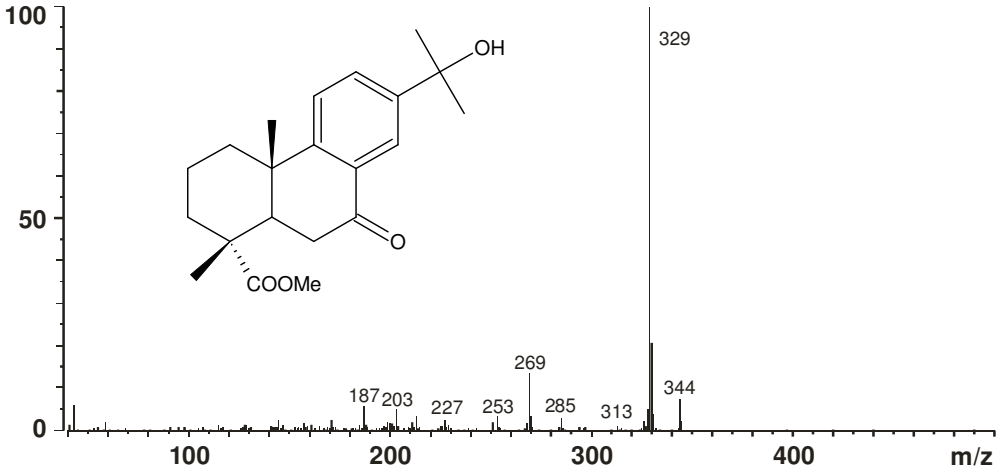
6 MW=386 7-oxo-DHA, TMS ester



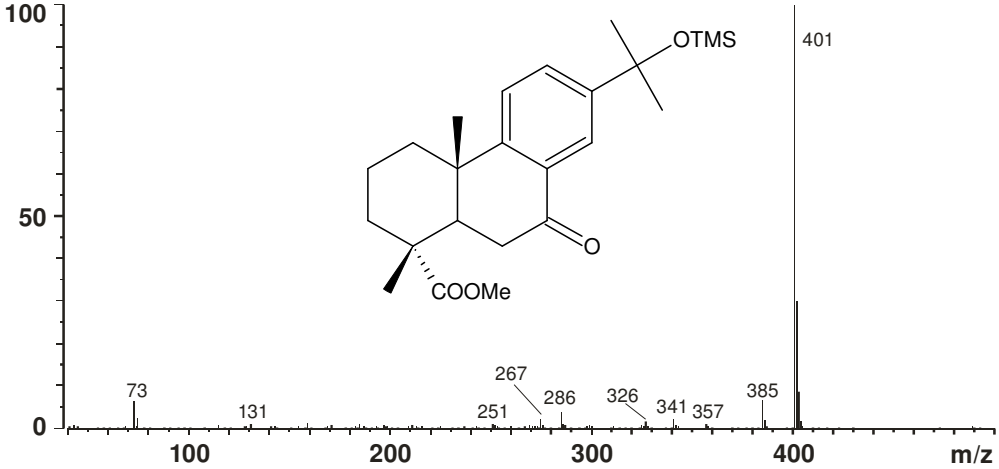
7 MW=342 7-oxo-DHA, dimethylated derivative



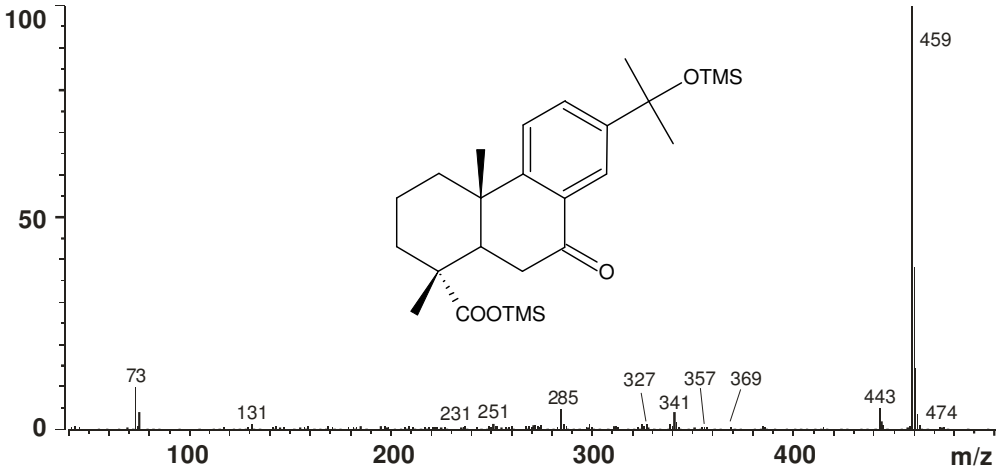
8 MW=356 7-oxo-DHA, trimethylated derivative



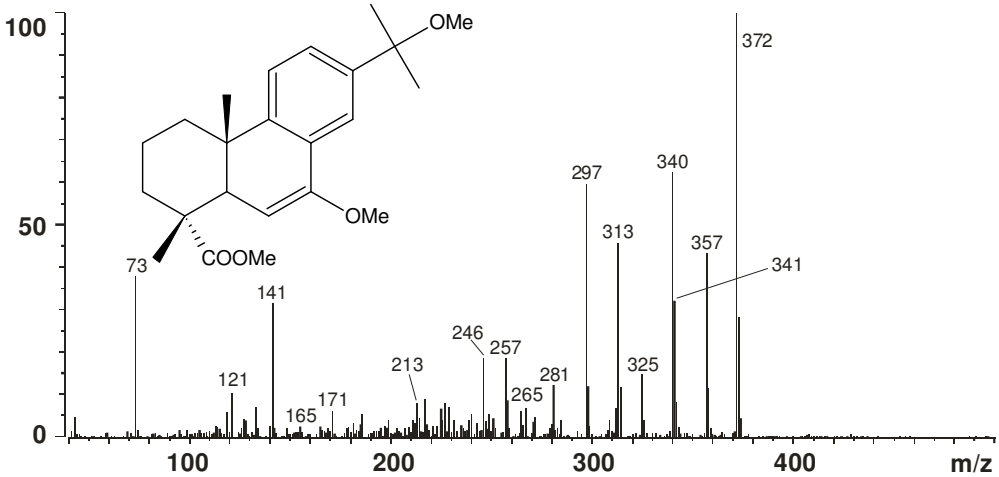
9 MW=344 15-hydroxy-7-oxo-DHA, methyl ester



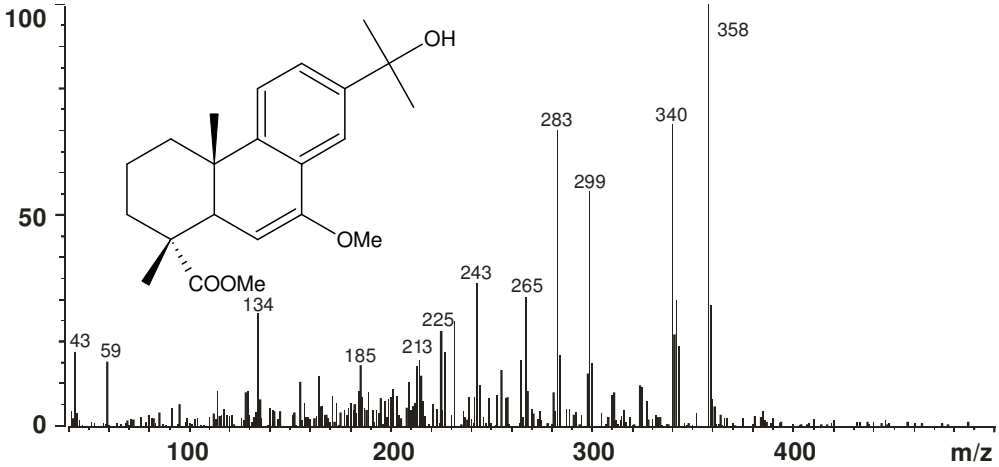
10 MW=416 15-hydroxy-7-oxo-DHA, TMS ether, methyl ester



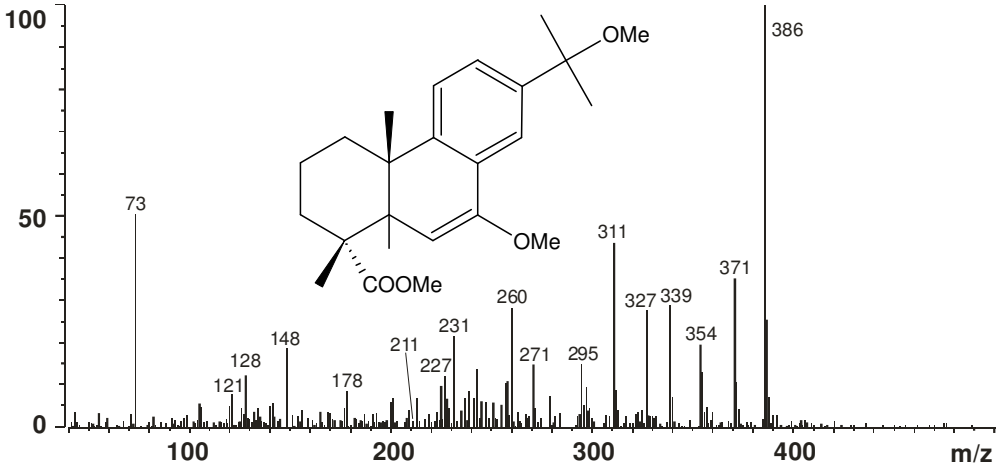
11 MW=474 15-hydroxy-7-oxo-DHA, TMS ether, TMS ester



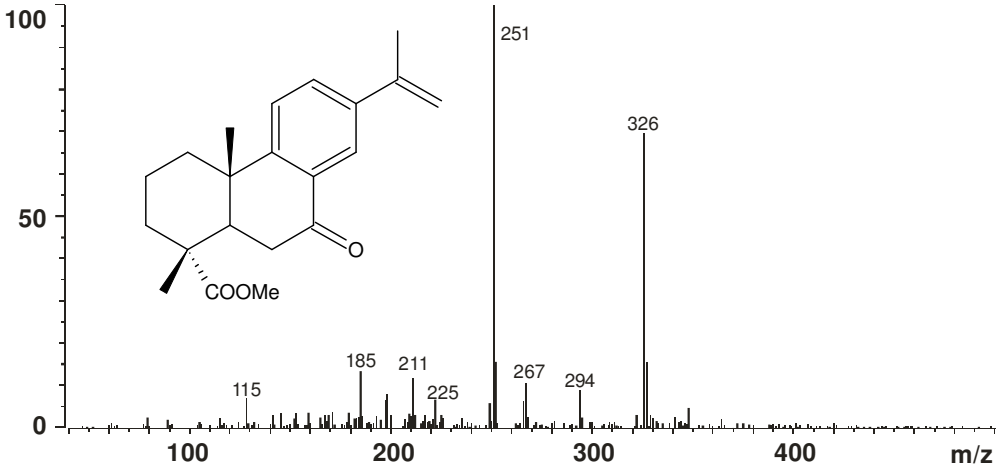
12 MW=372 15-hydroxy-7-oxo-DHA, trimethylated derivative



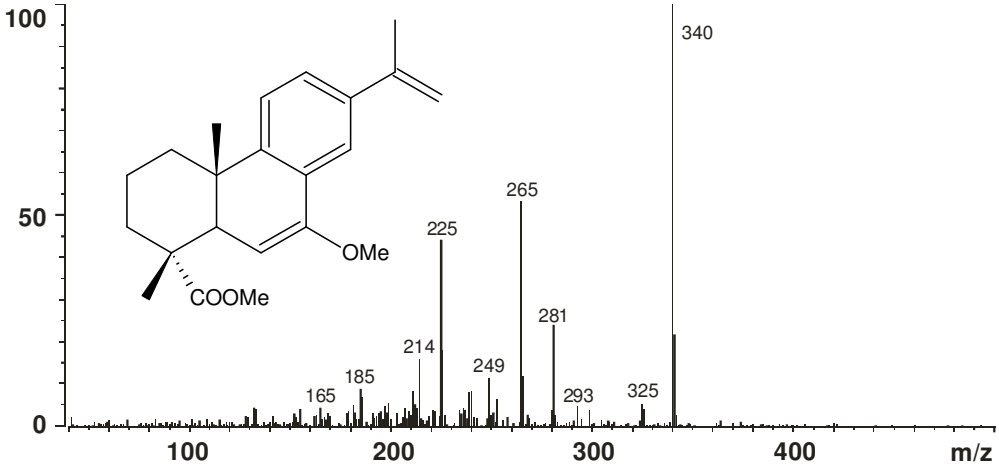
13 MW=358 15-hydroxy-7-oxo-DHA, dimethylated derivative



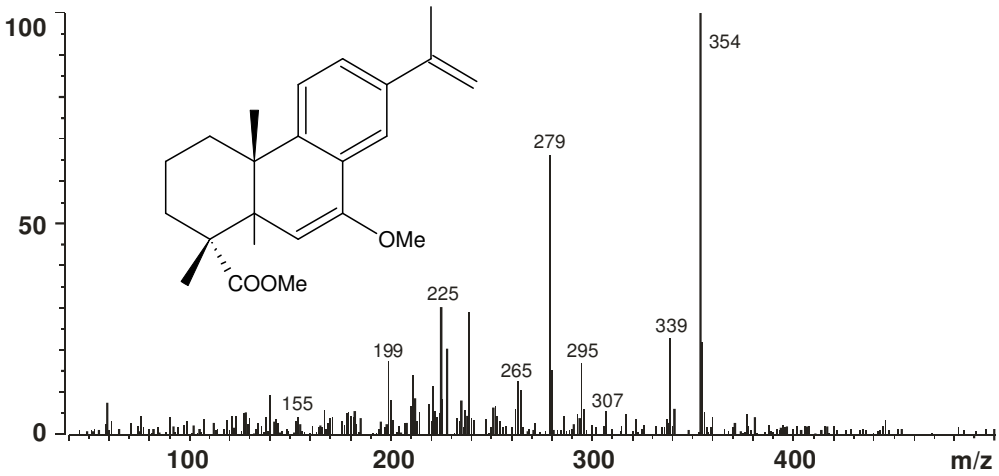
14 MW=386 15-hydroxy-7-oxo-DHA, tetramethylated derivative



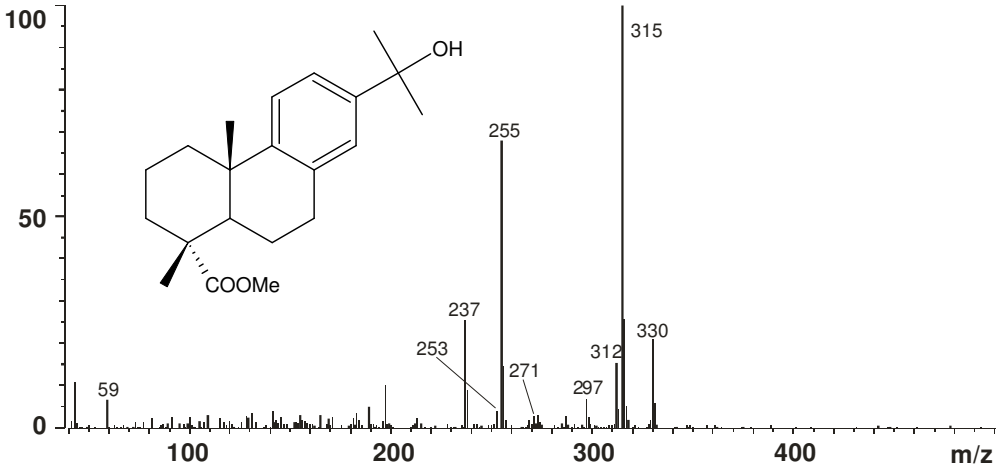
15 MW=326 15-hydroxy-7-oxo-DHA, methyl ester (dehydrogenated)



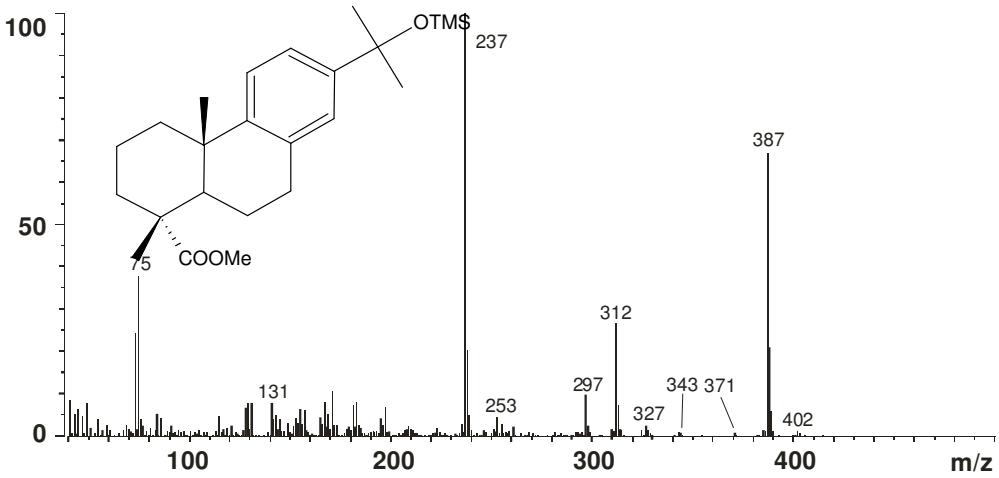
16 MW=340 15-hydroxy-7-oxo-DHA, dimethylated derivative (dehydrogenated)



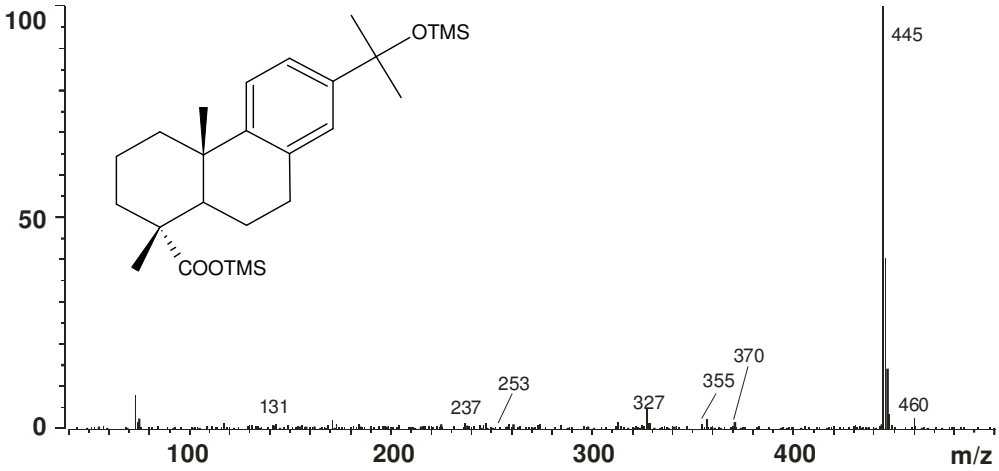
17 MW=354 15-hydroxy-7-oxo-DHA, trimethylated derivative (dehydrogenated)



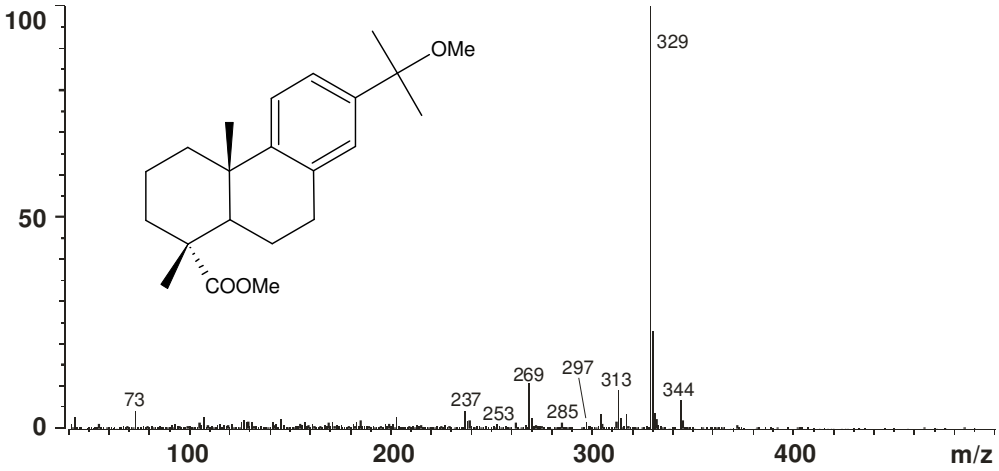
18 MW=330 15-hydroxy-DHA, methyl ester



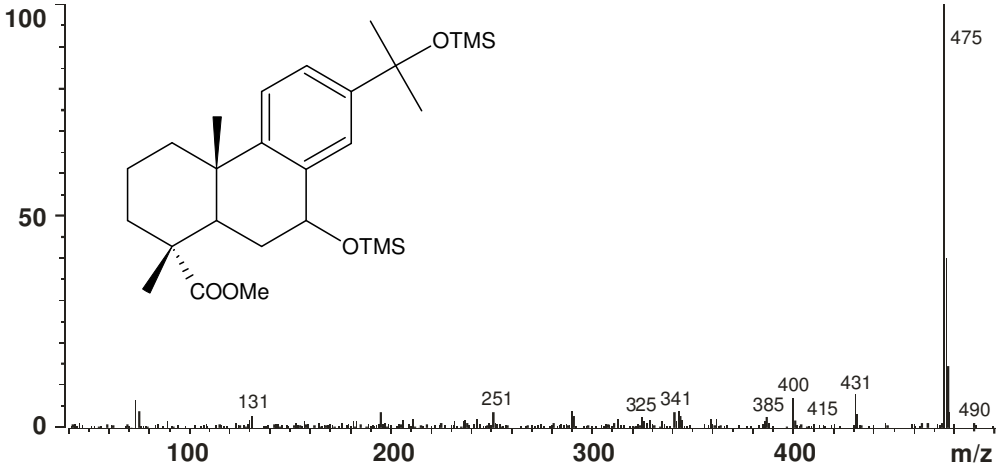
19 MW=402 15-hydroxy-DHA, TMS ether, methyl ester



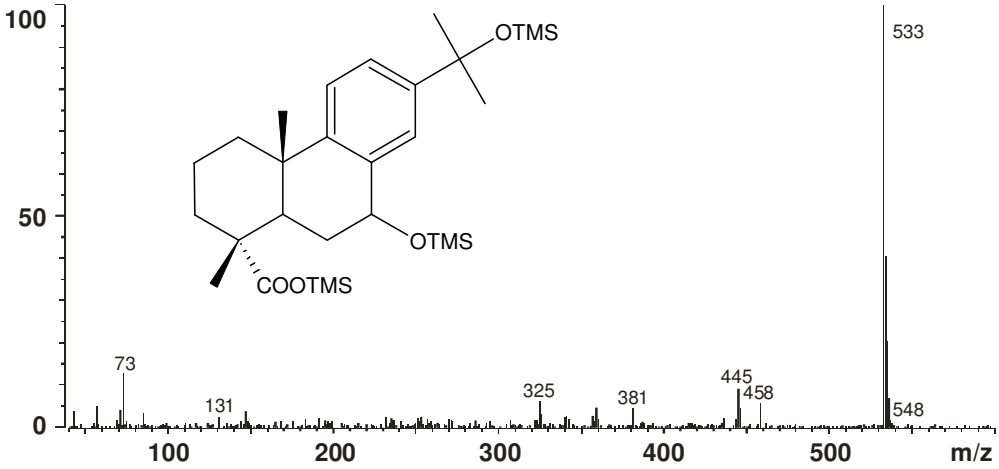
20 MW=460 15-hydroxy-DHA, TMS ether, TMS ester



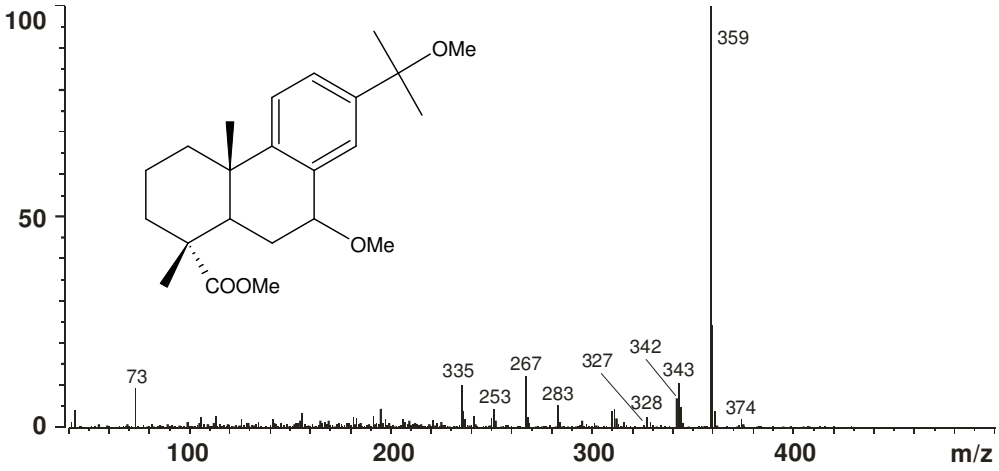
21 MW=344 15-hydroxy-DHA, methyl ether, methyl ester



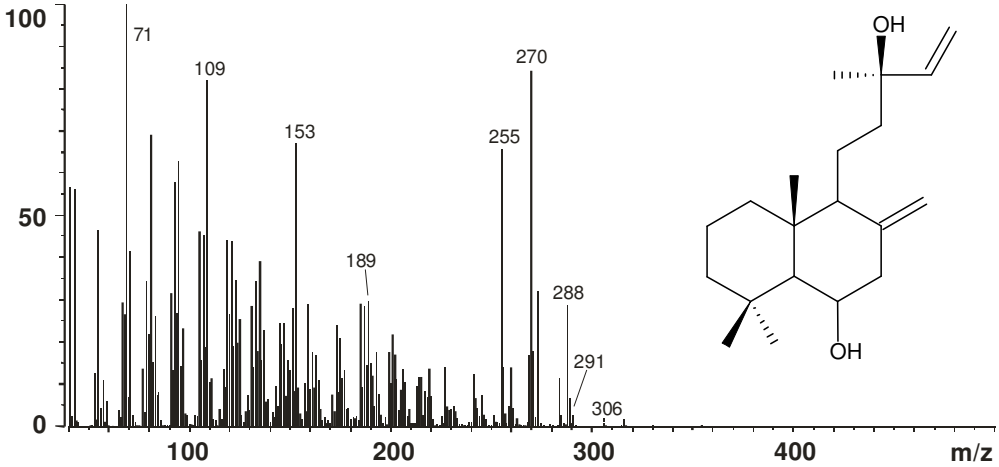
22 MW=490 7,15-dihydroxy-DHA, di-TMS ether, methyl ester



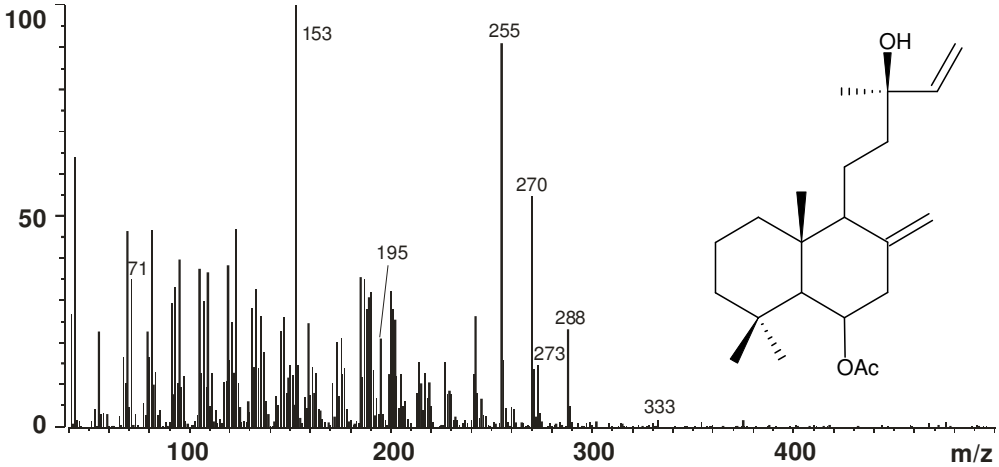
23 MW=548 7,15-dihydroxy-DHA, di-TMS ether, TMS ester



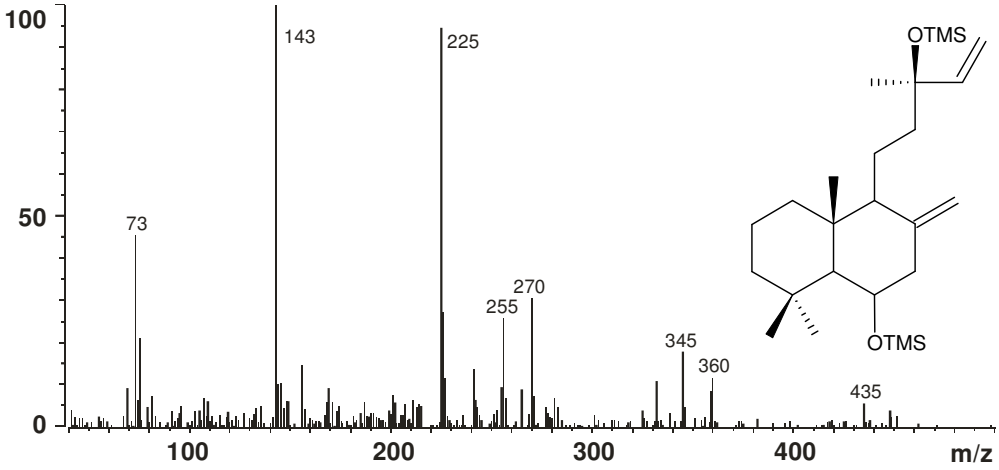
24 MW=374 7,15-dihydroxy-DHA, dimethyl ether, methyl ester



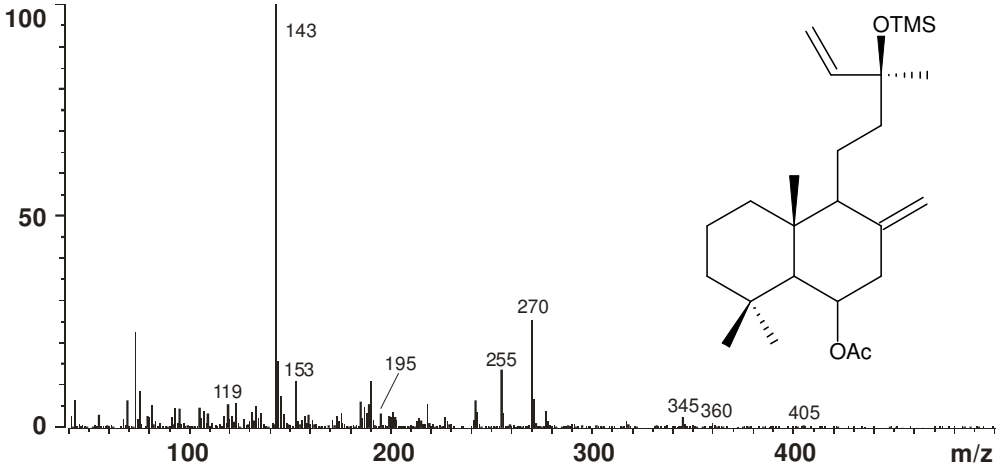
25 MW=306 Larixol



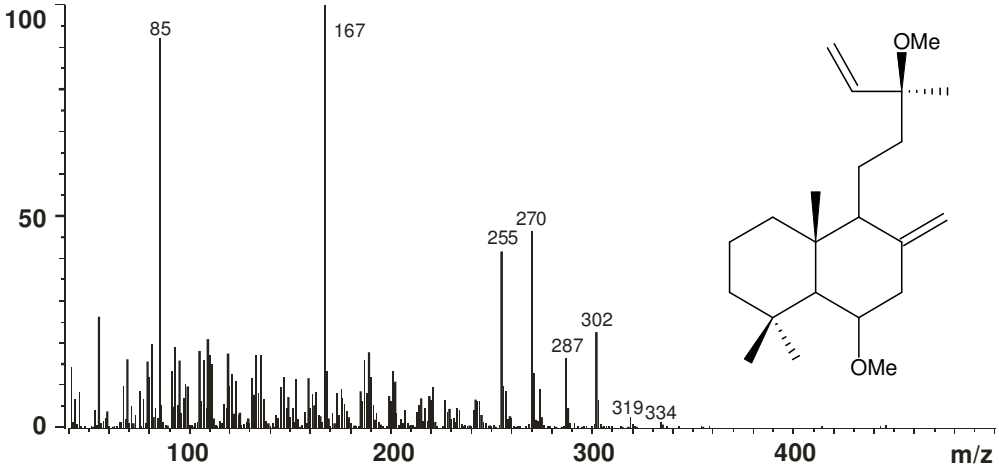
26 MW=348 Larixyl acetate



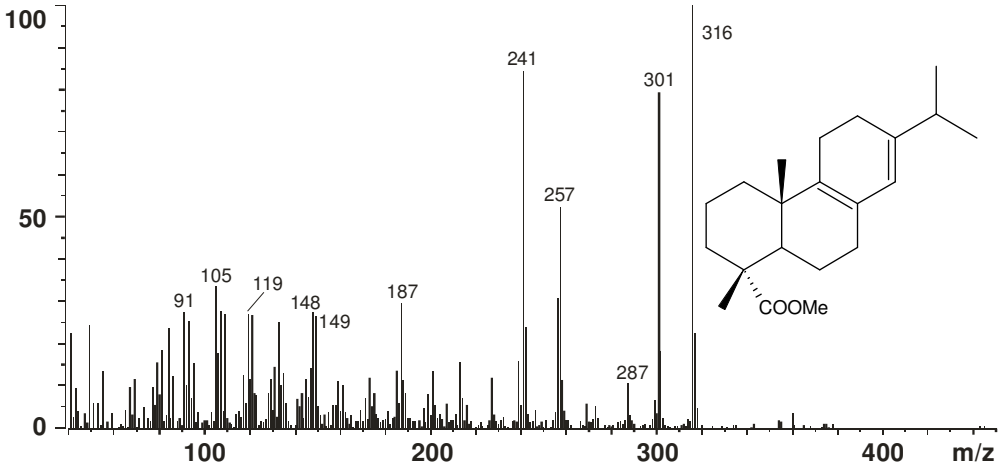
27 MW=450 Larixol, di-TMS ether



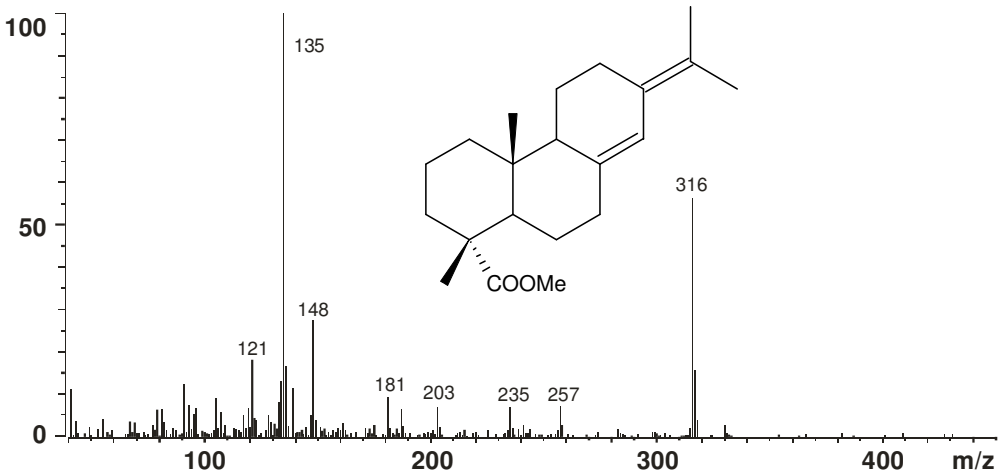
28 MW=420 Larixyl acetate, TMS ether



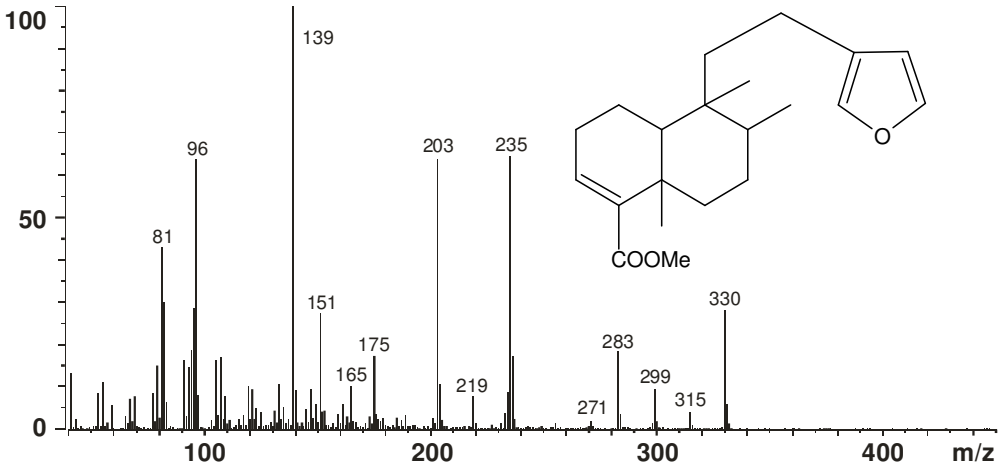
29 MW=334 Larixol, dimethyl ether



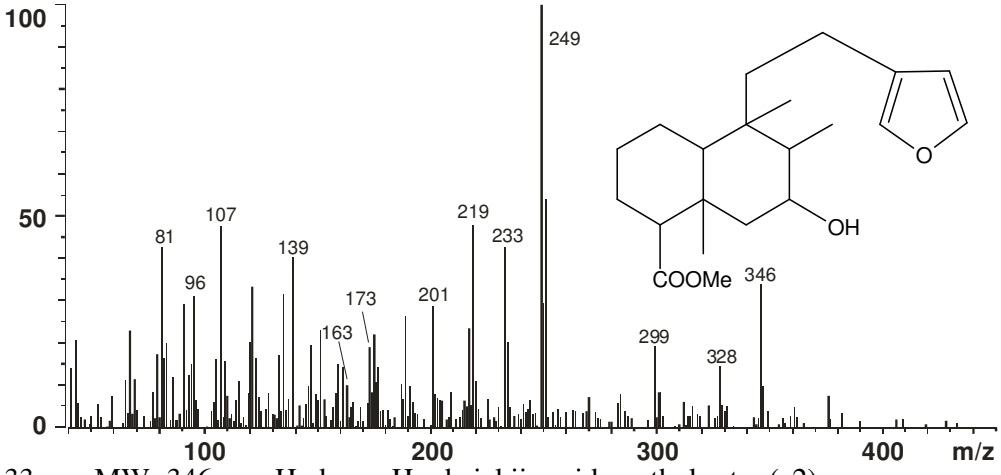
30 MW=316 Palustric acid, methyl ester (a1)



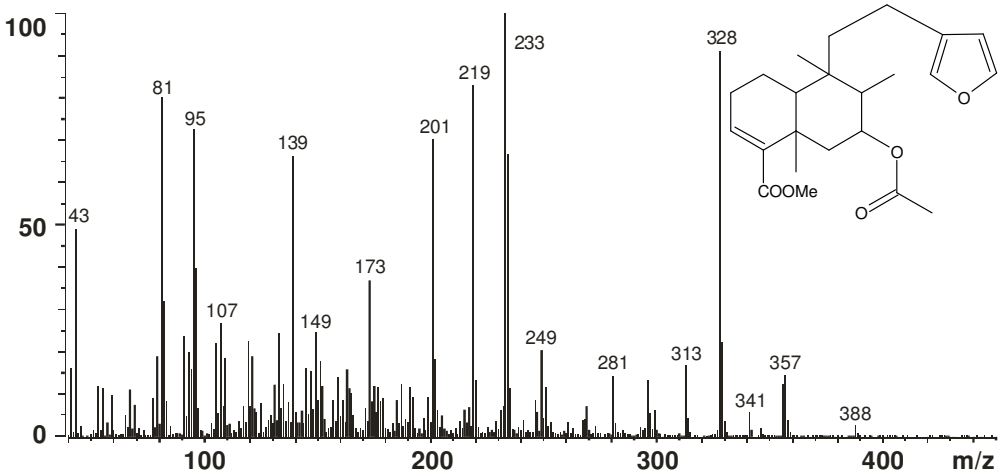
31 MW=316 Neoabietic acid, methyl ester (a4)



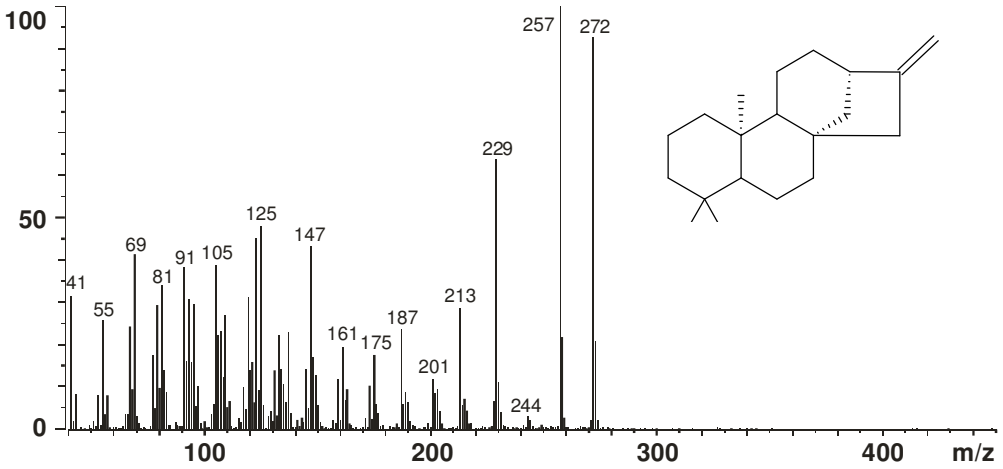
32 MW=330 Hardwickiic acid, methyl ester (c1)



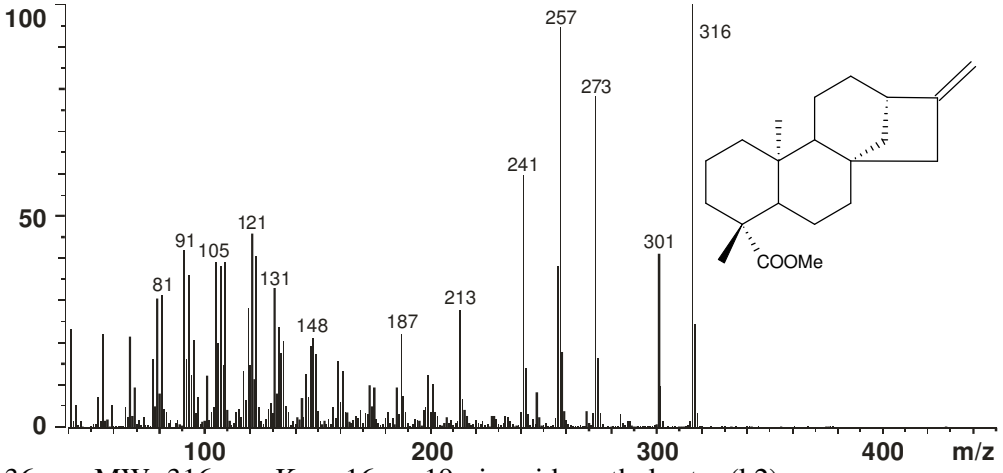
33 MW=346 Hydroxy-Hardwickiic acid, methyl ester (c2)



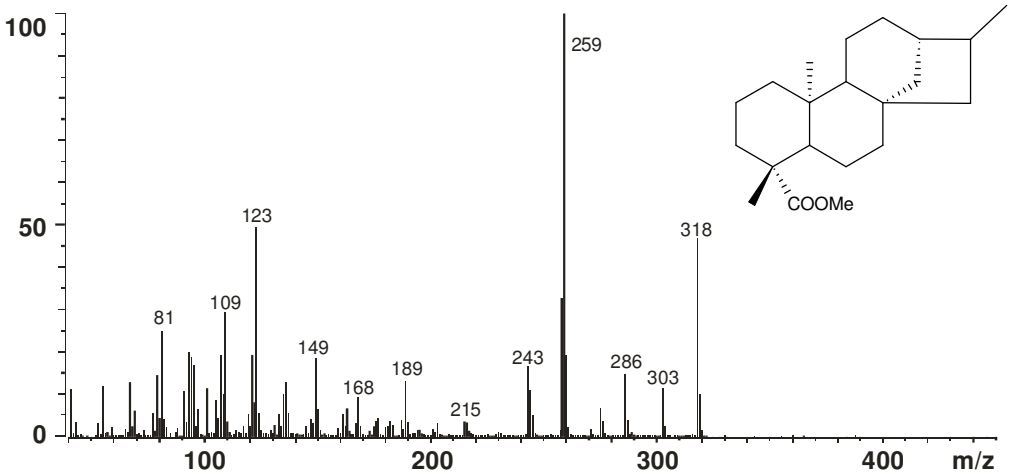
34 MW=388 Acetoxy-Hardwickiic acid, methyl ester (c3)



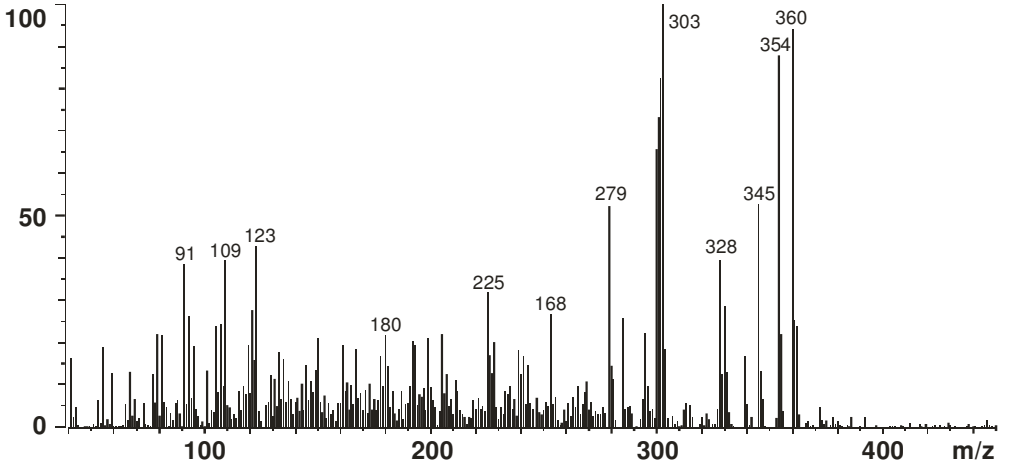
35 MW=272 (-)-16-kaurene (k1)



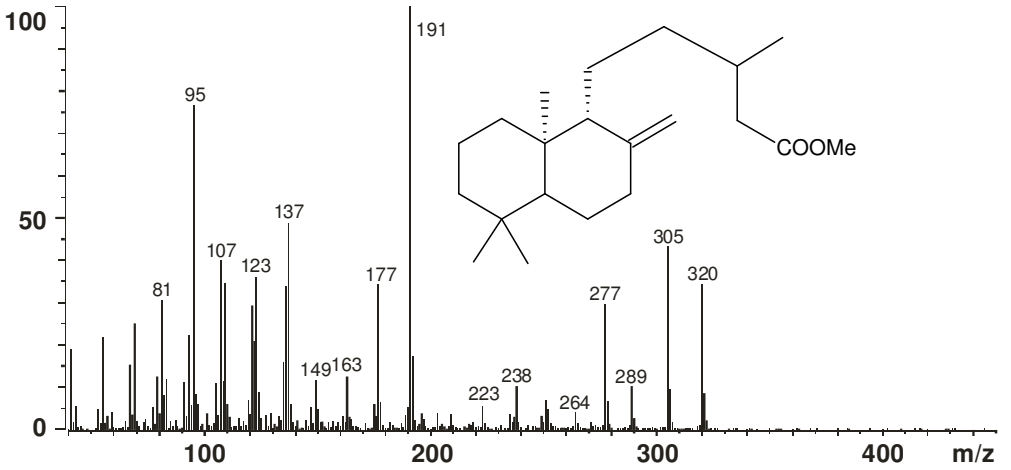
36 MW=316 Kaur-16-en-19-oic acid, methyl ester (k2)



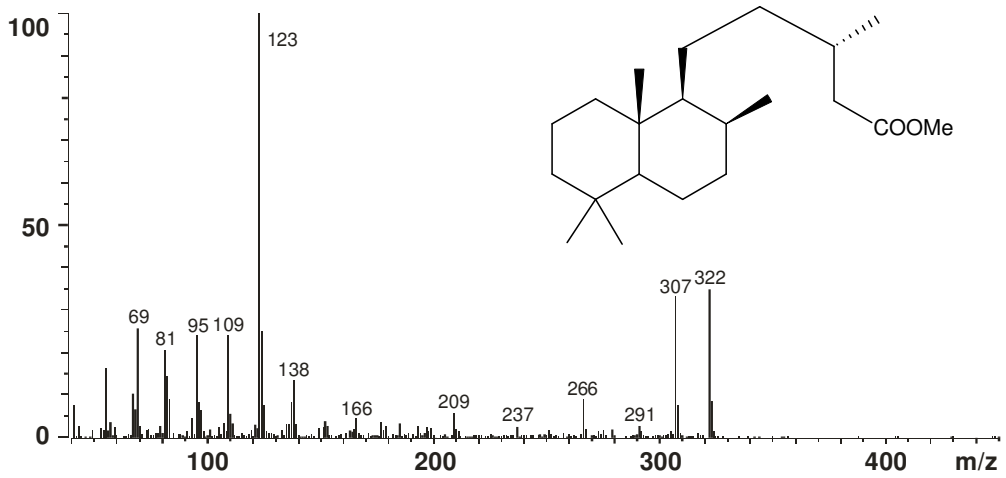
37 MW=318 16ß-kauran-19-oic acid, methyl ester (k3)



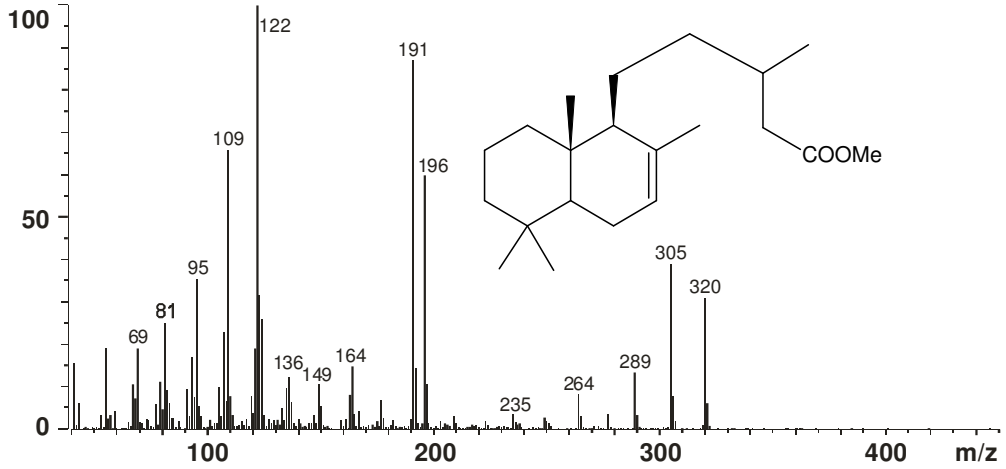
38 MW=360 Unidentified kaurane (k4) NB: not pure; e.g. m/z 354 and 279 probably derive from a co-eluting oxidised dehydroacbietic acid with M = 354



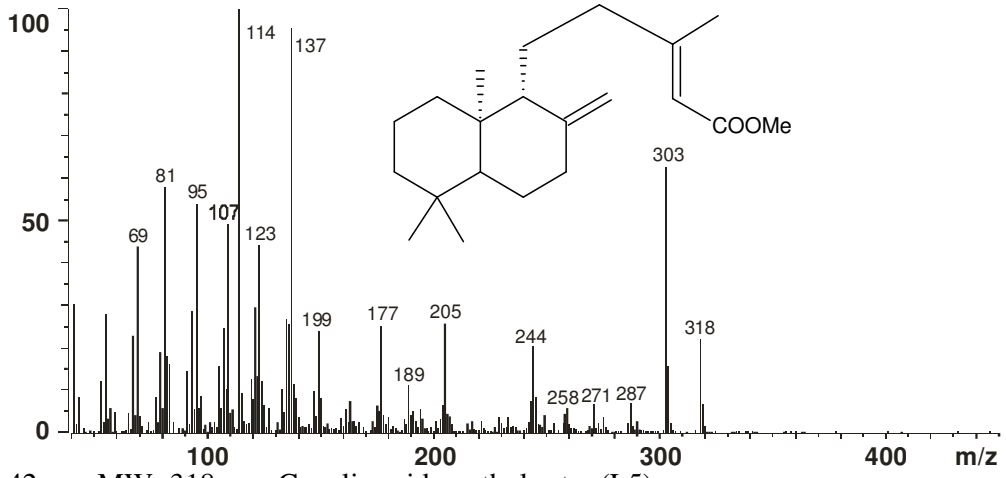
39 MW=320 Eperuic acid, methyl ester (L1)



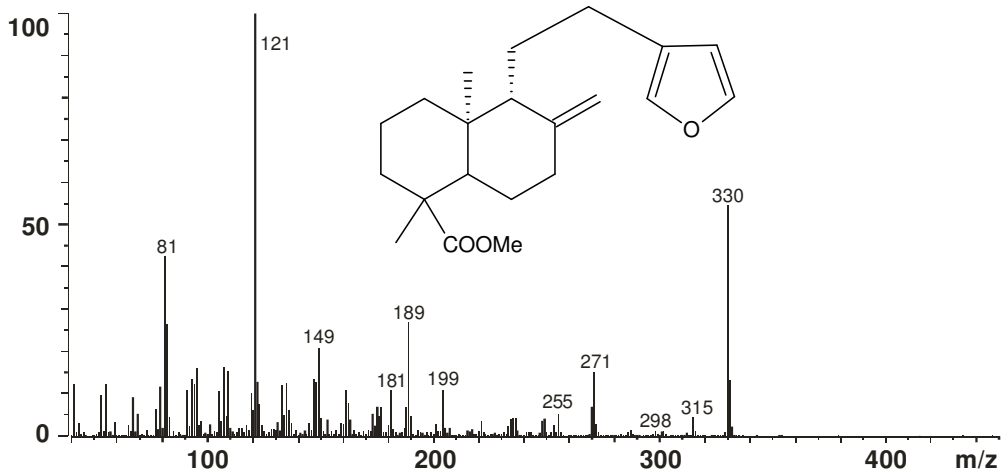
40 MW=322 Labdanic acid, methyl ester (L2)



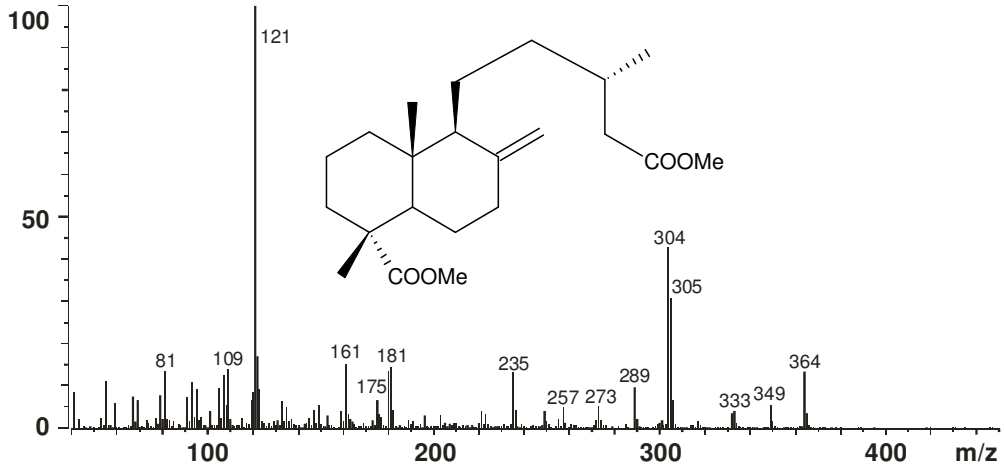
41 MW=320 Cativic acid, methyl ester (L3)



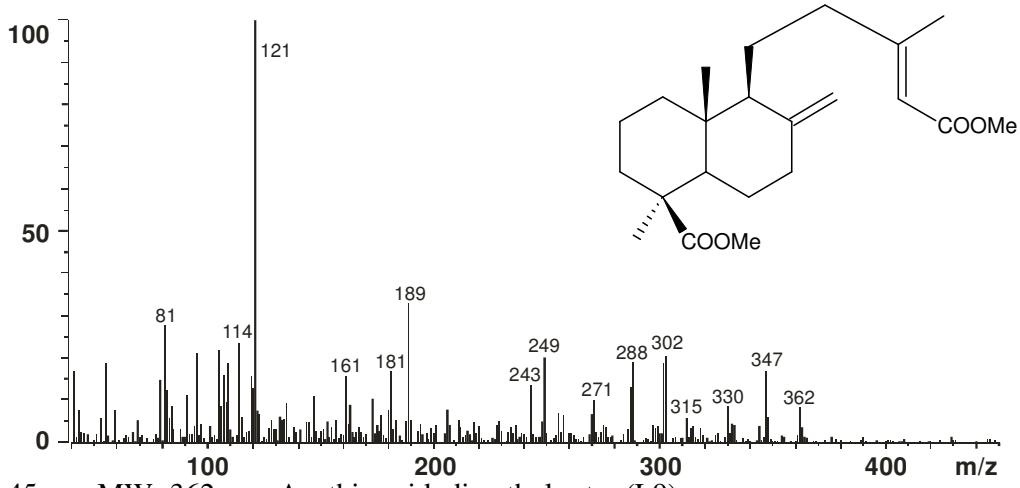
42 MW=318 Copalic acid, methyl ester (L5)



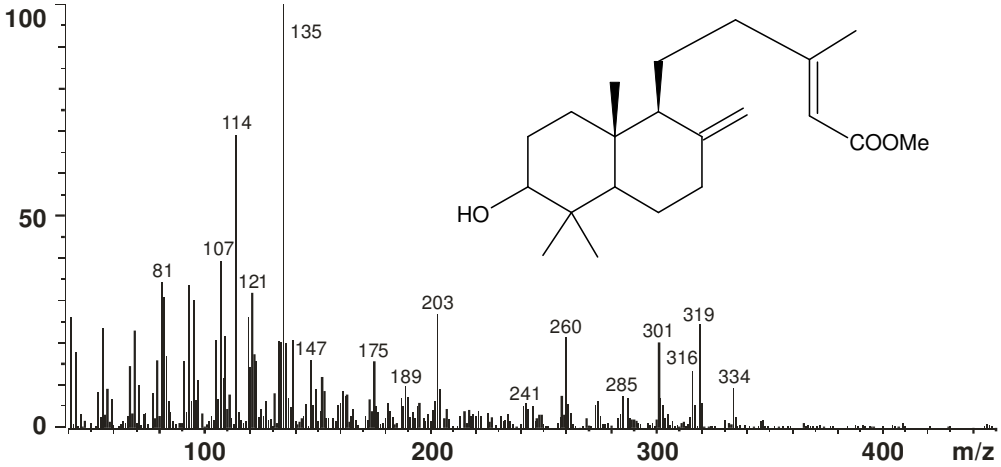
43 MW=330 Polyalthic acid, methyl ester (L6)



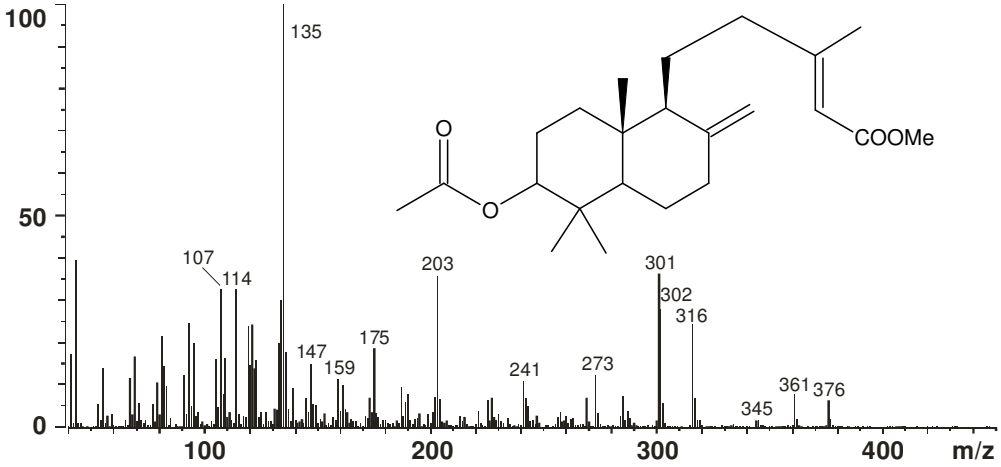
44 MW=364 Pinifolic acid, dimethyl ester (L8)



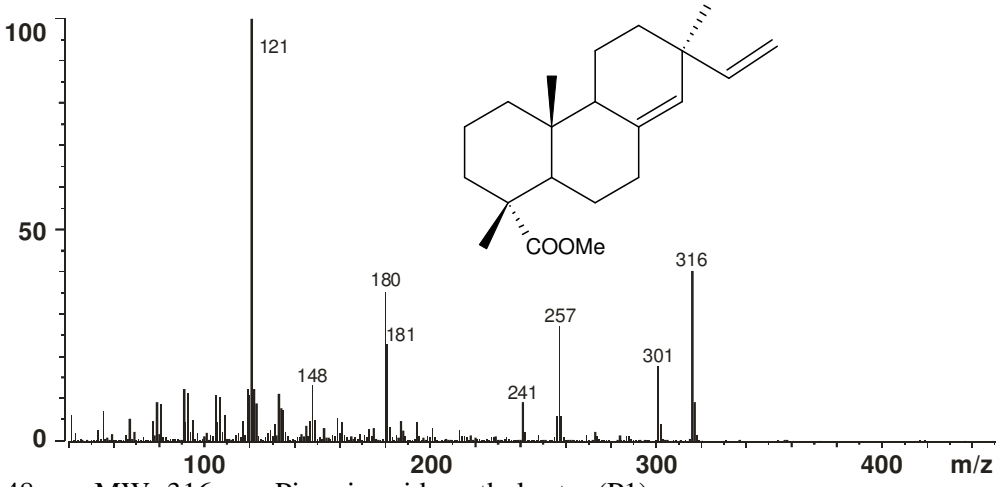
45 MW=362 Agathic acid, dimethyl ester (L9)



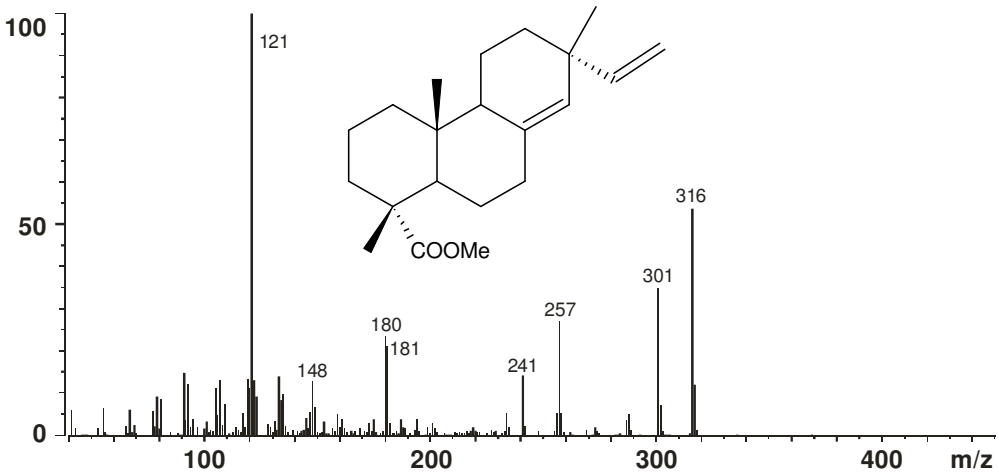
46 MW=334 3-hydroxy-copaiferic acid, methyl ester (L10)



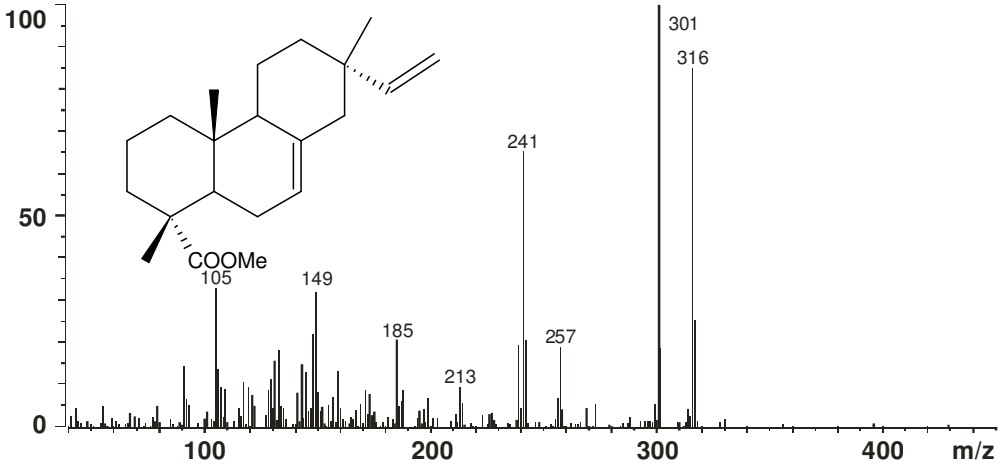
47 MW=376 3-acetoxy-copaiferic acid, methyl ester (L11)



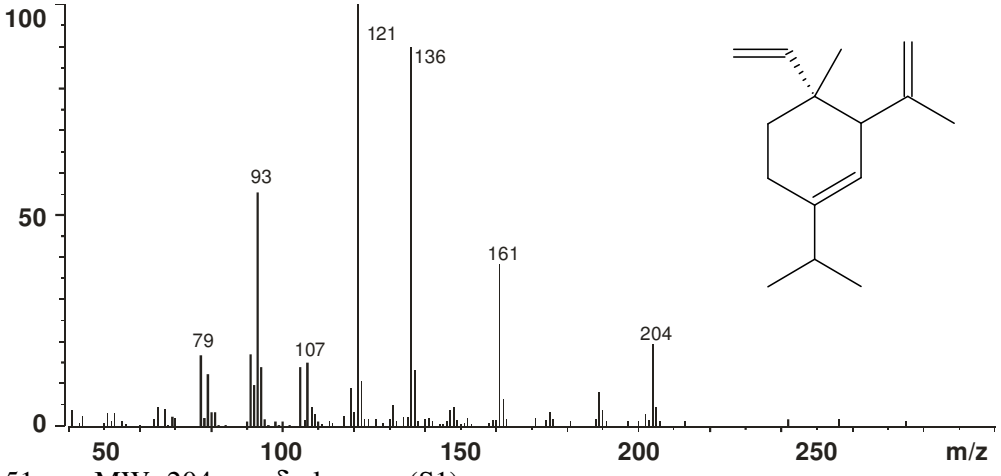
48 MW=316 Pimaric acid, methyl ester (P1)



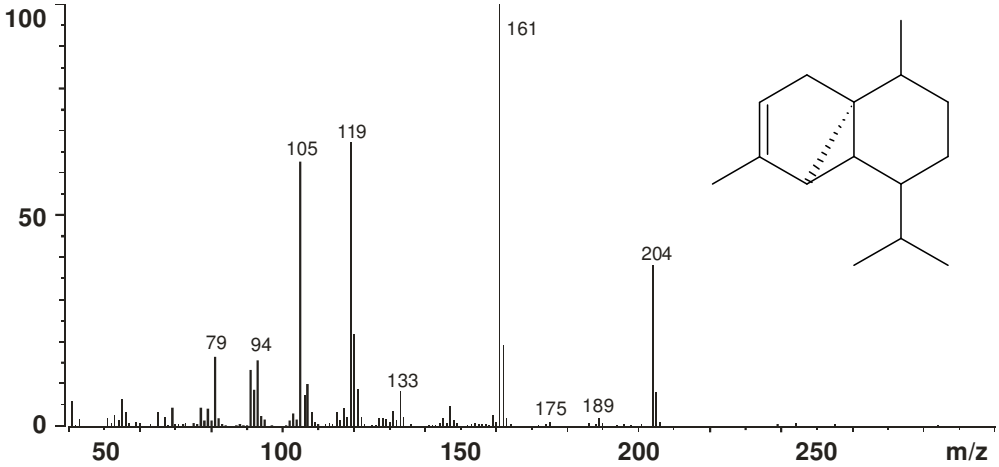
49 MW=316 Sandaracopimaric acid, methyl ester (P2)



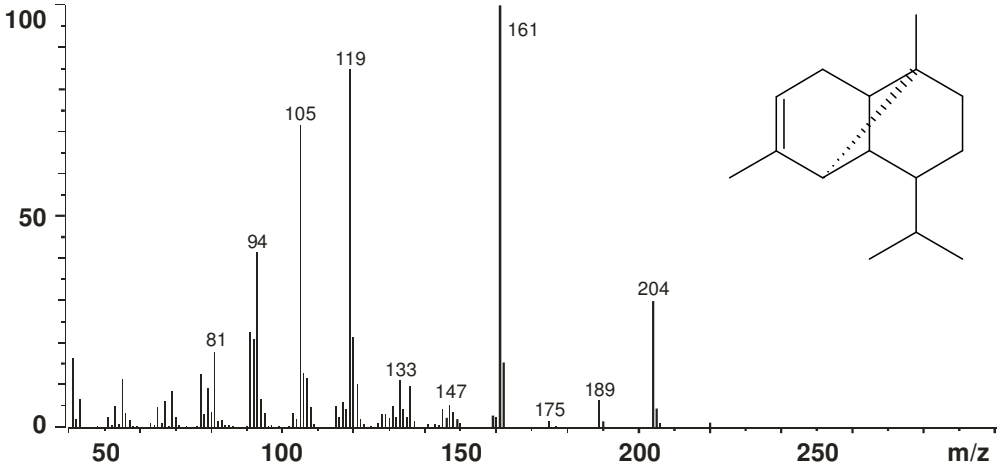
50 MW=316 Isopimaric acid, methyl ester (P3)



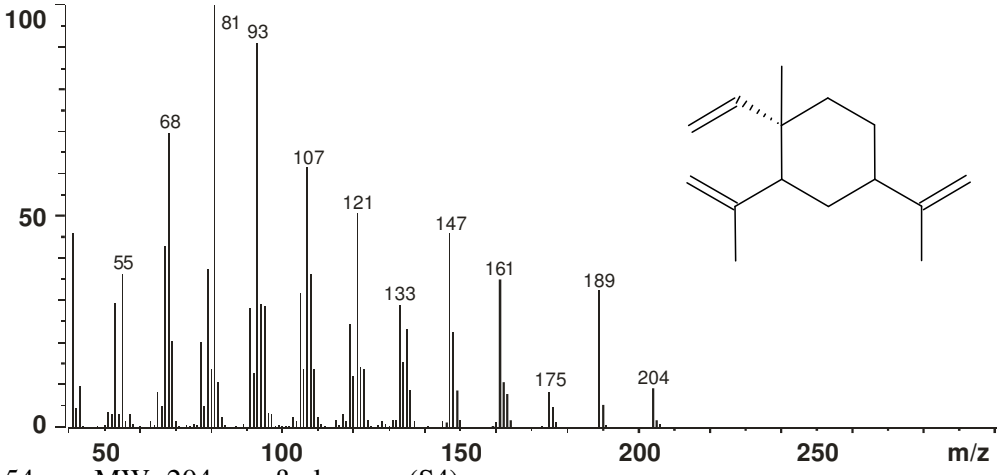
51 MW=204 δ-elemene (S1)



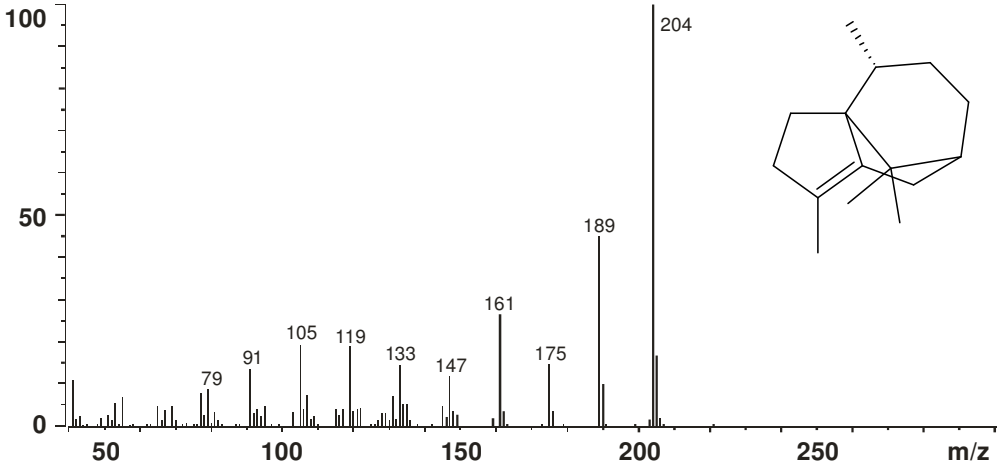
52 MW=204 α-cubebene (S2)



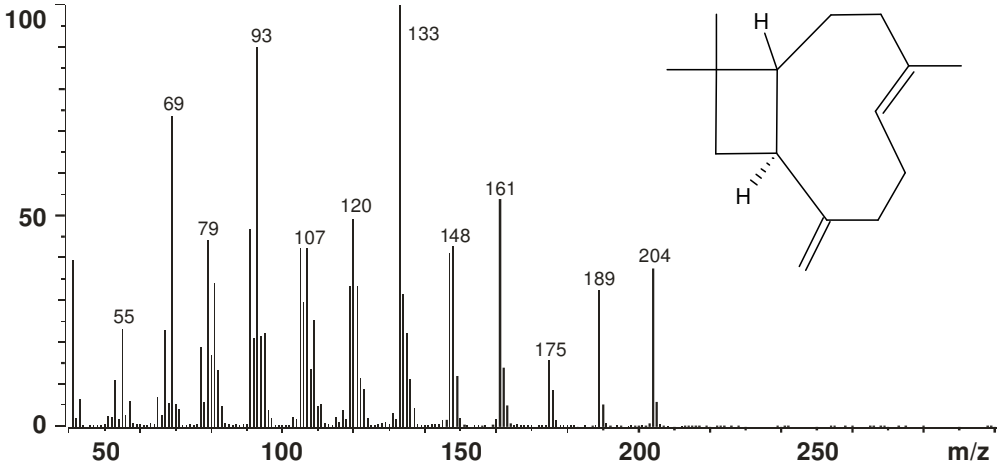
53 MW=204 α-copaene (S3)



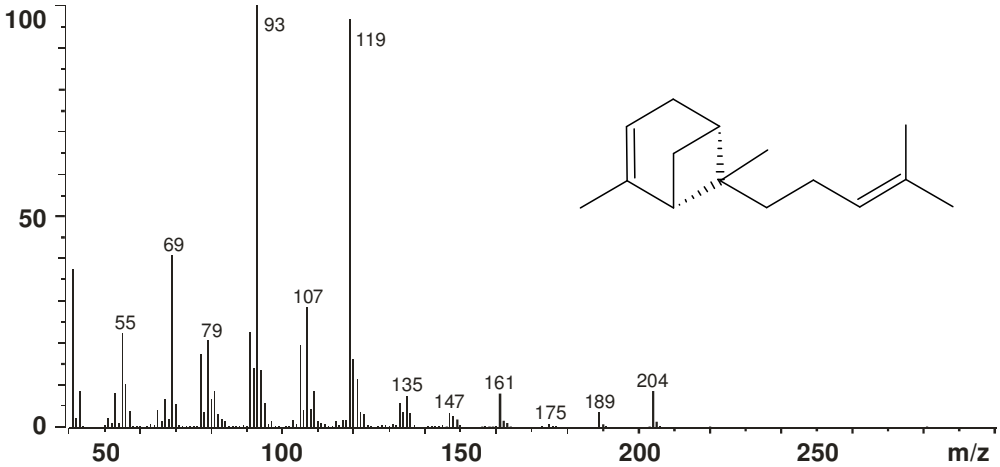
54 MW=204 ß-elemene (S4)



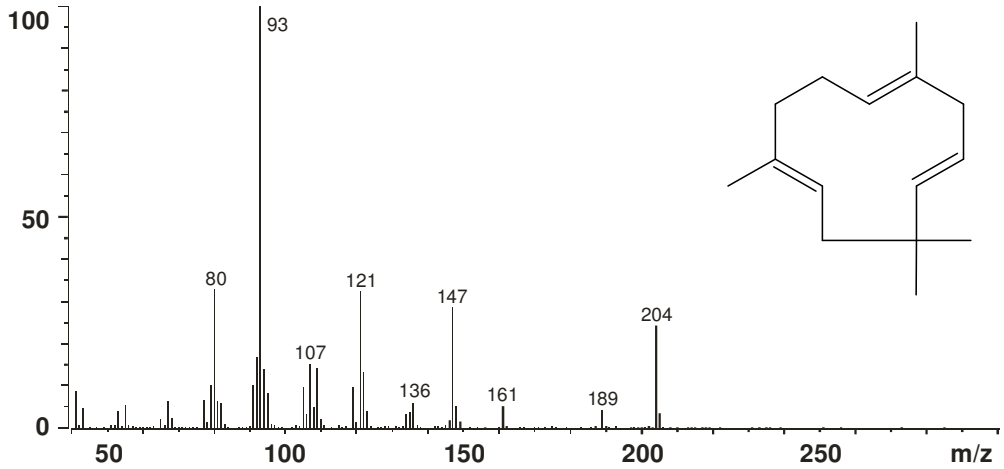
55 MW=204 Cyperene (S5)



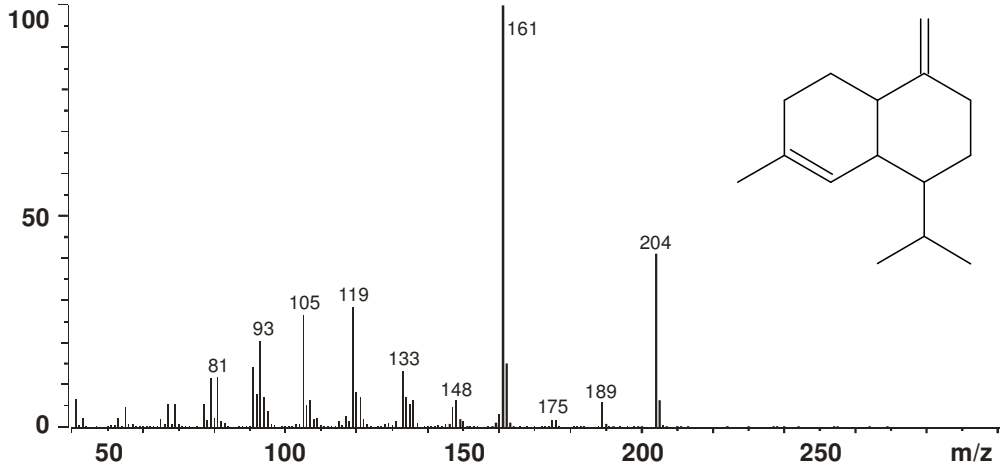
56 MW=204 Trans-caryphyllene (S6)



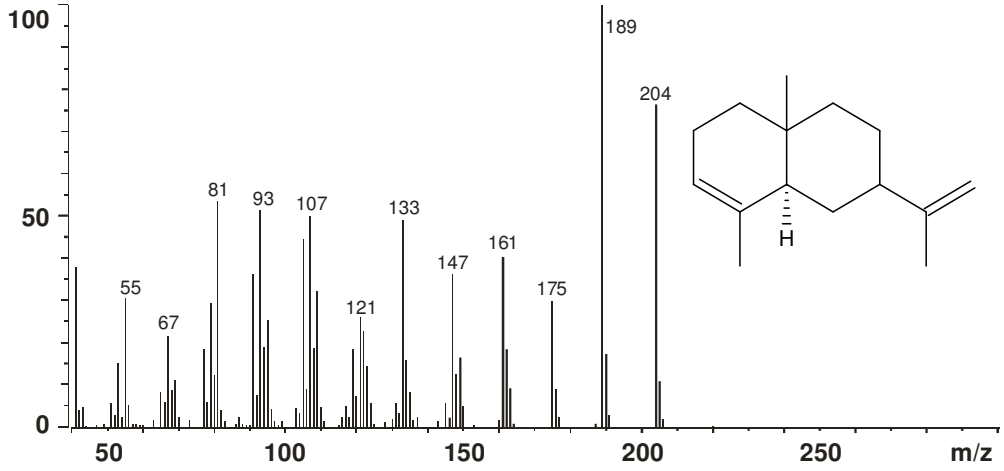
57 MW=204 α-bergamotene (S7)



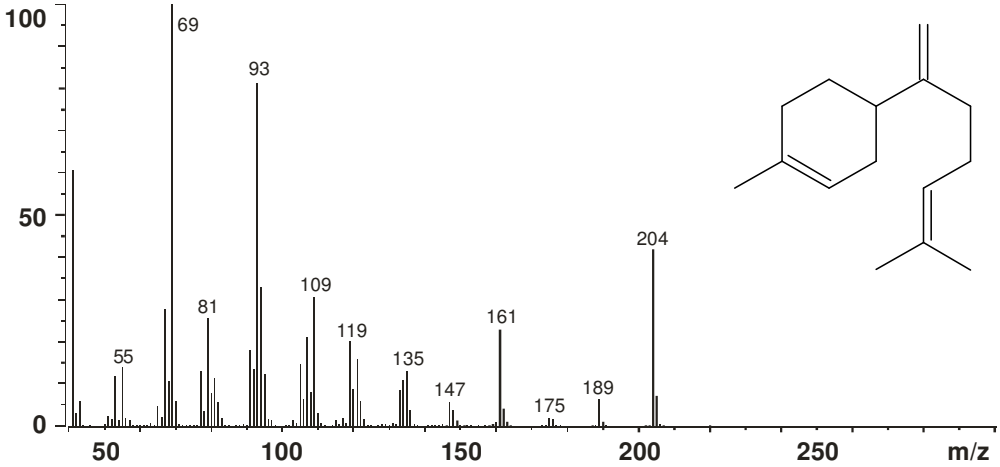
58 MW=204 α-humulene (S8)



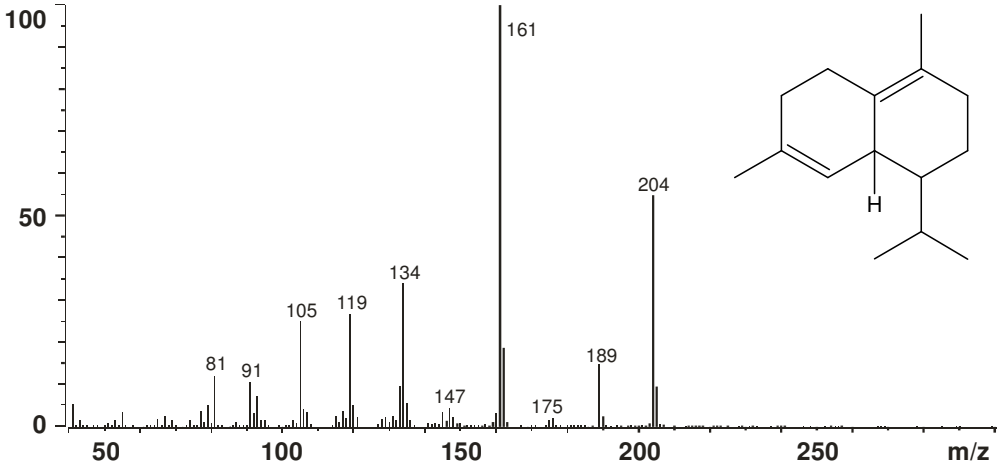
59 MW=204 γ-cadinene (S9)



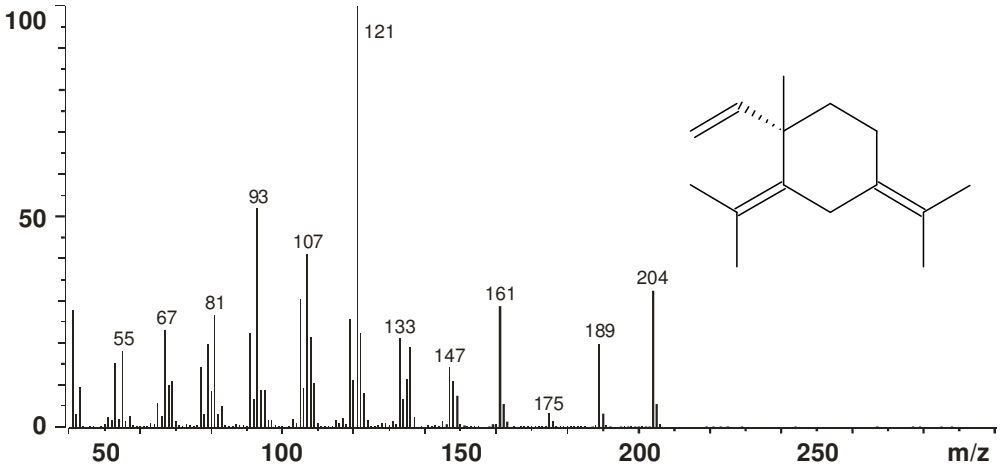
60 MW=204 α-selinene (S10)



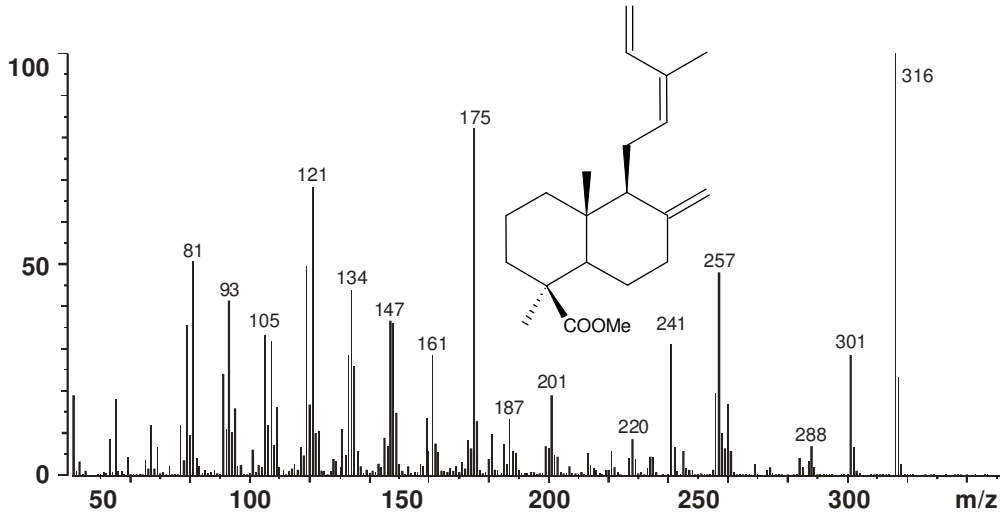
61 MW=204 ß-bisabolene (S11)



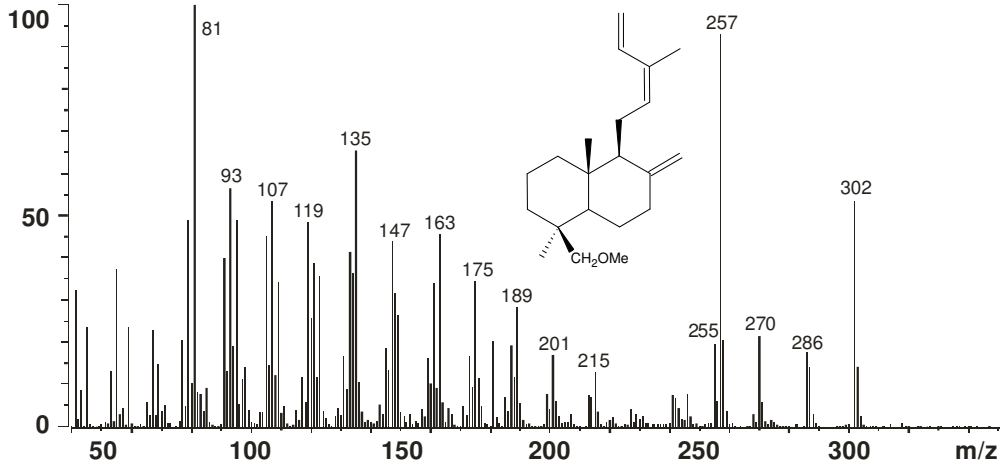
62 MW=204 δ-cadinene (S12)



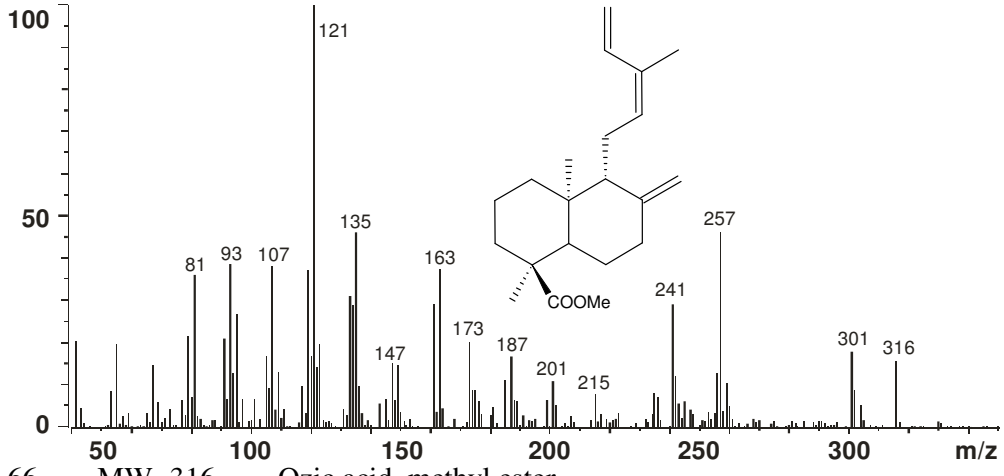
63 MW=204 γ-elemene (S13)



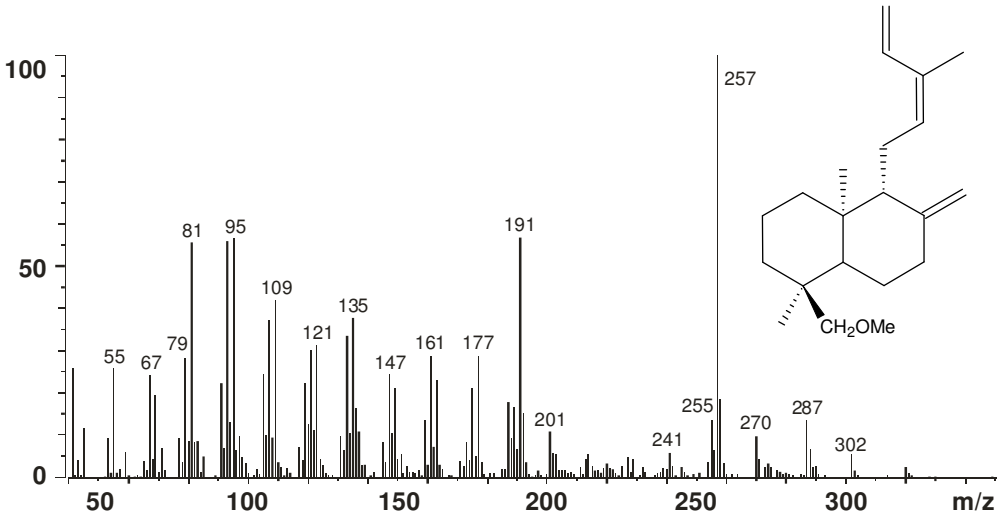
64 MW=316 Communic acid, methyl ester



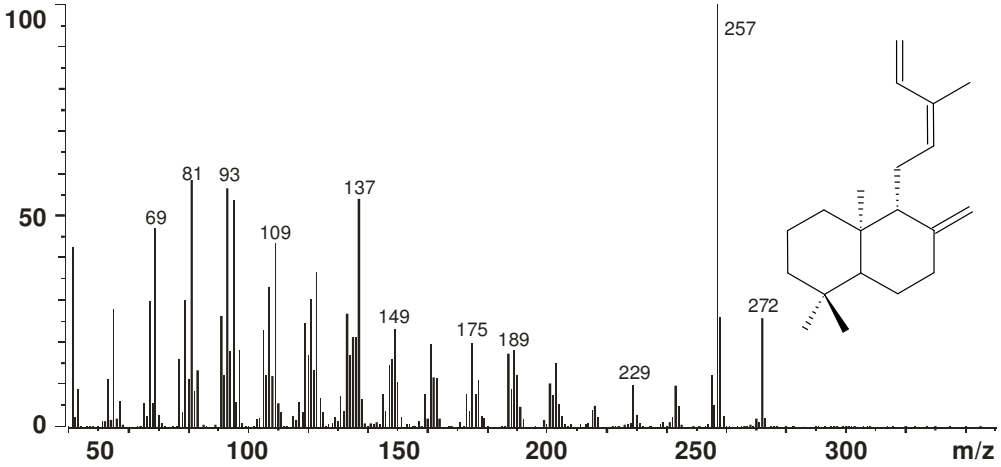
65 MW=302 Communol, methyl ether



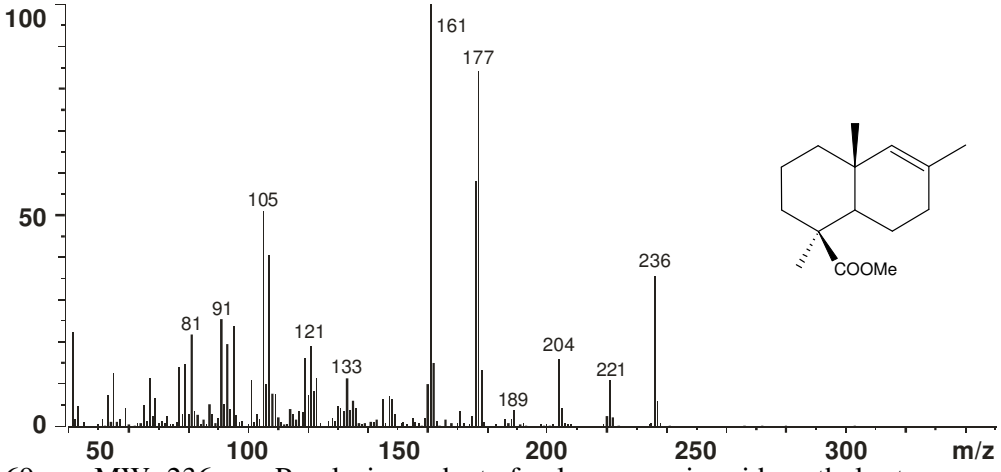
66 MW=316 Ozic acid, methyl ester



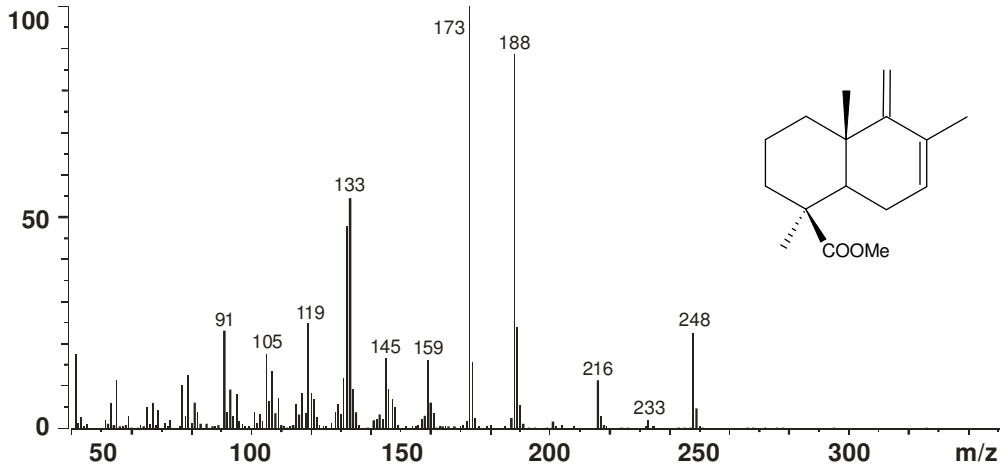
67 MW=302 Ozol, methyl ether



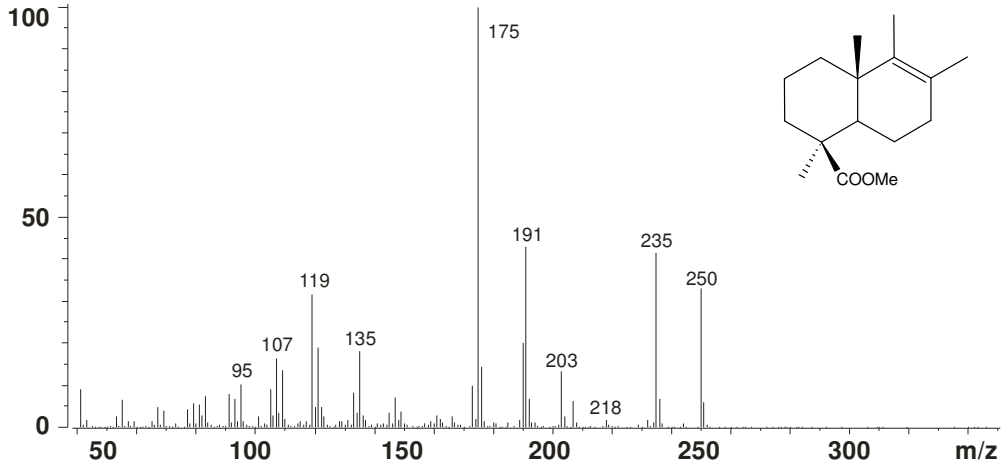
68 MW=272 e*nantio*-biformene



69 MW=236 Pyrolysis product of polycommunic acid, methyl ester



70 MW=248 Pyrolysis product of polycommunic acid, methyl ester



71 MW=250 Pyrolysis product of polycommunic acid, methyl ester