Exploring analytical pyrolysis; Characterisation of organic binding media in Ethiopian Christian art using pyrolysis gas chromatography mass spectrometry

Master thesis in heritage science

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Cover illustration: Paint sample from Mota Giyorgis (marked by an orange circle) applied (face-down) on filament of Pyrola®2000 filament pyrolyser (Pyrol AB), photo by Marie-Louise Tambo Magni

Dedicated to Pär, Lilly Olea and Enar Peter

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Preface

Interdisciplinary fields, such as heritage science, have always been of great interest to me. It evokes a peculiarly rewarding feeling to be investigating common denominators between fields often considered non-overlapping, such as the fields of art and science. Heritage science is a newer scientific field, which continues traditions of established fields, such as conservation science and archaeometry. The aim is to form and strengthen bridges between natural sciences and humanities and to safeguard our cultural heritage. A dedicated peer-reviewed journal, "Heritage Science", was published for the first time in 2013.

Cultural heritage is related to our collective identities and essentially answers questions like "Who am I?", "What am I a part of?" and "Where did I come from?". Cultural heritage is, therefore, much more than objects to protect and pass on to future generations. Both tangible and intangible heritage exists, and UNESCOs list of heritage to safeguard continues to grow, now also including traditions and languages. A natural scientist, however, only has the tools to investigate tangible matter, such as buildings, objects and materials of cultural interest. Investigations are therefore often related to materials: How old are they? Where are they from? From what are they made?

This thesis provides a glimpse into this vastly diverse field and touches a broad spectre of themes. Whilst some aspects will be thoroughly investigated, others will only be superficially explained. A few theses and projects related to cultural heritage have been undertaken at the Department of Chemistry (IKJ) at the Norwegian University of Technology and Science (NTNU), but no particular research group is dedicated to cultural heritage studies at IKJ. These theses,

including mine, are therefore somewhat “satellite theses” without the benefits of an internal research group to lean on. This thesis was one of the first to explore analytical pyrolysis using a newly purchased instrument (a Pyrola®2000 pyrolyser from Pyrol AB) and the first to investigate organic materials in paint samples here at IKJ/NTNU. Starting up a new field of investigation is demanding, and the initial attitude towards the challenges, yet to be faced, was somewhat naïve. Therefore, much resources were used to navigating through this broad scientific field as well as becoming acquainted with Pyrola®2000 and pyrolysis in general. The experimental work, thus, bid on many challenges related to both the sample preparations, the instrumental pyrolysis-GC/MS setup and interpretation of results.

Throughout the difficulties faced, I have hoped that this thesis would provide a solid starting point for future investigators of organic binding media in paint, and that it could be helpful to future operators of the Pyrola®2000 pyrolyser at IKJ, regardless of scientific field.

Sammendrag

Karakterisering av lipid- og protein-baserte bindemidler ble utforsket ved hjelp av pyrolyse-GC/MS (analytisk pyrolyse). Markører ble identifisert i seks malingsreplikaer av bindemiddelprøver uten pigmenter (linolje, bivoks, eggeplomme, eggehvite, animalsk lim og kasein) samt seks malerprøver fra etiopisk, kristen kunst, pyrolysert med en derivatiseringsagent tilstede (tetrametylammonium hydroksid, TMAH). I tillegg ble markører identifisert for tre replikaer av bindemiddelprøver (linolje, eggeplomme og animalsk lim) og en etiopisk, kristen maleprøve analysert uten derivatisering. De to analytiske tilnærmingene (med eller uten derivatisering) ble sammenlignet, og vurdert som komplementære: analyser med derivatisering passet bra for lipid-markører, mens analyser uten derivatisering passet bra for protein-markører. I tillegg til karakteriseringsprosessen, ble reproduserbarheten av det sensitive analytiske system evaluert. Karakteriseringsmetoden, som hadde blitt foreslått fra tidligere undersøkelser, ble justert basert på utfordringer knyttet til reproduserbarhet.

Abstract

Analytical pyrolysis was explored using pyrolysis-GC/MS with a high precision resistively filament pyrolyser (Pyrola®2000, Pyrol AB) for characterisation of lipid-based and proteinaceous binding media. Markers were identified for six paint replicas (mock samples) of binding media without pigments (linseed oil, beeswax, egg yolk, egg white, animal glue and casein) as well as six paint samples of Ethiopian Christian art, which were pyrolysed in presence of a derivatising agent (tetramethylammonium hydroxide, TMAH). Additionally, markers were identified for three paint replicas (linseed oil, egg yolk and animal glue) and one Ethiopian Christian paint sample analysed without derivatisation. These two analytical approaches (with and without derivatisation) were compared and deemed complementary: Analyses with derivatisation were suited for lipid markers, while analyses without derivatisation were suited for proteinaceous markers. In addition to the characterisation process, the reproducibility of the powerful, but sensitive analytical system was evaluated. The characterisation method, suggested by previous investigators, was adjusted based on reproducibility-related challenges encountered.

Contents

Supplementary data

Abbreviations and symbols

*Note: Abbreviations of marker compounds are presented in **bold***

AG	Animal Glue
Az	**Azelaic acid (dimethylated, when presence of TMAH)**
B	**Benzoic acid, methyl ester**
BW	Beeswax
Cas	Casein
Ch	Chalk
C	**Cholesterol**
cps	Counts per second
δ	With derivatisation/presence of TMAH
DKPs	2,5-diketopiperazines
DK-Py	**Diketodipyrrole (Pyrocoll)**
E	**Ethylbenzene**
EI	Electron ionisation
eV	Electronvolt
EW	Egg white
EY	Egg yolk
FA	Fatty acid
FAME	Methylated fatty acid
GC	Gas chromatography
GC/MS	Gas chromatography mass spectrometry
HMDS	Hexamethyldisilazane
I	**Indole**
I_1	Electric current at t_1, TTP parameter
I_2	Electric current at t_2, TTP parameter
IA	**Isobarbituric acid**

IC21B	Icon21 Brown
IC21G	Icon21 Green
IC9	Icon9
IKJ	Department of Chemistry
LO	Linseed Oil
MG	Mota Giorgis
MS	Mass spectrometry
ms	Milliseconds
NIST	National Institute of Standards and Technology
NTNU	Norwegian University of Science and Technology
O	**Oleic acid (methylated, when presence of TMAH)**
P	**Palmitic acid (methylated, when presence of TMAH)**
PA	**Phosphoric acid, trimethyl ester**
Pro-Ala	**Cyclo(proline-alanine), 2,5-diketopiperazine**
Pro-Gly	**Cyclo(proline-glycine), 2,5-diketopiperazine**
Pro-Hyp	**Cyclo(proline-hydroxyproline), 2,5-diketopiperazine**
Pro-Pro	**Cyclo(proline-proline), 2,5-diketopiperazine**
Py	**Pyrrole (methylated, when presence of TMAH)**
QIT	Quadrupole ion trap
QMF	Quadrupole mass analyser
R0	Resistance of filament when not in use
RT_p	Resistance of filament during pyrolysis
S	**Stearic acid (methylated, when presence of TMAH)**
ScA	Scanning range A
ScB	Scanning range B
ScC	Scanning range C
Se	**Sebacic acid (dimethylated, when presence of TMAH)**
SIM	Simulated Ion Monitoring

St	**Styrene**
Su	**Suberic acid (dimethylated, when presence of TMAH)**
T	**Toluene**
t_1	Temperature rise time (TRT), TTP parameter
t_2	Pyrolysis time, TTP parameter
T_c	Pyrolysis chamber temperature
T_D	TTP measured by photo diode
TIT	Total ion thermograms
TMAH	Tetramethylammonium hydroxide, $N(CH_3)_4OH$
T_p	Pyrolysis temperature
T_R	TTP measured by resistance of filament
TP1	Temperature programme 1
TP2	Temperature programme 2
TRT	Temperature rise time
TTP	Temperature Time Profile
µsc	Microscans
WCOT	Wall Coated Open Tubular
WCW	Wukero Cherkos white
WCY	Wukero Cherkos yellow
ø	Without derivatisation/non-derivatised

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Chapter 1: Introduction

> *Everyone has the right freely to participate in the cultural life of the community, to enjoy the arts and to share in scientific advancement and its benefits.*
>
> United Nations General Assembly, 1948

This thesis operates in the interdisciplinary field heritage science, a newly established scientific field continuing traditions of others, such as conservation science and archaeometry. In this research area, bridges between natural sciences and humanities are formed and strengthened, where natural sciences can contribute to investigations related to e.g. ancient materials: Dating how old they are, investigating where they are from (provenance), and characterising the materials.

The subject of this thesis is a case of the latter (material characterisation) and came about in the aftermath of the PhD of Kidane Fanta Gebremariam (2016). He analysed samples of cultural interest (Ethiopian Christian paintings) with a broad spectrum of analytical methods. Most analyses he performed himself, but pyrolytic analyses on binding media in paint were undertaken externally at the Norwegian paint company, Jotun. The Department of Chemistry (IKJ) here at the Norwegian University of Science and Technology (NTNU) lacked the required equipment: A pyrolysis unit for gas chromatography (GC).

With an aim for self-sufficiency, a Pyrola® 2000 resistively heated filament pyrolyser (Pyrol AB) was purchased by IKJ in 2015. An exchange student, Tim Lauschke, investigated a method for characterisation of binding media in paint employing the newly purchased instrument in a Chemistry Specialisation Project (Lauschke, 2016) supervised by Lise Kvittingen and Rudolf Schmid. The specialisation project was the starting point of this thesis and binding media in paint samples collected during the doctoral thesis of Gebremariam were investigated in both this and the preceding project. The Chemistry Specialisation Project of Tim Lauschke (2016) will in the following will be referred to as "the Lauschke project".

The pyrolysis-GC/MS setup at NTNU/IKJ consisted of the platinum filament pyrolyser (Pyrola®2000, Pyrol AB), coupled online to a gas chromatography system and ion trap mass spectrometer (Trace GC Ultra ITQ 1100, Thermo Scientific). In this thesis, "the pyrolysis-GC/MS setup at IKJ/NTNU" refers to this particular system.

Paint consist of colourful components, pigments, dispersed in binding media, which enable the pigments to adhere to a surface of choice. Pigments themselves are powders of colourful substances of both organic and inorganic nature (Masschelein-Kleiner, 1995; Smith, 2003, p. 11; Bonaduce and Andreotti, 2009). Historically, painting techniques were mainly based on organic binding media (except "fresco"[1]) and these are continuously investigated in heritage science. Of the constituent in paint, binders are the most prone to degradation, and their decay can be prevented or slowed, when information on organic constituents in an object is available. This information can, thus, aid restoration, conservation and long-

[1]A special (mural) painting technique where the pigments are introduced directly into wet, fresh lime plaster before it dries. However, even some frescos have been found to contain binders as additives (Casadio, Giangualano and Piqué, 2004).

term preservation. Knowledge of the organic constituents can assist evaluations of storage or display conditions, thereby minimising further decay. The information acquired when a binding medium is characterised can also aid analyses of painting techniques of specific artists or historical eras or shed light on possible falsifications (Smith, 2003; Colombini *et al.*, 2010). Successfully characterising binding media is therefore potentially valuable to many partakers.

Several scientific methods are used to investigate binding media in paint, and many of these involve mass spectrometry, as research heavily rely on structural information on a molecular level (Colombini, 2009). Paint samples require pre-treatments before analysis, and "wet treatments" (chemical treatments and extractions into fractions) is currently a popular alternative (Degano *et al.*, 2018). The technique, however, involves risks of contamination or sample loss when performing analyses, and these are time consuming procedures. It was developed by *Chemical Science for Safeguard of Cultural Heritage Group* (Colombini *et al.*, 2010; Lluveras *et al.*, 2010) led by Maria Perla Colombini at the University of Pisa. In this thesis, the group will be referred to as "the Colombini Group", a name they use themselves as well.

This project investigated pyrolysis-GC/MS for characterising binding media in paint, in which the "pre-treatment" is pyrolysis. This alternative is continuously investigated in the scientific community as well, with the hope to avoid the disadvantages of wet treatments, as very little sample material and less sample preparation is needed. In characterisation investigations, the aim is to identify so-called markers, which are compounds specific for a material. In pyrolysis-GC/MS, these are detected as pyrolysis products, and MS fragment patterns are analogous to a fingerprint of a given compound.

Three sample types are needed to identify possible markers: Mock samples, paint samples and reference samples. Mock samples, also known as paint reconstructions or replicas, are samples of binding media which mimic paint.

Mock samples in this thesis were colourless (without pigments). Paint samples were collected by Kidane Fanta Gebremariam from Ethiopian Christian artwork. Reference samples were purchased standard materials.

The aim of this project was to review and further develop the method established in the Lauschke project for identification of binding media in paint using pyrolysis gas chromatography mass spectrometry (pyrolysis-GC/MS).

The following stages were outlined to evaluate and finetune the method set up in the Lauschke project:

1) To repeat analyses of mock samples of binding media in paint and identify possible markers in these.

2) To analyse paint samples and identify possible markers in these.

3) To investigate the possibility of simultaneously detecting more than one group of organic material in the same sample run. The groups investigated in this thesis were proteins and lipids.

As the experiments advanced, an additional objective arose: To improve reproducibility of analyses, when employing the newly purchased pyrolysis unit (Pyrola®2000, Pyrol AB).

1.1 Organic paint binders

For any creative, crafty and engineering purpose Man has always used what is at hand, preferably easily obtainable in large quantities and cheap. Paint materials are no exception. The main purpose of binders in paint is to facilitate adhesion between the pigment (dispersed in the binder) and the surface of which the paint is to be applied, as well as cohesion between adjacent paint layers. (Masschelein-Kleiner, 1995, p. 1; Colombini and Modugno, 2009).

Binders are also known as binding media or adhesives. For a material to be suitable as an organic paint binder, certain physico-chemical properties must be met. It must form a fluid, homogenous paste when mixed with pigments, the binder-pigment mixture must dry when applied to the desired surface (the support) and generate a solid, elastic, non-sticky layer with filming properties. This solid layer must be stable through aging (Bonaduce and Andreotti, 2009). The way in which the pigments are dispersed in the media is important to the properties in the dried film. In a well-mixed paint, each pigment particle is present separate from each other, completely surrounded ("wetted") by binding media (Masschelein-Kleiner, 1995, pp. 2–5; Smith, 2003).

Through the ages, diverse organic materials have met the criteria of a good binder. These include various plant and animal products such as vegetable oils, tree gums, various waxes, eggs, milk and boiled skin/bone material. Now, synthetic polymers such as acrylic polymers are widely used (Masschelein-Kleiner, 1995, p. 33; Smith, 2003; Casadio, Giangualano and Piqué, 2004; Colombini and Modugno, 2009).

Since the Industrial Revolution "pre-mixed paint" has been commercially available (Levin, 1991). Before this time period, naturally occurring organic materials were employed as binding media for pigments, mixed by artists themselves. Many of these materials, such as egg, oil and milk, were foodstuffs.

These are categorised based on their structural similarities. This thesis focuses on two groups of organic material; proteinaceous and lipid-based material.

In the Lauschke project, linseed oil, beeswax, egg yolk, whole egg, egg white and animal glue were investigated. Since the resulting pyrograms of whole egg and egg yolk were indistinguishable, only egg yolk was analysed in this thesis. In addition, casein (produced from milk) was later included. Of the binders analysed in this project, egg white (EW), egg yolk (EY), animal glue (AG) and casein (Cas) are considered proteinaceous material, whereas linseed oil (LO) and beeswax (BW) are considered lipid-based binders (Colombini and Degano, 2018).

Of organic materials in paintings, these are among the most often encountered and represent common painting techniques used over the centuries: *Oil painting* (made from drying oils), *proteinaceous tempera* including *egg tempera* (made from proteinaceous materials), *tempera grassa* (made from a mixture of drying oils and proteinaceous materials) and *the encaustic painting technique* (made from beeswax). In addition, *polysaccharide tempera* (e.g. water colours and gouache, made primarily from plant gums) were also common. Resins, another lipid-based material, were used as additives or varnish (Bonaduce and Andreotti, 2009).

Although not investigated in this thesis, it is still important to be aware of other organic materials one may encounter when analysing paint samples. Polysaccharides or resins could be encountered in this project as well, as ancient paint samples of unknown composition were investigated.

The classification of binding media is based on the group of organic material most prominent in the material, but compounds associated with other groups can also be found. A proteinaceous material, for example, does not exclusively contain proteins and polysaccharide compounds are not restricted to polysaccharide material. Especially proteinaceous binders often contain constituents of other

groups. Casein, for instance, is made from milk, and is classified a proteinaceous material, but also contains the disaccharide lactose and fatty acids (Mills and White, 1994c). In general, mixes containing both water-soluble substances such as proteins and lipids such as fatty acids, are called emulsions. Considering painting techniques, *tempera* is just another word for an emulsified paint binder (Smith, 2003).

In paintings, organic materials can be found all the way from the surface layer of the painting and to material of which is was applied (Fig. 1.1) (Colombini, 2009). The miniscule sample size used in pyrolysis-GC/MS analyses is, therefore, a complicating factor, as organic material can be present in several layers of a paint sample. It can be applied as a binding medium in the ground layer (often made from animal glue or casein and white pigments), a binding medium in one or several paint layers and/or coating or varnish in an outer protective layer (often waxes or resins) (Colombini and Gautier, 2009; Colombini and Modugno, 2009). Fig. 1.1 illustrates the stratigraphy of a real painting, as described above, but for a repainted painting (two layers of grounding, paint and varnish). If an organic material is identified in a pyrolytic profile, the layer(s) from which it originates would be unknown, as all layers are analysed together when pyrolysing micrograms of a paint sample. Thus, it does not necessarily stem from the binding medium in the *paint,* specifically.

The aim of analysing mock samples is to identify marker compounds specific for a material (*1.3.2 Markers*). To understand the sources of pyrolytic markers obtained when performing pyrolysis-GC/MS, knowledge on the chemical composition of the organic materials are essential. When paint dries, the components present in the binding media undergo chemical reactions and forms an elastic film (Masschelein-Kleiner, 1995, p. Introduction, no page number; Bonaduce and Andreotti, 2009). To identify markers of paint from a specific binder, knowledge of this film formation is important as well. The compounds

present in a paint film is, however, also influenced by degradation through aging and interactions with other components in the paint mixture, such as pigments. Aged organic samples, such as ancient paint, are therefore challenging to analyse, as illustrated in Fig. 1.4 (*1.3 Pyrolysis-GC/MS analyses of binding media*).

1.1.1 Lipid-based material

The most common lipid-based material found in paint are glycerolipids, such as oil. Waxes and resins are lipid-based as well, but less common. In this project, linseed oil (a drying oil) and beeswax (a natural wax) were investigated. Drying oils and (to some degree) waxes require derivatisation before GC-analysis (Husek, 2000; Bonaduce and Andreotti, 2009; Regert, 2009) (See *1.3.3 Derivatisation*).

1.1.2 Linseed oil – a drying oil

Oil painting became a popular painting technique in Europe in the fifteenth century. The oils used, primarily vegetable oils e.g. from linseeds, poppy seeds or walnuts, are so-called "drying oils", with a high content of polyunsaturated acids. As all drying oils contain more or less the same fatty acids, it is difficult to distinguish between them, but linseed oil was popular, and is therefore often analysed as a representative for drying oils (Mills and White, 1994b; Smith, 2003).

Fresh linseed oil contains oleic acid (C18:$1^{\Delta 9}$, 14-24%), linoleic acid (C18:$2^{\Delta 9,12}$, 14-19%) and linolenic acid (C18:$3^{\Delta 9,12,15}$, 48-60%) in addition palmitic acid (C16:0, 6-7%) and stearic acid (C18:0, 3-6%). The high content of polyunsaturated fatty acids is the basis for the good drying properties of linseed oil (Mills and White, 1994b, p. 33).

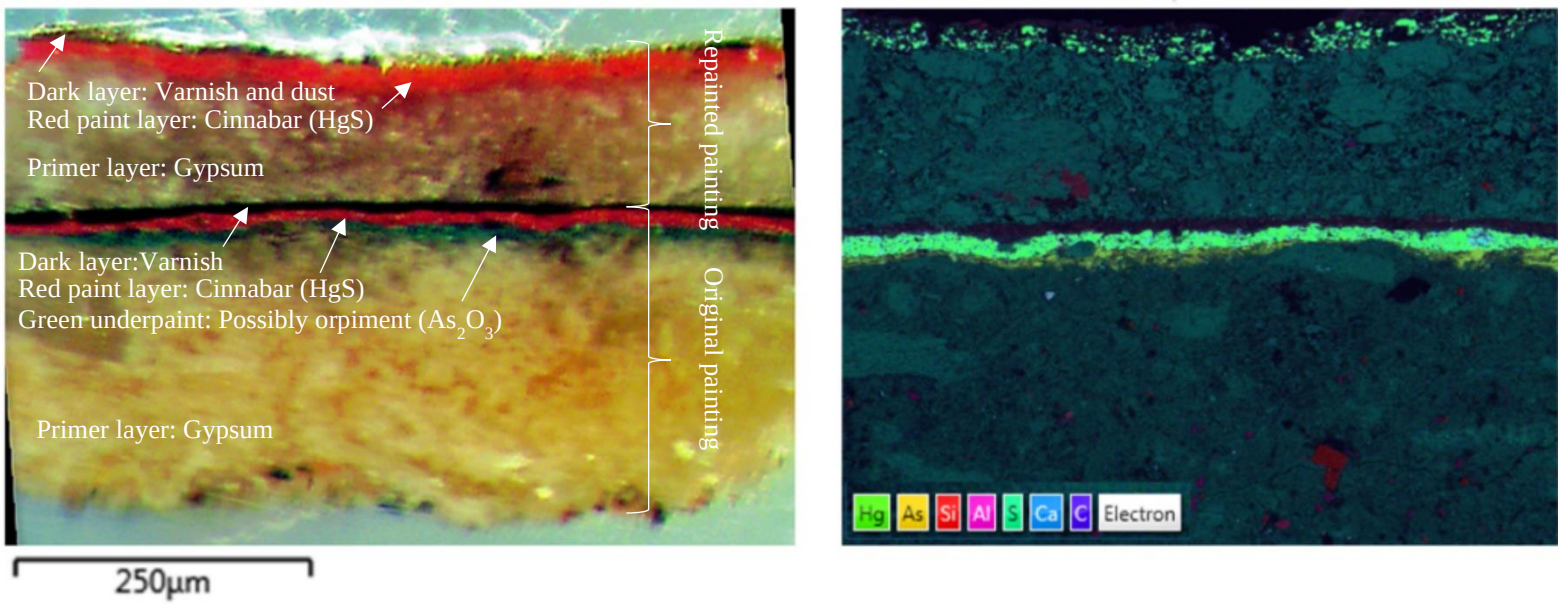


Figure 1.1: Stratigraphy of repainted paint sample. Cross section of a paint sample from an icon. The fragment is photographed by a normal light microscope (left) and analysed using elemental mapping in Scanning Electron Microscopy with Energy Dispersive Spectroscopy (SEM-EDS) (right). The original icon had been repainted, and therefore the stratigraphy is repeated. From the bottom the layers are: A large layer of the primer gypsum ($CaSO_4 \cdot 2H_2O$, white-yellow), orpiment underpaint (As_2O_3, yellow-green) and cinnabar paint (HgS, red), varnish (dark), a new layer of gypsym primer, cinnabar, varnish and dust. Photos by Kidane Fanta Gebremariam.

The term “drying oil” is somewhat misguiding, as it can give the impression of evaporation of water/moisture or other constituents in the material when applied. During paint film formation in drying oils, free radical chain reactions occur, resulting in a polymerisation of the fatty acid molecules of the triglycerides. In this process, diacids are formed in reactions between oxygen and the polyunsaturated fatty acids in the oil (Mills and White, 1994b, pp. 36–38). Diacid products of the polymerisation reactions are pimelic acid (C7), suberic acid (C8), azelaic acid (C9) and sebacic acid (C10).

The main oxidation product of the polymerisation reactions of glycerolipids is azelaic acid, which is usually the main peak in pyrolytic profiles of drying oils and therefore reported as the main marker. It is sometimes detected in pyrolytic profiles of egg yolk as well, but in much lower abundance. To distinguish egg yolk from drying oils, the ratio between azelaic acid and palmitic acid (Az/P) is suggested as an indicator. A ratio lower than 0.3 suggests presence of a non-drying fat, mainly egg yolk, and a ratio of more than 1 suggests presence of drying oil (Mills and White, 1994a, p. 171; Bonaduce *et al.*, 2016, p. 305).

Certain pigments, such as lead white ($2PbCO_3 \cdot Pb(OH)_2$), can inhibit the formation of azelaic acid as the metal ions produce carboxylate ions (soaps). Thus, the amount of azelaic acid present in a paint sample mixed with these specific pigments is lower compared to a paint sample without them (Chiavari, Fabbri and Prati, 2005). Linseed oil can therefore, in special cases, be present in a paint sample, despite having an Az/P ratio below 1.

1.1.3 Beeswax – a natural wax

Natural waxes, such as beeswax, lanolin, carnauba and paraffin wax, is a non-homogenous group of lipid-based materials, which are mixes of lipids and hydrocarbons. Unlike oils, which are liquid at room temperature, waxes are solids,

often soft and mouldable. They are hydrophobic and consist of various homologous compounds such as fatty acids, *n*-alkanes, *n*-alcohols and esters of long chain carboxylic acids (Tulloch and Hoffman, 1972; Masschelein-Kleiner, 1995, p. 43; Colombini and Modugno, 2009; Colombini and Degano, 2018).

Waxes have had many applications in paint through the ages e.g. as protective layers/coatings, stabilisers in oil painting and as main binder (*encaustic painting technique*) (Smith, 2003, p. 43; Bonaduce and Andreotti, 2009; Colombini and Modugno, 2009).

The composition of beeswax and other waxes have been thoroughly investigated, and identification in analytical pyrolysis is based on overall pyrolytic profiles (Bonaduce *et al.*, 2016). Regarding the required derivatisation, methylation results in better diagnostic profiles than silylation (Bonaduce and Andreotti, 2009).

The markers of beeswax are hydrolysed mono- and polyesters as well as methyl esters of acids and methyl ethers of alcohols (Asperger, Engewald and Fabian, 1999; Bonaduce and Andreotti, 2009). For non-derivatised samples, most acids and alcohols are supposedly not detected. Although a diagnostic pyrolytic profile of linear hydrocarbons can be detected, occurrence of other lipid materials in a sample could prevent correct interpretation, due to similar pyrolysis products (e.g. compared with glycerolipids) (Bonaduce and Andreotti, 2009).

Asperger, Engewald and Fabian (1999) have reported the following markers for beeswax pyrolysed in presence of TMAH: Even-numbered methyl esters of fatty acids (C_{16}-C_{32}), odd-numbered hydrocarbons (C_{23}-C_{33}), even-numbered alkyl methyl ethers (C_{24}-C_{34}). In addition, methyl esters of methoxy fatty acids and dimethoxyalkanes were reported.

1.1.4 Proteinaceous material

Proteins are water-soluble compounds present in various animal sources, such as egg, milk and glues, and some vegetable sources as well, such as garlic (Bonaduce and Andreotti, 2009). The proteinaceous materials, analysed in this thesis, were egg yolk, egg white, animal glue and casein. Egg yolk, animal glue and casein were frequently used as paint binders in the tempera painting technique, and *egg tempera* was the most common tempera type, often mixed from egg yolk or whole egg (Colombini and Modugno, 2009). Egg white ("glair") was often used as varnish or glue for gilding but has historically also been described as main binder in tempera paint (Casadio, Giangualano and Piqué, 2004; Colombini and Gautier, 2009). Additionally, some proteinaceous materials, such as animal glue and casein, were often constituents in a preparatory ground layer applied before paint (Fig. 1.1), e.g. a mixture of glue and gypsum (Colombini and Modugno, 2009).

Proteinaceous material can, therefore, be present in several layers in a paint sample: As adhesive in a ground layer/priming, as binding media in one or more paint layers and as varnish in an outer protective layer (Colombini and Gautier, 2009). It is seldomly possible to distinguish between the different layers in a microsample and if markers of proteinaceous material are detected, the layer(s) from which they originate would therefore be unknown.

Identifying proteins is generally challenging, regardless of analytical approach (Colombini and Modugno, 2004; Bonaduce and Andreotti, 2009; Dallongeville *et al.*, 2016; Orsini, Parlanti and Bonaduce, 2017). The pyrolytic profiles of proteinaceous materials are very complex and peak-rich and demand a targeted approach when searching for markers (Vinciguerra *et al.*, 2019). The challenge is to determine which marker compounds to target. As proteins are made up of a limited number of amino acids, identification is based on quantitative evaluations of the contents of these and associated compounds (Bonaduce *et al.*, 2016; Orsini, Parlanti and Bonaduce, 2017).

Markers of proteinaceous binding material are related to the products of the chemical reactions occurring during film formation after paint application. During the initial drying process, a large amount of the water present in the freshly applied paint quickly evaporates. The loss of water continues until an equilibrium with the humidity in the surroundings is achieved. As water evaporates empty pockets between the molecules present in the paint are formed forcing the proteins to rearrange their structures. This rearrangement is essentially denaturation, where "the peptide chains lose their ordered state, and become unfolded and uncoiled" (Karpowicz, 1981, p. 153). This process leads to functional groups of sidechains being exposed, and available for chemical reactions.

Through aging, chemical reactions will "continue through the centuries to create proteinaceous material hardly comparable to proteins in their native state" (Karpowicz, 1981, p. 157). The proteins will undergo both physical changes and chemical processes dependent on the physical environment of the surroundings (pH, temperature, relative humidity), contact with micro-organisms and presence of pollutants, which will affect the aging proteins by accelerating the degradation processes. Additionally, other components in the mixture may contribute to the change, including metallic cations e.g. from pigments (forming complexations) (Colombini and Gautier, 2009) and glycerolipids (condensation and cross-linking reactions) (Colombini and Modugno, 2009). These degradations reactions will not be described further but are briefly mentioned to provide an illustration of their complexity.

Proteins have been investigated both with and without derivatisation in studies of cultural heritage objects. Currently, either small aromatic compounds or 2,5-diketopiperazines (DKPs) and DKP-related compounds are used as markers (Chiavari and Galletti, 1992; Bonaduce and Andreotti, 2009; Fabbri *et al.*, 2012; Dallongeville *et al.*, 2016; Orsini, Parlanti and Bonaduce, 2017). In recent literature (Orsini, Parlanti and Bonaduce, 2017; Orsini, Duce and Bonaduce,

2018), the thermal behaviour of the proteinaceous materials was investigated with evolved gas analysis mass spectrometry, where total ion thermograms (TITs) of egg white, animal glue and casein were recorded with a temperature program (starting from 50°C increased by 10°C/min to 700°C) (Orsini, Parlanti and Bonaduce, 2017). The two categories of proteinaceous markers reflect two thermal degradation steps of proteins occurring during pyrolysis identified in these studies.

The researchers monitored the formation of compounds during thermal degradation based on temperature. From the TITs recorded, two peaks where identified and classified as two steps in the "depolymerisation" process of proteins: One around 300-350°C, associated mainly with DKP formation, and another around 420-430°C, associated with the formation of aromatic compounds from amino acid side chains and aromatic amino acids. The proteinaceous markers are categorised correspondingly in this thesis.

DKPs are cyclopeptides found in abundance in nature. These compounds are formed from two neighbouring amino acids during natural degradation of polypeptides and are found in many types of processed food and beverages (Sammes, 1975; Borthwick, 2012; Borthwick and Da Costa, 2017). The most important pathway to DKPs in food is by chemical reaction during thermal processing (Borthwick and Da Costa, 2017), and DKPs are formed during pyrolysis as well (Chiavari and Galletti, 1992; Hendricker and Voorhees, 1998; Orsini, Parlanti and Bonaduce, 2017), which is not surprising, as pyrolysis is an extreme type of thermal degradation (Jones, 2000).

Proline-based DKPs are the most common cyclodipeptides in food, as the stereochemical structure of proline particularly facilitates DKP formation (Borthwick and Da Costa, 2017). In the reviewed literature, mainly proline-containing DKPs were identified as markers for proteinaceous binding media

(Chiavari *et al.*, 2003; Orsini, Parlanti and Bonaduce, 2017; Orsini, Duce and Bonaduce, 2018).

DKPs were traditionally reported for non-derivatised samples or samples pyrolysed in presence of hexamethyldisilazane (HMDS), a milder derivatising agent (Bonaduce and Andreotti, 2009). In this project, the applied derivatising agent was a strong alkaline, tetramethylammonium hydroxide (TMAH, *1.3.3 Derivatisation*), and DKPs have not been reported *for binding media*, when pyrolysed in presence of this reagent.

However, the mechanisms occurring when some selected single amino acids were pyrolysed in presence of TMAH have been investigated, and direct methylation, deamination, homolyses, decarboxylation, dehydration, dimerization and cyclisation (including DKPs) where concluded to occur (Gallois, Templier and Derenne, 2007). Still, analysing single amino acids and dipeptides can result in different products (Templier, Gallois and Derenne, 2013), and a thorough investigation of the compounds formed, under these conditions, is therefore important for identification of markers of proteins. The complex reactions between TMAH and amino acids during pyrolysis, causies the resulting pyrolytic profiles to be even more challenging to interpret. Therefore, the advice, currently, is to avoid pyrolysis-GC/MS analyses of proteinaceous materials due to the difficulties of interpreting these pyrolytic profiles (Colombini *et al.*, 2010) – the non-derivatised samples are complicated enough.

The other category of markers, small aromatic compounds e.g. pyrrole and indole, were traditionally presented as specific markers for proteinaceous binding media but are, now, often considered too unspecific to be employed as single markers. An example is pyrrole, which is derived from hydroxyproline and therefore regarded specific for animal glue (Chiavari and Galletti, 1992) (*1.1.6 Animal glue*). It can also be derived from proline, which is found in all proteins (although often in much lower quantities), and additional markers are therefore needed.

The challenge of identifying proteinaceous materials is an open issue in the scientific community (Bonaduce *et al.*, 2016; Orsini, Parlanti and Bonaduce, 2017). Egg white, casein and animal glue was thoroughly investigated in a study by Orsini, Parlanti and Bonaduce (2017), and the resulting pyrolytic profiles of egg white and casein were difficult to distinguish. In a following study, they investigated the main protein in egg white, ovalbumin, to understand how this particular protein reacts during pyrolysis (Orsini, Duce and Bonaduce, 2018) and have identified new types of possible markers: Unsaturated DKPs, 3,5-alkyl-3,4-dihydro-2H-pyrrole-2,4-diones (ADPDs) and 3-alkenyl-5-alkyl-pyrrolidine-2,4-diones (AAPDs). Although these are not investigated here, it is interesting to note that this field of investigation, currently, is in development, and that non-derivatised proteinaceous samples are investigated in detail.

Additionally, the markers for proteinaceous material are dependent on the pyrolyser and the instrumental setup used (*1.2.1 Reproducibility*). Due to the diversity of reported markers, it is important to build a reference library of pyrographic profiles (Bonaduce and Andreotti, 2009).

1.1.5 Egg

Egg tempera was common in Europe until *oil painting* gained popularity in the fifteenth century. In this painting technique, either egg white, egg yolk or whole egg is applied (Casoli, Berzioli and Cremonesi, 2012). Although egg white can be applied as binder, it was, historically, more common to use egg yolk or whole egg (Colombini and Modugno, 2009), and egg white, also referred to as *glair*, had additional applications, such as glue for gilding or varnish (Colombini and Gautier, 2009). Of the three egg components, only egg yolk and egg white will be described in the following, as whole egg was not analysed.

Egg yolk is a natural emulsion of lipids in water (Sugino, Nitoda and Juneja, 1997; Smith, 2003, p. 170). In dried form it contains 33% lipids and the remaining (water-soluble) components are proteins (16.5%) and small amounts of carbohydrates and minerals (Mills and White, 1994b, p. 42; Sugino, Nitoda and Juneja, 1997).

Some claim that it is incorrect to consider egg yolk a proteinaceous material (Casoli, Berzioli and Cremonesi, 2012), as proteinaceous markers are not reported for egg yolk, while lipid markers are extensively reported: Fatty acids/diacids, alkyl nitriles (formed in reactions between lipids and proteins), cholesterol and phosphoric acid (Mills and White, 1994a, 1994b; Chiavari, Fabbri and Prati, 2001, 2005; Chiavari and Prati, 2003; Bonaduce and Andreotti, 2009; Melucci *et al.*, 2011; Orsini, Parlanti and Bonaduce, 2017). Egg allegedly has the same drying properties as drying oil (although not as strong), and undergo the same oxidative polymerisation reactions as oil (Casoli, Berzioli and Cremonesi, 2012).

Egg yolk is reported to have an especially high phosphorus content (0.9%), which is derived from phosphoproteins (e.g. phosphovitin and lecithin), and phospholipids (29% of the lipids in egg). Phosphorous compounds are, therefore, suggested as strong marker for egg yolk (Mills and White, 1994c, p. 88; Chiavari and Prati, 2003).

Egg white contain no lipids (Mills and White, 1994b, p. 42), in contrast to egg yolk, animal glue and casein, but 10% proteins. The amino acids in these are the source of markers, and the main proteins are ovalbumin, conalbumin and lysozyme accounting for about 50%, 15% and 3% of the protein content respectively (Mills and White, 1994c, pp. 86–87, 1994b, p. 42).

Traditionally, indole and methyl indole were reported as main markers of egg white (Chiavari *et al.*, 1993; Bonaduce and Andreotti, 2009). These originate from the amino acid tryptophan, which is present in relatively high abundance in lysozyme (6.0%) (Chiavari and Galletti, 1992; Chiavari *et al.*, 1993; Mills and

White, 1994c, p. 87). Unfortunately, tryptophan is also present in milk proteins (up to 6.6%, depending on the protein). Samples containing casein (milk proteins) could therefore contain a high amount of tryptophan and thereby display a high indole content. Indole and derivatives thereof are not specific for egg white and additional markers are needed.

When investigating non-derivatised egg white, the markers reported were small aromatic compounds such as toluene, styrene, phenol, benzenepropanenitrile, indole and methyl indole, and the 2,5-diketopiperazines cyclo(Pro-Val) and cyclo(Pro-Ile) (Orsini, Parlanti and Bonaduce, 2017). In addition, cyclo-octasulphur has been identified, a compound not mentioned in relation to other protein-based binding media. This compound could potentially be a specific marker for egg white. However, its origin or specifics remain somewhat mysterious. It was only reported by name, peak number in pyrogram (of high intensity) and mass spectra in a table seemingly DKP-related, or at least mentioned in a table together with DKPs.

All other markers reported for egg white, both aromatic compounds and DKPs, have also been reported present in either animal glue, casein or both. The reappearance of possible marker compounds in several binding media is problematic for both proteinaceous material and glycerolipid material. For the latter, ratios between specific components are evaluated. This approach is also used, qualitatively, for proteinaceous materials (Colombini and Modugno, 2004), where comparisons of DKP contents were the basis for identification of proteinaceous material in several articles (Chiavari and Prati, 2003; Chiavari *et al.*, 2003; Orsini, Parlanti and Bonaduce, 2017).

1.1.6 Animal glue

Animal glue is an adhesive material, obtained by boiling skin, connective tissue and bones from mammals and fish. It was widely used as binding medium in both grounding (preparatory layer) and paint (Fig. 1.1) and sometimes denoted "gelatine glue" or just "gelatine". As fresh, these animal parts consist of collagen, a fibrous, insoluble protein, which degrades into gelatine when the protein is suspended in water and heated. Gelatine then leaches into the water. Less prolonged boiling gives lighter-coloured materials known as gelatines, while "glue" tends to be darker, thicker and more turbid (Mills and White, 1994c, p. 86; Colombini and Modugno, 2004).

Collagen is characterised by its high content of glycine, proline and hydroxyproline (Mills and White, 1994c; Colombini and Modugno, 2009). The latter is formed through hydroxylation of proline, and this amino acid together with compounds associated with it, such as pyrrole and diketodipyrrole[2], are identified as markers of collagen (Stankiewicz *et al.*, 1997; Colombini and Modugno, 2004; Bonaduce and Andreotti, 2009; Dallongeville *et al.*, 2016; Orsini, Parlanti and Bonaduce, 2017). Diketodipyrrole is derived from two neighbouring hydroxyprolines. The dehydration product of these amino acids is similar to the dehydration product of two neighbouring prolines, but with an additional loss of two water. These are formed from the two hydroxyl groups of hydroxyproline and two adjacent protons (Chiavari and Galletti, 1992).

Hydroxyproline-containing proteins can, in rare cases, be present in polysaccharidic gums (e.g. 3% proteins in arabic gum), which have been used as binding media in paint as well. One should therefore be cautious if identifying

[2] Diketodipyrrole is a compound with an ambiguous name. The mechanism in which this compound is formed is described by Chiavari and Galletti (1992). It seems that it is also known as "Pyrocoll" (Colombini and Modugno, 2004)

animal glue based on presence of hydroxyproline alone (Colombini and Modugno, 2004).

Traditionally, the main marker for animal glue was. This compound is formed from proline and hydroxyproline during pyrolysis and appear as high intensity peaks in analyses both with and without derivatisation (Chiavari and Galletti, 1992; Stankiewicz *et al.*, 1997; Chiavari *et al.*, 1998; Bonaduce and Andreotti, 2009; Dallongeville *et al.*, 2016). As with other binding media, these main markers ought to be accompanied by other specific markers, in order to strengthen the identification of a binding medium.

For non-derivatised samples, the most abundant small aromatic compounds reported are pyrrole, methyl pyrrole, ethyl pyrrole, toluene and ethylbenzene. Toluene and ethylbenzene were reported in high intensities for egg white and casein as well, but pyrrole and pyrrole derivatives were exclusively detected for animal glue (Orsini, Parlanti and Bonaduce, 2017).

DKPs and related compounds were also detected in a non-derivatised sample and the main DKP-related markers were cyclo(proline-glycine), diketodipyrrole, cyclo(proline-hydroxyproline) and cyclo(proline-proline) in addition to Cyclo(proline-alanine) and cyclo(proline-valine). Cyclo(proline-hydroxyproline), cyclo(proline-alanine) and cyclo(proline-valine) were reported in more than one stereochemical conformation (Orsini, Parlanti and Bonaduce, 2017).

For samples pyrolysed in presence of TMAH, reports of DKPs and other products formed from proteins or amino acids have been documented (Hendricker and Voorhees, 1998; Gallois, Templier and Derenne, 2007; Tsuge, Ohtani and Watanabe, 2012; Templier, Gallois and Derenne, 2013), but none of the reviewed articles were dedicated to identification of the particular DKPs present in

proteinaceous binders. The main markers in these samples are, therefore, small aromatic compounds.

1.1.7 Casein

Casein tempera, casein glue or milk tempera were often used instead of egg for certain pigments (e.g. blue) and for mural paintings, or added as binder/glue in the grounding (Mills and White, 1994c; Casadio, Giangualano and Piqué, 2004; Colombini and Gautier, 2009). Milk is an aqueous emulsion of proteins and lipids and dry cow milk contains about 26% proteins and 26% lipids in addition to smaller amounts of sugars, such as lactose (Mills and White, 1994c; Colombini and Modugno, 2009).

There are two mayor groups of milk proteins: Caseins (80%), which are phosphoproteins, and whey proteins (20%) (Colombini and Gautier, 2009). To obtain casein glue ("curds"), milk is subjected to acidic, enzymatic or thermal treatments, which is the same process as milk being curdled to make cheese (Mills and White, 1994c; Colombini and Modugno, 2009). Casein is then filtered after precipitation and dried, and the white powder is applied after being dissolved in aqueous alkaline solutions. Traditionally casein tempera was made from skimmed milk and lime (Colombini and Gautier, 2009).

The main amino acids present in casein are glutamic acid (glutamine), lysine, proline, leucine and valine (Mills and White, 1994c). Glutamic acid and lysine are hydrophilic and therefore inhibit DKP-formation (Orsini, Parlanti and Bonaduce, 2017).

Casein contains about 1% phosphorus, which mostly stems from phosphoric acid esterifying hydroxyl groups of amino acids such as serine and glutamic acid (Mills and White, 1994c). Despite that, phosphoric acid has not been detected in samples of casein (Chiavari and Prati, 2003).

For samples analysed with TMAH, pyrrole, pyrroline (both in low intensities) and methylated fatty acids have also been reported as markers (Bocchini and Traldi, 1998; Bonaduce and Andreotti, 2009). Saccharide-related markers have been suggested as well, e.g. mono-, di- and trimethoxybenzenes. These are formed during thermal degradation of carbohydrates in presence of TMAH. And example is trimethoxybenzene, which was identified in samples of carbohydrates pyrolysed with TMAH. Markers associated with saccharides are suggested as specific for casein, as these are not present in any other proteinaceous samples (Fabbri, Chiavari and Galletti, 1996; Chiavari *et al.*, 1998).

In non-derivatised samples, DKPs, which are associated with proteins, are the most abundant. The most prominent DKPs were cyclo(proline-valine), cyclo(proline-leucine) and cyclo(proline-isoleucine), while the most prominent small aromatic compounds were toluene, methyl-phenol and indole. Markers recorded for egg white coincide (Orsini, Parlanti and Bonaduce, 2017).

1.2 Analytical pyrolysis

Analytical pyrolysis is defined as "the characterization, in an inert atmosphere, of a material or a chemical process by a chemical degradation reaction(s) induced by thermal energy" (IUPAC, 1993), and is also known as thermal degradation or thermal fragmentation (Jones, 2000; Wampler, 2007a).

During pyrolysis a substance is transformed into a series of volatile, characteristic products, usually of lower molecular weights than the initial molecules (Poole, 2003a; Lettieri and Al-Salem, 2011). The product of a pyrolysis is called the pyrolysate (IUPAC, 1993). The fragmentation that occurs during pyrolysis is analogous to the fragmentation occurring during mass spectrometry, where energy is added to a system, molecules break apart and stable fragments are formed. The reaction mechanism, free radical degradation, is initiated by dissociations of chemical bonds after heat transfer (Poole, 2003a; Wampler, 2007a).

Coupling pyrolysis to gas chromatography (pyrolysis-GC) for analysis of cultural heritage samples have been studied since mid-1960, originally using flame ionization detectors. With mass spectrometry (MS), the technique gained a broader range of application (Dallongeville *et al.*, 2016). In gas chromatography, analytes are separated when they differ in vapor pressure and/or in interactions with the stationary phase, provided suitable chromatographic parameters during analysis. The analytes (or some convenient derivatives of them) must also be thermally stable at the required vaporization temperature. The single most important physio-chemical property is therefore the vapor pressure of the constituents in the sample (Poole, 2003b).

Solid samples, such as paint, were conventionally unsuitable for GC. They cannot be analysed using GC/MS without pre-treatments, as they are polymerised materials. These need to be reduced to low molecular molecules in a preliminary

step (Colombini *et al.*, 2010), and pyrolysis is one way to achieve this. Coupling GC to pyrolysis greatly expanded the application range of this sensitive and powerful diagnostic tool (Bonaduce and Andreotti, 2009; Tsuge, Ohtani and Watanabe, 2012).

The resulting chromatogram of a pyrolysate is called a pyrogram (IUPAC, 1993), and the "pyrolytic profile" refers this molecular profile.

Advantages of analytical pyrolysis is little need for pre-treatment and miniscule sample amounts, which reduce time consumption and risk of contamination (Colombini *et al.*, 2010). A disadvantage is that the molecular profile produced through thermal degradation (during pyrolysis) can be difficult to interpret, as the final pyrolysis products are not always straightforwardly related to the chemical structures of the molecules originally present in the sample (Bonaduce and Andreotti, 2009; Colombini *et al.*, 2010).

1.2.1 Reproducibility

In analytical pyrolysis it is possible to study both this behaviour of molecules during pyrolysis (degradation mechanism) and the resulting molecular fragments (pyrolysis products) (Wampler, 2007a). This thesis is a case of the latter. The fragmentation is characteristic for the original molecule, based on the relative strengths of the bonds between its atoms. If the energy parameters (temperature, heating rate and time) are controlled in a reproducible way, the same distribution of smaller molecules will be produced each time an identical sample is heated in the same manner (Wampler, 2007a).

Traditionally, analytical pyrolysis was criticised for being unreliable, as neither apparatus nor practice were standardised. Operating conditions varied and homemade pyrolysis instruments were more widely used, which inevitably

affected both inter- and intralaboratory reproducibility (Jones, 2000). When analytical pyrolysis came into use it was somewhat stigmatised, as "dirty analyses" as residues are often present in the pyrolysis zone after analysis, requiring thorough cleaning procedures.

As the potential of the technique was recognised, pyrolyser designs have been standardised and now a handful of different pyrolysers are commercially available. Factors influencing the reproducibility in analytical pyrolysis are related to each of the stages in a pyrolytic analysis: Sample preparation, actual pyrolysis (the pyrolyser), transfer of pyrolysate to the analytical instrument and analysis of pyrolysate (Wampler and Levy, 1987; Wampler, 2007b).

For sample preparation contaminations and sample size, homogeneity and geometry are important for the reproducibility. During pyrolysis, the sample should be heated uniformly and rapidly, with as low thermal gradient through the sample as possible. To achieve this, the sample should be small (5-25 µg) and preferably a thin, homogenous film. Samples can be homogenised by grinding them to powder, and a thin film can be created by melting the sample to a film or dissolving it in suitable solvent (Wampler and Levy, 1987; Jones, 2000; Wampler, 2007b). In special cases, larger samples are preferred, e.g. when they are non-homogenous or low in organic content (Wampler, 2007b). This is the case for paint samples.

Regarding contaminations, even small amounts can have a significant effect on the reproducibility when analysing miniscule samples like these. Sources of contaminations could be fatty acids from fingers or cross contaminations from the tools involved. It is therefore important to avoid skin contact and standardise cleaning procedures for all tools and instrumental parts in contact with samples (Wampler and Levy, 1987).

For the actual pyrolysis, it is important to consider the design of the pyrolyser, in particular the pyrolysis zone, as it dictates how the pyrolysate is produced. How

uniformly and quickly the pyrolysate is produced is a major concern in analytical pyrolysis. Rapid pyrolysate formation is important to avoid secondary degradation reactions, which would affect the reproducibility negatively (Wampler and Levy, 1987; Wampler, 2007b). The heating time, called the temperature rise time (TRT), is defined as the time required for a pyrolyser to increase temperature from initial to final temperature (IUPAC, 1993). It is measured in milliseconds, and for a Pyrola®2000 filament pyrolyser (Pyrol AB), the default TRT is set to 8.0 ms. After reaching the final temperature it can be held for several seconds (i.e. the pyrolysis time), and the temperature over time is therefore also important to the reproducibility. A graphical representation of the temperature over time is called a temperature time profile (TTP) (IUPAC, 1993).

There are multiple ways of heating a sample sufficiently to break chemical bonds, and the configuration of the pyrolyser is, therefore, important for the reproducibility of the results. Three commercialised designs are currently widely used: Isothermal furnaces (microfurnaces), inductively heated filaments (Curie-point pyrolysers) and resistively heated filaments (pyroprobes) (Wampler, 2007b). The different designs have their particular advantages and disadvantages, but these three types are standardised, in contrast to the somewhat stigmatised "plethora of home-made pyrolysers", which were used in the early years of analytical pyrolysis (Jones, 2000). Curie-point pyrolysers were not encountered in the literature review, and will, therefore, not be described further here.

The Pyrola®2000 platinum filament pyrolyser (Pyrol AB) is a pyroprobe. This pyrolyser type may be heated to any temperature over its usable range and at a variety of rates, which enable investigations of degradation processed by e.g. thermogravimetric analyses, where the sample is heated at low rates. Disadvantages with pyroprobes are the need for a heated zone, in which the filament (with sample) is inserted, as the heating may alter samples before pyrolysis. In addition, the dependency on the physical connection to a control unit

for temperature control (example in *1.2.2 Pyrolysis-GC/MS setup at IKJ/NTNU*), can affect the temperature produced, if the connection is damaged.

Pyrola® pyrolysers were "elegantly" designed to tightly control the temperature rise time and final pyrolysis temperature (Tydén-Ericsson, 1973; Ericsson, 1980, 1985; Wampler and Levy, 1987). Pyrola®2000 is marketed as a "high precision pyrolyser", where the sample is in direct contact with the heating element (the platinum filament), thus facilitating "extremely fast" temperature rise and precise temperature control (Pyrol AB, 2019b).

Pyrolysers from Pyrol AB were seldomly encountered in the reviewed literature. The most frequent pyrolysers were different models of pyroprobes from Chemical Data System (CDS) and microfurnaces from Frontier Laboratories. For both these, solid samples are placed in a container, e.g. a quarts tube for pyroprobes and small stainless steel cups for microfurnaces. In pyroprobes, the container is in direct contact with a heating element (a coiled metal filament), while in microfurnaces, the sample container is placed in a isothermally heated volume/furnace (Wampler, 2007b, pp. 29–40). It is not possible to know the TRT of the sample in microfurnace pyrolysers, as the furnace is isothermally heated (Wampler, 2007b, p. 32). For pyroprobes, the TTP for a sample analysed using a CDS pyroprobe is illustrated by Ronsse *et al.* (2012, fig. 2), and is rather complex compared to the TTP obtained when operating a Pyrola®2000 (examples given in Appendix IX).

For the pyrolysate transfer, the coupling between the pyrolyser and the analytical instrument influences the reproducibility of results, as it effects the transfer of pyrolysates from the pyrolysis zone. The pyrolyser can be either directly coupled, which is the case for the device operated in this thesis, or it can be connected by a transfer line. All transfers in analytical systems bares a risk of irregular sample transfer, as the transitions are prone to leaks and (partial) condensation of heavier

pyrolysates in zones with temperature differences, known as cold spots (Wampler and Levy, 1987).

For the analysis of the pyrolysate, the more convoluted and hyphenated the analytical system becomes, the more likely it is to be a source of irreproducibility (Wampler and Levy, 1987). It contributes to the reproducibility of the system as a whole, but will not be reviewed further here, except for the spectral differences related to quadrupole ion traps (QITs) described in *1.2.2 Pyrolysis-GC/MS setup at IKJ/NTNU*.

1.2.2 Pyrolysis-GC/MS setup at IKJ/NTNU

The pyrolysis-GC/MS setup at IKJ/NTNU (Fig. 1.2) consisted of a Pyrola®2000 pyrolyser installed on a GC/MS system from Thermo Scientific (a Trace Ultra GC coupled to an ITQ1100 3D ion trap MS).

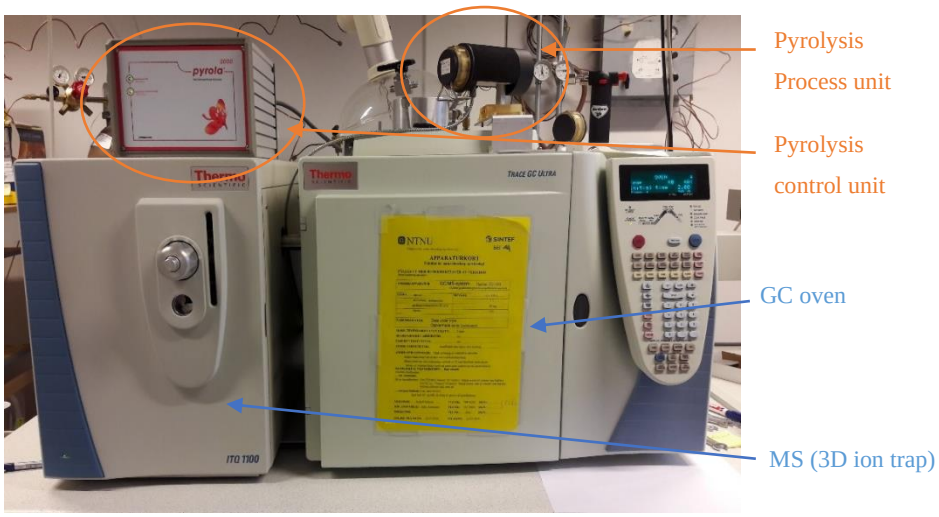


Figure 1.2: The pyrolysis-GC/MS setup at IKJ/NTNU. A pyrolyser with a platinum filament (Pyrola®2000, Pyrol AB), coupled online to a gas chromatography system and ion trap mass spectrometer (Trace GC Ultra ITQ 1100, Thermo Scientific).

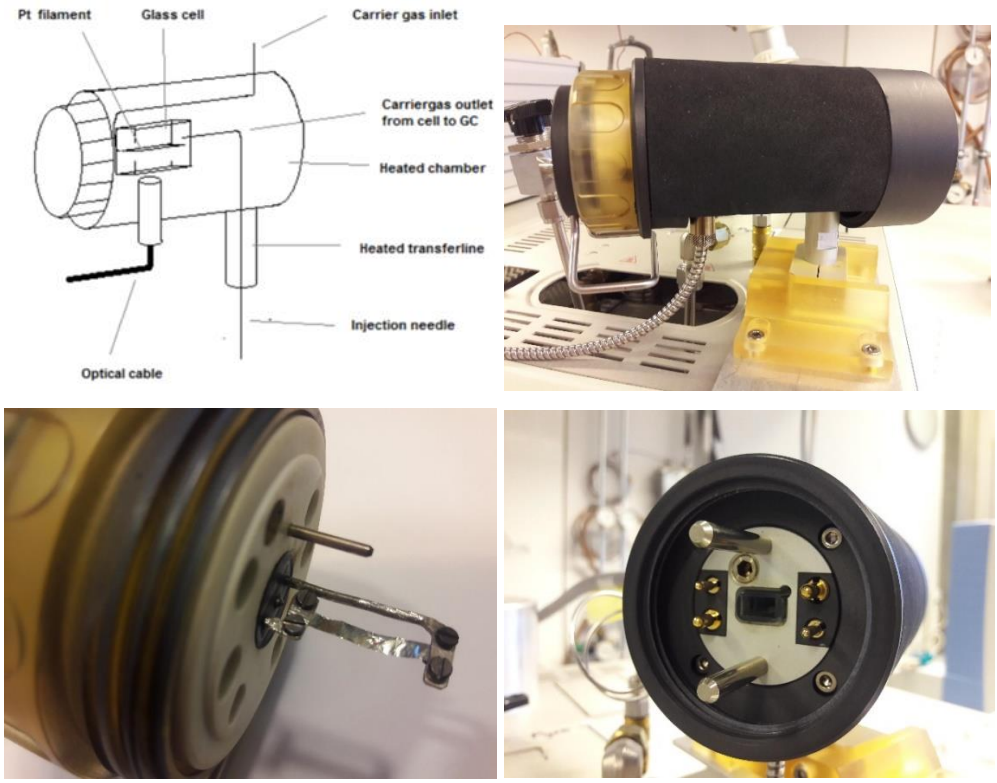


Figure 1.3: **Illustrations and pictures of the Pyrola®2000 (Pyrol AB) process unit**. Upper left: Schematic diagram (Östman and Näsström, 2012). Upper right: Pyrolysis process unit: Probe and chamber assembled. Lower left: Pyroprobe with platinum filament (heating source and sample application site) removed from pyrochamber. Lower right: Exposed pyrochamber with glass cell inserted.

The pyrolyser is comprised of a process unit (mounted on the GC) and a control unit (Fig. 1.2). The process unit consists of a pyrolysis chamber and probe with a platinum filament as both sample application site and heating source (Fig. 1.3). The filament is heated by two constant current pulses defined by their durations (t1, t2) and current amplitudes (I1, I2). These are produced by the control unit, which is controlled by a PC with Pyrola®2000 software installed. The control unit also measures the temperature of the pyrolysis chamber and the filament. The latter is measured using both and resistance of the filament and the signal from a

photodiode monitoring the filament, coupled to the control unit with an optic cable (PyrolAB, 2013, p. 6).

The chamber contains a quarts cell (Fig. 4.4), which protects the GC from non-volatile pyrolysis products and enable emitted light from filament to be detected by the photo diode. The temperature of this chamber is usually set to 150-175°C, and the higher the temperature, the more of the high boiling pyrolysis products will be transported to the column (PyrolAB, 2013, p. 6). The chamber is enclosed with either a pyroprobe or "probe dummy" (pyroprobe without filament, see Appendix VIII), and is only exposed when removing/inserting pyroprobe and/or glass cell during sample application or cleaning.

A special feature for the Pyrola pyrolysers is that they measure the actual ("true") pyrolysis temperature of the filament during pyrolysis, presented by the software as temperature time profiles (TTPs) immediately after every pyrolysis (examples in Appendix IX) (Pyrol AB, 2019a).

In addition to an uncommon pyrolyser, the mass analyser of the pyrolysis-GC/MS setup at IKJ/NTNU, was relatively uncommon as well. It was a 3D ion trap, also known as a quadrupole ion trap (QIT), but "standard" quadrupole mass analysers (QMFs) are most often preferred. Ions are stored for longer before detection in QIT than QMFs and they, additionally, may collide with helium (buffer gas) before detection. As ions have a finite stability, they might degrade before detection in QIT, resulting in the spectral differences compared to QMF (Sparkman, Penton and Kitson, 2011).

The NIST08 library, embedded in the software for the GC/MS system, is recorded using QMF. Comparisons between the mass spectra recorded in the experiments and the library could therefore be difficult, if not impossible. Partial matches in the NIST08 library can indicate possible identifications, but fully identifying a

marker as a specific compound would probably require a reference sample run for comparisons.

1.3 Pyrolysis-GC/MS analyses of binding media

Analytical pyrolysis is a valuable tool in analyses of artistic and archaeological materials, when based on "very thorough and systematic research programs rather than sporadic efforts" (Shedrinsky and Baer, 2007, p. 107). Investigating these materials involve many complicating factors and practical difficulties, due to e.g. the complicated nature of artistic samples, where the artist often used mixtures. From the initial mixing of binding media and pigments to the recording of a pyrogram, the material will undergo several alterations, such as during film formation, degradation through aging and pyrolysis (Fig. 1.4). Many complicating factors makes it easy to overlook minor, significant peaks, when interpreting of pyrograms, as these small peaks are overshadowed by peaks of major, less significant components (Shedrinsky and Baer, 2007).

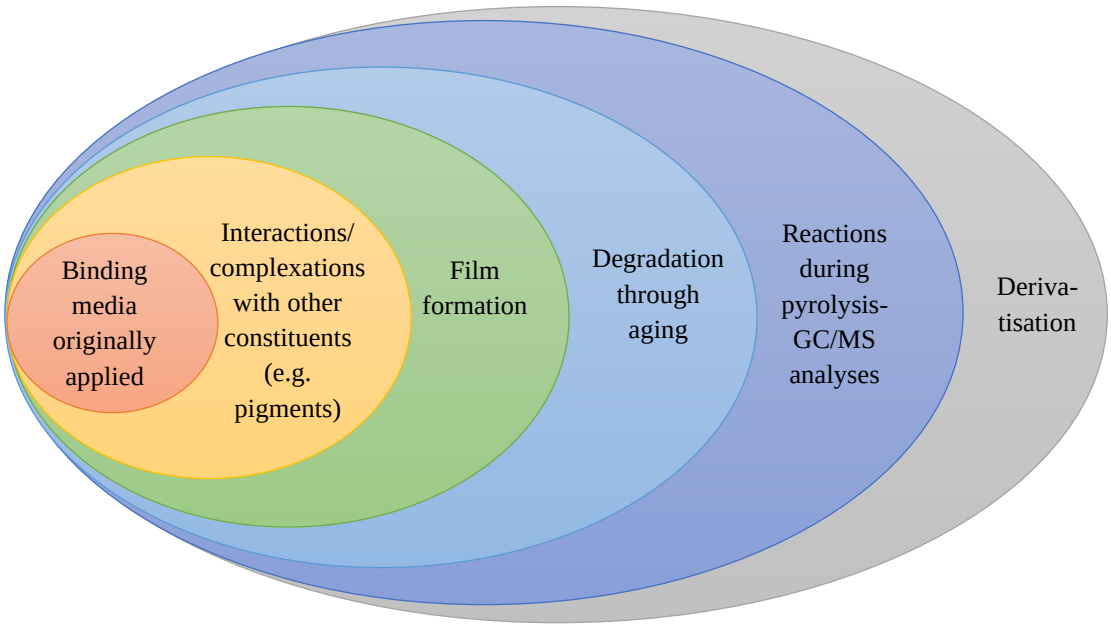


Figure 1.4: Alterations of binding media material from application to detection. Possible factors altering the binding media applied before detections. These complicate the identification of binding media in the analysed paint. When interpreting pyrograms, an understanding of these aspects is important.

There are, currently, two main focuses for analysing binding media in paint: To simultaneously detect more than one class of organic material and to get an overview of degradation and interferences of inorganic material in the paint (e.g. pigments). This thesis focuses on the former, but it is important to mention the latter, as paint samples can contain inorganic binders affecting the structure of the organic media in the paint (Bonaduce *et al.*, 2016). These are primarily inorganic pigments containing metallic cations, which can form complexes with for example amino acids and glycerolipids (Bonaduce and Andreotti, 2009; Colombini and Gautier, 2009). In a study, Colombini *et al.* (2010) stress that understanding of the degradation processes and inorganic interferences are fundamental for correct characterisation of binding media in paint and that this can only be achieved if 1) model paint replicas (mock samples) are used correctly, 2) appropriate artificial aging protocols are developed and 3) collaborations between research groups are set up. The latter points to sharing reference materials and databases as well as performing interlaboratory exercises and "round-robin analyses" on shared samples to validate and compare analytical procedures.

1.3.1 Mock samples

Mock samples are reconstructions of paint, which, in this thesis, were prepared as described in *2.1.3 Mock samples*.

For proteinaceous materials, standardised paint reconstructions and standardised artificial aging protocols recently have been introduced (Duce et al., 2012, 2013; Ghezzi et al., 2015; Pellegrini et al., 2016). Paint reconstructions of rabbit glue, casein or ovalbumin (the main protein in egg white) were each prepared differently, probably according to (historical) recipes. Ovalbumin was dissolved in water, and casein was left to expand in water, then an ammonia solution (30% *w/w*) was added drop by drop to obtain a clear solution (Duce *et al.*, 2012, 2013).

Rabbit glue was dissolved in water and heated until a clear/fluid solution was obtained (Ghezzi *et al.*, 2015; Pellegrini *et al.*, 2016). Paint reconstructions were made both with and without pigments, and when the reconstruction was to contain pigments, they were mixed in after fluid solutions were obtained. The paint was then applied on microscope glass slides with a brush. In the article by Ghezzi *et al.* (2015) they specify that the paint is applied in two fine coats of about 1 mm, and that the first layer was left to dry before the next layer. In the first of these four studies (Duce *et al.*, 2012), the samples had been prepared the summer 2010, and the article was received in November 2011, whilst the storage time for the paint replica samples was one week for the article published in 2015 (Ghezzi *et al.*, 2015). For the two remaining articles (Duce *et al.*, 2013; Pellegrini *et al.*, 2016), storage conditions were not specified. The reconstructed paint samples were analysed both before and after "indoor artificial aging conditions", where the samples were placed in a Solarbox. The exposure conditions were the same for all four publications, e.g. 720 h at 25°C and 50% relative humidity (Duce *et al.*, 2012, 2013; Ghezzi *et al.*, 2015; Pellegrini *et al.*, 2016). These recipes reveal the ongoing experiments aiming to overcome the challenge of proper reference materials and could be useful for future investigators.

1.3.2 Markers

Markers are compounds, which are specific for a material. The aim of analysing mock samples is to identify these "target molecules". There are three categories of markers (Bonaduce *et al.*, 2016):

1) A single marker: One specific compound characteristic for a specific organic material

2) An overall profile: A specific pattern of several compounds characteristic for a specific material

3) Quantitative evaluations of ratios: Quantitative relations between amounts of multiple significant compounds characteristic for a specific material

Some markers (mainly the proteinaceous) are reported differently for various pyrolysis instruments. Therefore, it is important to create a reference library for the specific instrument in use (Bonaduce and Andreotti, 2009).

Most organic materials (proteins, glycerolipids and polysaccharides) consist of the same building blocks (amino acids, fatty acids and saccharides respectively). Thus, different proteins can seldomly be distinguished from each other based on specific markers, as they all contain the same amino acids. The same is true for glycerolipids, which mostly contain the same fatty acids (Bonaduce *et al.*, 2016).

Despite the only semi-quantitative nature of pyrolysis-GC/MS, the basis for identification of lipid and proteinaceous material is comparisons of the quantities of certain compounds. For lipids, the ratio between azelaic acid and palmitic acid which is used to distinguish between drying oils and the lipid components of egg yolk (Colombini *et al.*, 2010; Bonaduce *et al.*, 2016; Degano *et al.*, 2018). For proteins, such ratios are yet to be defined.

1.3.3 Derivatisation

As fatty acids and dicarboxylic acids are important markers in the identification of glycerolipid material (*1.1.3 Linseed oil - a drying oil*), a suitable derivatising agent is required. Derivatisation increases detection in GC, as less polar derivatives are analysed (Husek, 2000). Pyrolysis performed in the presence of a derivatising agent (often an organic alkaline), is also known as reactive pyrolysis (Tsuge, Ohtani and Watanabe, 2012)

The Lauschke project investigated different derivatisation techniques (methylation and silylation reagents). For pyrolysis in presence of a methylating

reagent, tetramethylammonium hydroxide (TMAH) and trimethylphenylammonium hydroxide (TMPAH) were tested. For pyrolysis in presence of a silylating reagent, hexamethyldisilazane (HMDS) alone as well a mixture of HMDS, trimethylchlorosilane and pyridine in a 3:1:9 ratio were tested. Lipid-containing mock samples analysed in presence of a silylating reagent mostly resulted in non-derivatised samples (fatty acids or other expected markers were not identified) and therefore, the silylation approach was deemed unsuccessful, even for samples suspended in HMDS for an hour before analysis. Fatty acids were, however, detected in samples pyrolysed in presence of TMAH, and it was decided to move forward with this derivatisation reagent alone in this project.

Chapter 2: Materials and methods

Science is simply the word we use to describe a method of organising our curiosity.

Tim Minchin, 2013

This chapter provides an overview of materials, instrumental setup, experimental procedure as well as adjustments to the experimental procedure implemented gradually throughout this project.

Samples were either reference samples, mock samples and paint samples. Mock samples mimic ("mock") dry paint (illustrated in Fig. 2.1). Paint samples are microsamples (μg-range) collected by Kidane Fanta Gebremariam from Christian Ethiopian artwork (samples illustrated in Fig. 2.2 and artwork illustrated in Appendix VI). Reference samples are standard materials of chemical compounds, suspected to be present in the mock samples or paint samples. These were run to confirm the identities of suspected markers.

2.1 Materials

2.1.1 Derivatisation reagent

Tetramethylammonium hydroxide (TMAH; in 25 % in water, nitrogen flushed, Acros Organic) was used for thermally assisted derivatisation. Most analyses were performed using TMAH. Exceptions will be mentioned explicitly.

2.1.2 Reference samples

Small aromatic compounds: Pyrrole (98%, Sigma Aldrich), Toluene (Merck), Indole (99+%, Sigma Aldrich).

Fatty acids (FA, mix of solid compounds): Lauric acid (C12:0; Merck KGaA), Pentadecanoic acid (C15:0; Approx. 99 %, Sigma Chemical Company), Palmitic acid (C16:0; "Rein", Riedel-de Haën), Stearic acid (C18:0; "Rein", Riedel-de Haën).

Methylated fatty acids (FAME; in toluene; 2 µL/100 µL): Methyl myristate (Me-C14:0), Methyl palmitate (Me-C16:0), Methyl oleate (Me-C18:1 Δ^{9}), Methyl linoleate (Me-C18:2 $\Delta^{9,12}$). "Puriss. CHR", Koch-Light Laboratories Ltd – Colnbrook Bucks England.

Methylated diacids (diFAME, mix of pure liquid compounds): Dimethyl suberate (diMe-C8; 99%), Dimethyl azelate (diMe-C9; ≥98,5%), Dimethyl sebacate (diMe-C10; Analytical standard). Sigma Aldrich.

Alkanenitriles (mix of solid samples): Octadecanenitrile (CN-C18; Technical grade ≥ 90%), Heptadecanenitrile (CN-C17; ≥ 95.0%, GC). Sigma Aldrich.

Amino acid mixture (solid sample): Glycyl-L-Proline (Gly-Pro; >96 %, TCI)

Sterols (solid sample): Cholesterol (Chol; Sigma Aldrich, ≥ 99%).

2.1.3 Mock samples

Proteinaceous: Egg yolk (EY) and egg white (EW) from eggs of a local supermarket in Trondheim, Norway, animal glue (AG) from Zecchi, Firenze, Italy (Rabbit glue) and casein (Cas) of unknown origin provided from Archaeology Museum in Stavanger.

Lipid: linseed oil mixed with chalk (LO+Ch) and beeswax (BW), were both of unknown origin.

Mock samples were provided and prepared by Kidane Fanta Gebremariam. Some materials (LO+Ch, EY and EW) had been dried on glass plates (object glass), scraped off and grinded. Linseed oil was analysed with chalk as unmixed linseed oil samples were not present in the mock sample collection at NTNU[3].

Some mock samples were fine homogenous grains (AG and Cas), others rougher mixed size grains (BW, EY, EW and LO+Ch) (Fig. 2.1).

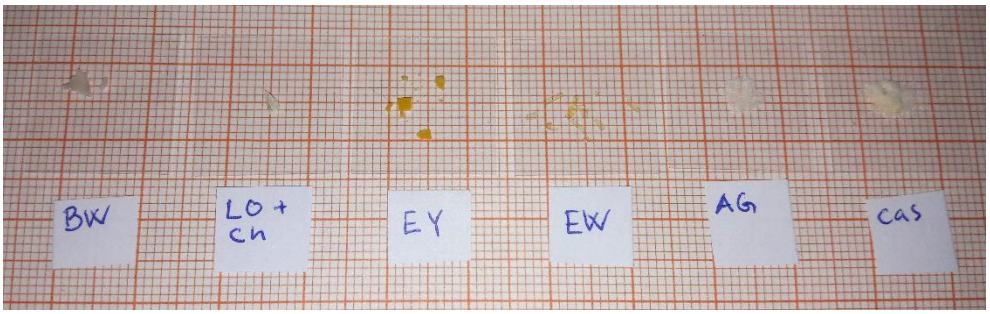


Figure 2.1: Presentation of mock samples. From left: Beeswax (BW), Linseed Oil + Chalk (LO+Ch), Egg Yolk (EY), Egg White (EW), Animal Glue (AG), Casein (Cas).

[3] Chalk had been added to linseed oil to mimic grounding material, but this allegedly had little or no influence on the results. The practice was therefore discontinued before this thesis.

2.1.4 Paint samples

In total, six paint samples of Ethiopian Christian art were investigated (Fig. 2.2). These samples had been collected by Kidane Fanta Gebremariam from the Institute of Ethiopian Studies (IES) and during fieldtrips in 2007-2012. Background information on sample origin was provided by Gebremariam (illustrated in Appendix VI).

Mota Giyorgis (MG)

Sample morphology: Thin, flat, red paint sample with little support material/primer attached.

Sample origin: An icon at the collections at IES, originally from an 18th century church, Mota Giyorgis (MG). The icon could be from 18th or 19th century.

Two analyses were performed on fragments from this sample: MG-S (smaller fragment, 0.5-1mm^2) and MG-L (larger fragment, 1.5mm^2).

Icon 21 (IC21)

Sample morphology: Two paint samples, brown (B) and green (G). The samples were quite hard, and therefore difficult to divide. Both samples had medium amounts of support/primer.

Sample origin: A 15th century icon from the collections at IES. In this special case the artist is known: Nicoló Brancaleon, known as Merqoriwos in Ethiopia.

Three analyses were performed on the two samples from this icon: IC21B (2 mm^2), IC21G (1 mm^2) and IC21G-ø (1.5 mm^2). IC21G-ø was analysed without derivatisation ("ø" = mathematical symbol for "null").

Icon 9 (IC9)

Sample morphology: Small sample with medium amount of support/primer.

Sample origin: A 15th century icon from the collections at IES.

One analysis was performed on the sample from this icon*:* IC9 (0.5mm^2).

Wukero Cherkos (WC)

Sample morphology: Two paint samples, white (W) and yellow (Y). These were large and consisted mostly of support material (wall/ceiling) and/or primer. It was difficult to distinguish between the light paint and light preporatory øayer/wall material.

Sample origin: Paintings on ceiling and walls at Wukero Cherkos Church. This is one of the oldest rock hewn churches (excavated from stone) in Ethiopia. It is claimed to be from the 10th century or before. The paintings are assumed to be from the same time period and were in a poor state of conservation (see Appendix VI). One analysis was performed on each of the two samples: WCW (2.5 mm^2) and WCY (1 mm^2).

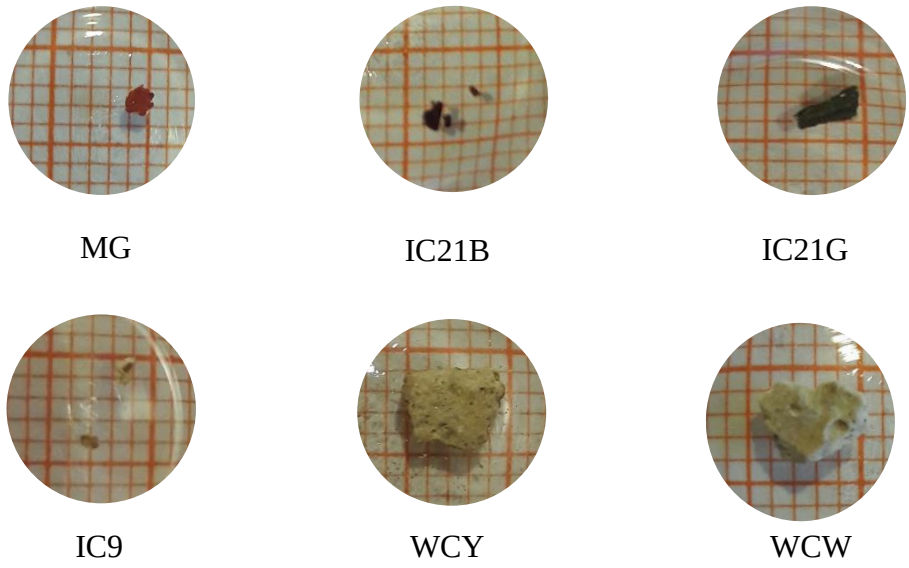


Figure 2.2: Presentation of paint samples before sample preparation. Samples placed in a small glass bowl on millimetre paper. After picture was taken, the samples were divided and/or crushed to obtain a sample size between 0.5 and 2.5 mm^2. From top left: Mota Giyorgis (MG), Icon21 Brown (IC21B), Icon21 Green (IC21G). From bottom left: Icon9 (IC9), Wukero Cherkos Yellow (WCY), Wukero Cherkos White (WCW). For the latter two, picture is, probably, taken with paint facing down.

2.2 Instrumental parameters

Analyses were performed using a Pyrola®2000 (Pyrol AB) resistively heated pyrolyser with a platinum filament, coupled online to a Trace GC Ultra gas chromatography system and ITQ 1100 ion trap mass spectrometer (Thermo Scientific). Thermo Xcalibur 3.0.63 software was used for data collection.

2.2.1 Pyrolysis

Chamber temperature, T_c, was kept at 175°C when the pyrolysis unit was not in use. Pyrolysis temperature, T_p, was programmed to be 700°C during analyses.

Calibration of the Temperature Time Profile (TTP) of the pyrolyser was performed when TTP values had changed substantially from preceding calibrated values. In addition, calibration was performed when parts were replaced (e.g. filament, quarts cell, septa) and when the pyrolysis unit was (re)installed.

A few reference samples were analysed on GC/MS without pyrolysis. This will be explicitly mentioned when relevant.

2.2.2 Gas chromatography (GC)

DB5-MS fused silica WCOT columns (Agilent Technologies) were used. Column dimensions: 30m x 0.25mm, 0.250μm film, 5 % phenyl 95 % dimethyl arylene siloxane. A split/splitless injector with a narrow liner (0.75 mm inner diameter) dedicated for pyrolysis was held at 280°C in split mode with split ratio 1:15. Carrier gas (HiQ® Helium 6.0) was used with a constant flow rate of 1.5mL/min (not vacuum corrected value). The transfer line from GC to MS was kept at 300°C.

Two temperature programmes for the GC oven were used; Temperature Programme 1 (TP1) for sample runs and Temperature Programme 2 (TP2) for blank runs (clean, empty filament):

$$40°C\ (5min) \xrightarrow{5°C/min} 230°C \xrightarrow{10°C/min} 300°C(5min) \qquad (TP1)$$

$$40°C \xrightarrow{15°C/min} 300°C(6min) \qquad (TP2)$$

2.2.3 Mass spectrometry (MS)

The mass spectrometer (MS) was equipped with a 3D ion trap operating with automatic gain control and using electron ionisation (70 eV). Analyses were run in full scan mode with accumulating 5 μsc per spectrum at 0.88 sec/scan.

Three scanning ranges were employed. Scanning range A (ScA) and Scanning range B (ScB) for TP1 (samples) and Scanning range C (ScC) for TP2 (blanks):

45-600 *m/z* (2 min) → 60-600 *m/z* (ScA)

60-600 *m/z* (2 min) → 45-600 *m/z* (ScB)

40-600 *m/z* (ScC)

In ScA the MS scans from 45 *m/z* the first 2 minutes, then from 60 *m/z* the remaining time. ScB is inverse of ScA as it filters out 0-60 *m/z* the first two minutes, then scans from 45 *m/z* thereafter.

2.2.4 Data system: Identification

Preliminarily, mass spectra of possible markers were compared with mass spectra from NIST 08 MS Library (version 2.0f, 2008) in Xcalibur software and/or suggested markers in literature (see section *1.1 Organic paint binders* under each material type).

Identity of a marker was confirmed through comparison with MS fragment pattern of a refence sample in an in-house collected reference library created for this purpose. This reference library contained pyrograms and mass spectra of reference and mock samples (Appendix IV).

Selected markers without a matching reference were marked as “unknown”.

2.3 Experimental

As the investigation advanced, the procedure for analysing samples were continuously adjusted. In the following, the latest, ("final") experimental procedure is described in *2.3.1 Experimental procedure*. In Appendix VII, the procedure is described in a manual (incl. troubleshooting) for future operators.

The procedure for pyrolysis-GC/MS involved four steps: 1. Blank run, 2. Sample preparation, 3. Sample run and 4. Standard cleaning procedures (Fig. 2.3, next page). When needed, extensive cleaning and troubleshooting were performed.

Samples were analysed in six batches (batch number specified per sample in Appendix I). In between these analyses, the GC column, and occasionally the pyrolysis unit, were uninstalled and reinstalled for other operations or for maintenance (e.g. change of GC column).

2.3.1 Experimental procedure

On the following two pages, representations (flow charts) of the initial (Fig 2.3) and the final experimental procedure (Fig. 2.4) are shown. The initial procedure was employed for batch 1, and the continuous adjustments for the following batches (Table 2.1) resulted in the final experimental procedure for batches 5 and 6.

In the flow charts circles (○) indicate starting and ending points, diamonds (◊) indicate decisions to be made (evaluations) and squares indicate actions (□). The actions and starting/ending point are each described in the subsequent text. As some actions require certain decisions or evaluations (e.g. during sample preparation the preparation depended on the sample type), these flow charts are simplified versions of the experimental steps.

In addition, the flow charts are colour coded. Green indicates actions related to actual analysis of a sample. Yellow indicate steps performed before and after,

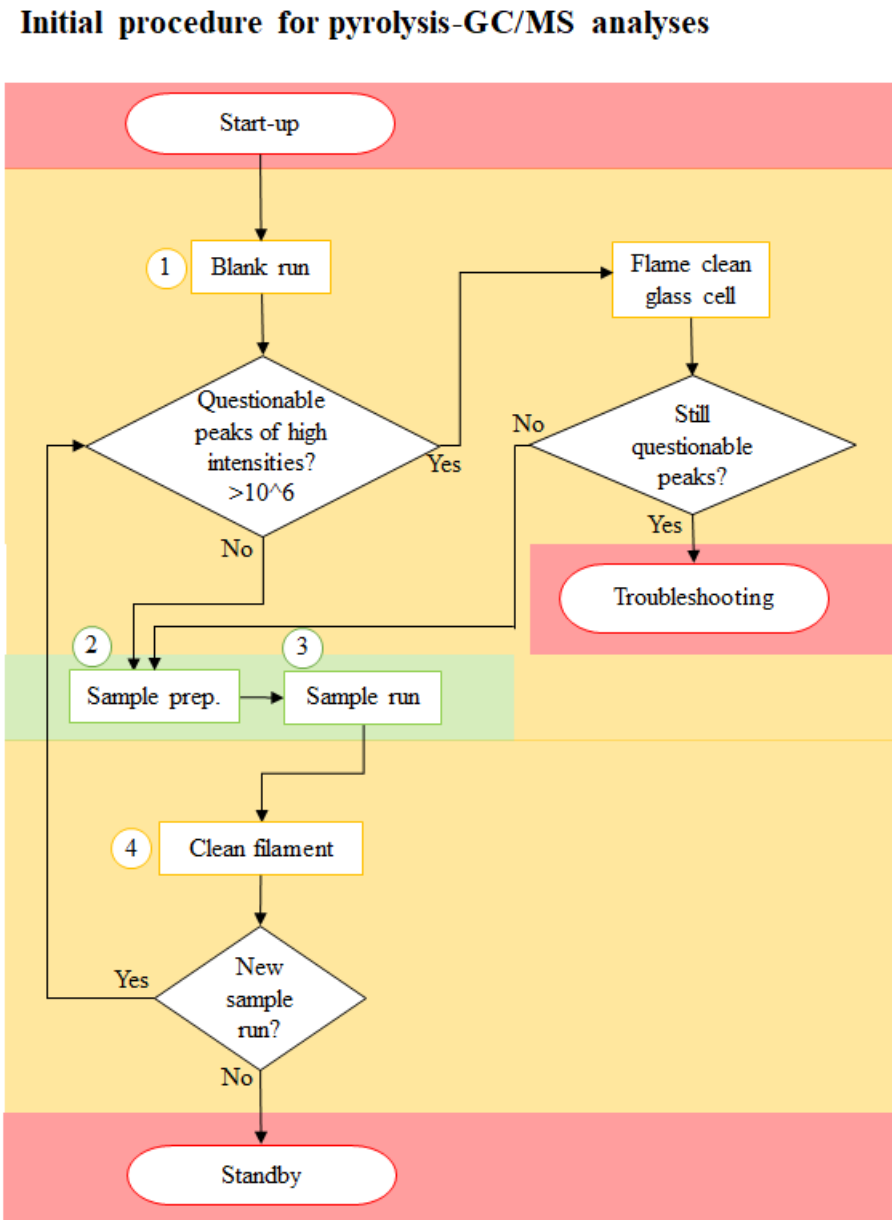


Figure 2.3: Initial experimental procedure for pyrolysis-GC/MS analyses. Flow chart of initial pyrolysis-GC/MS procedures (Batch 1). Circles (○) indicates starting and ending points, diamonds (◊) indicates decisions and squares indicate actions (□). Red indicates steps where the experiment is started or put on halt, yellow indicates steps performed before and after the actual analysis of the sample aiming to improve the reproducibility and green indicates actions related to actual analysis of a sample.

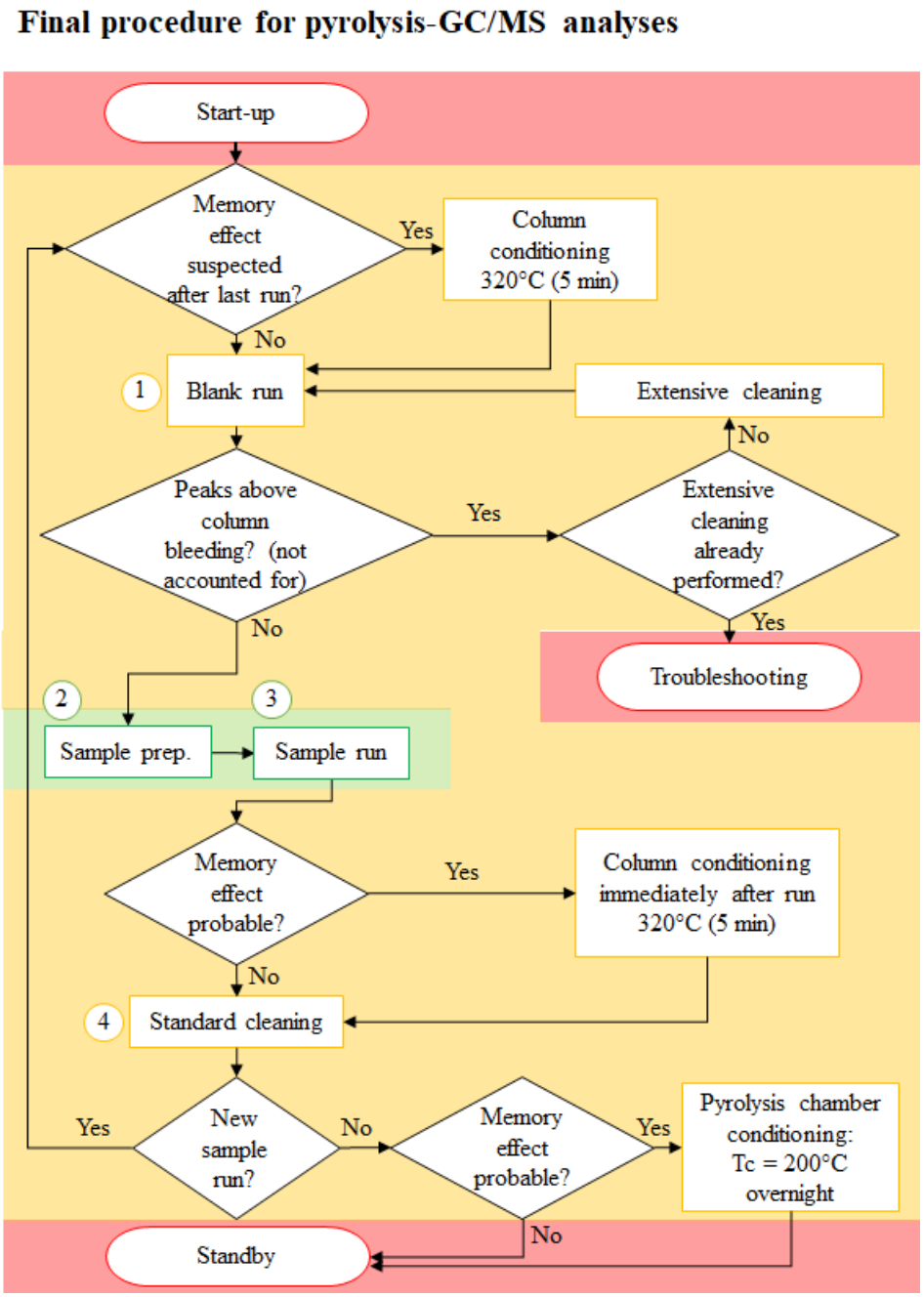


Figure 2.4: Final experimental procedure for pyrolysis-GC/MS analyses. Flow chart of the setup for pyrolysis-GC/MS procedures in the last batches (Batch 5 and 6). Circles (○) indicates starting and ending points, diamonds (◊) indicates decisions and squares indicate actions (□). Red indicates steps where the (Figure text continued) experiment is started or put on halt, yellow indicates steps performed before and

after the actual analysis of the sample aiming to improve the reproducibility and green indicates actions related to actual analysis of a sample.

aiming to improve the reproducibility. Red indicates steps where the experiment is started (start-up) or stopped (standby, troubleshooting).

In the following, the most recent version of the experimental setup is described (based on Fig. 2.4). When earlier versions of the setup deviate from the newest version, an asterisk (*) has been added to the given parameter. These adjustments are summed up in Table 2.1.

Start-up/standby

During start-up, these standard conditions were employed for the GC:
He flow (1.5mL/min), injector (280°C), transfer line (300°C)

When not in use (standby) these conditions were set:
He flow (0.8-1.0 mL/min), injector, (150°C), transfer line (150°C)

Pyrolysis chamber temperature, T_c, kept at 175°C both when in use and during standby, unless chamber had been conditioned overnight (200°C) to avoid memory effects (see "Extensive cleaning" in the following sections).

(1) Blank run

An empty, cleaned filament was analysed prior to every* sample run, using Temperature programme 2, Scanning range C. If a blank run contained peak(s), deviating from that of a typical blank pyrogram, extensive cleaning procedures were initiated.

(2) Sample preparation

All tools for sample manipulation and transfer were cleaned with water and acetone before use. Skin contact with sample was carefully avoided, and the filament was flame cleaned before sample application.

Sample size depended on sample type:

Reference samples: Samples were either undiluted liquids ("tip of a micro-syringe plunger"*), undiluted solids ("tip of a spatula") or solids/liquids dissolved in toluene (5µL). For gas phase references (batch 4) samples were extracted headspace (10µL) after heating in the GC oven and injected directly into the split injector of the GC (pyrolysis unit not installed).

Mock samples: Samples were either finely grained (animal glue or casein), where the tip of a spatula was applied or roughly grained (beeswax, egg yolk, egg white, linseedoil+chalk), where 0.5-2 mm^2 was applied.

Paint samples: Samples were divided and/or crushed to obtain a suitable fragment size (1-2.5 mm^2, specified per sample in section *2.1.4 Paint samples*). Fragments containing as much paint as possible were selected.

For both mock and paint samples flatter grains or pieces were preferred.

For derivatisation, TMAH (1µL*) was added to the sample on the filament, which then immediately was inserted in the pyrolysis chamber and analysed after completed reaction time (1 min in total from application)*.

(3) Sample run

Analyses were performed using Temperature programme 1, Scanning range B*.

(4) Standard cleaning

Sample residues were removed, and filament and quarts cell were flame cleaned*. For persisting stains, filament and/or quarts cell were washed with water and acetone using non-plastic cotton swaps.

Extensive cleaning

When suspecting persistent contaminants in the pyrolysis chamber (e.g. blown-off samples or other stains), the quarts cell, filament, filament holder, helium conduit and septa were carefully examined and wet cleaned (water and acetone on cotton swaps and/or precision wipes) followed by extra flame cleaning of quarts cell. To remove visible stains on filament holder after wet cleaning (e.g. melted samples), careful mechanical removal using a scalpel was performed. When chromatographic peaks showed intensities above 10^7 csp, memory effects were suspected. Generally, this was only an issue when running undiluted reference samples. Multiple steps were then initiated: Column conditioning (325°C for 5 min) immediately after sample run, pyrolysis chamber conditioning at 200°C overnight and eventually a new column conditioning (325°C for 5 min) before next run.

Troubleshooting

Troubleshooting was initiated when contaminants were still present after extensive cleaning procedures or when other problems occurred. This often involved replacing suspected contaminated or malfunctioning parts such as different septa, quarts cell, filament or GC column (some of which are illustrated in Fig. 4.4). These procedures are described in detail in the manual for the Pyrola®2000 (PyrolAB, 2013). Instrumental issues were solved according to a separate troubleshooting manual for the GC/MS system.

2.3.2 Adjustments (method development)

This section sums up adjustments to the method from batch to batch (chronologically) in an extensive table (Table 2.1). The columns are based on the experimetnal steps illustrated in Fig. 2.3 and 2.4, and describe the parameters changed within these steps: (1) Blank run, (2) Sample preparation, (3) Sample run (instrumental parameters) and (4) Cleaning (Standard and Extensive cleaning). The table is meant to illustrate progression in the method development. Most adjustments relate to cleaning or sample preparation.

The table provides details about specific individual samples have been run, and an overview of sample are found in Appendix I, where the batch number is mentioned for each sample.

Table 2.1: Overview of adjustments

Summation of adjustments to the experimental procedures in and between batches. In one batch (Batch 2) adjustments were implemented gradually throughout the sample runs of the batch. All other adjustments were implemented from the first run in a new batch and lasted throughout the batch (except for Batch 4).

An arrow ("→") indicates an adjustment and the change is underlined.

Batch	Blank run	Sample preparation	Parameters in sample run	Cleaning
1	Blank run before first run using TP2	*Reference samples* Diluted reference (5µl); Solid reference (tip of a spatula); *Mock samples* Roughly grained mock samples (0.5-2 mm^2) *Derivatisation* TMAH (3 µl); briefly heated to remove excess water; reaction time 1 min outside pyrolysis chamber	Samples run using TP1-ScA	*Standard* - Filament flame cleaned after every run. - Quarts cell flame cleaned after mock samples *Extensive* - Quarts cell and filament examined. - If visible stains: Wet cleaning and a new round of flame cleaning.

Batch	Blank run	Sample preparation	Parameters in sample run	Cleaning
2	→Blank run before every run using TP2	*Reference samples* - Diluted reference (5µl); - Solid reference (tip of a spatula); → - Liquid reference (tip of a spatula); *Mock samples* - Roughly grained mock samples (0.5-2 mm^2) → - Finely grained mock samples (tip of a spatula). *Derivatisation* - TMAH (3 µl) → TMAH (1 µl) - → Sample not heated to remove excess water - Reaction time 1 min outside pyrolysis chamber	Samples run using →TP1-ScB	*Standard* - Filament flame cleaned after every run. - → Quarts cell flame cleaned after every run *Extensive* → - Quarts cell and filament + filament holder, helium conduit and septa examined. - If visible stains: Wet cleaning and a new round of flame cleaning of quarts cell and filament.

Batch	Blank run	Sample preparation	Parameters in sample run	Cleaning
3	Blank run before every run using TP2	*Reference samples* - Diluted reference (5µl); - Solid reference (tip of a spatula); - Liquid reference (tip of a spatula) *Mock samples* - Roughly grained mock samples (0.5-2 mm^2) - Finely grained mock samples (tip of a spatula). →*Paint samples* (0.5-2.5 mm^2) *Derivatisation* - → Either no derivatisation or TMAH (1 µl) - Reaction time 1 min outside pyrolysis chamber	Samples run using TP1-ScB	*Standard* - Filament flame cleaned after every run. - Quarts cell flame cleaned after every run *Extensive* → Distinction between contaminants and memory effect - Memory effects, when intensities of chromatographic peak >10^7 cps: Column conditioning (325°C for 5 min), Conditioning pyrolysis chamber at 200°C overnight. - Contaminants: Quarts cell, filament, filament holder, helium conduit and septa examined: Wet cleaning and a new round of flame cleaning of quarts cell and filament. + Careful mechanical cleaning of filament holder when stains persisted both flame and wet cleaning.

Batch	**Blank run**	**Sample preparation**	**Parameters in sample run**	**Cleaning**
4	No blanks	*Reference samples (g)* Headspace extraction (10µl) after heating in GC oven.	Samples run using TP1-ScB	*(pyrolyser not in use)*
5+6	Blank run before every run using TP2	*Reference samples* - Diluted reference (5µl); - Solid reference (tip of a spatula); - Liquid reference (tip of a→ micro-syringe plunger) *Mock samples* - Roughly grained mock samples (0.5-2 mm^2) - Finely grained mock samples (tip of a spatula). *Paint samples* (1-2.5 mm^2) *Derivatisation* - Either no derivatisation or TMAH (1 µl) - → Reaction time 1 min inside pyrolysis chamber	Samples run using TP1-ScB	*Standard* - Filament flame cleaned after every run. - Quarts cell flame cleaned after every run *Extensive* - Memory effects, when intensities of chromatographic peak >10^7 cps: Column conditioning (325°C for 5 min), Conditioning pyrolysis chamber at 200°C overnight. - Contaminants: Quarts cell, filament, filament holder, helium conduit and septa examined: Wet cleaning and a new round of flame cleaning of quarts cell and filament. + Careful mechanical cleaning of filament holder when stains persisted both flame and wet cleaning.

Chapter 3: Results

Success is a science; if you have the conditions, you get the results.

Oscar Wilde

This chapter is divided in three categories of samples, presented in separate sections: *3.1 Lipid-based binding media*, *3.2 Proteinaceous binding media* and *3.3 Paint samples*. In these sections the pyrolytic profiles of the samples are each represented by

1. A pyrogram and a table identifying the selected markers
2. Mass spectra of these selected markers

1. Pyrograms

Some pyrograms were detailed (contain many peaks), others were simpler (few peaks). In simpler pyrograms, most or all peaks were selected as possible markers (e.g. Figs. 3.1 and 3.4), whilst in detailed pyrograms, only some were selected (e.g. Figs. 3.7 and 3.10). When selecting peaks as possible markers, these parameters weighed in:

- Peak intensity. High intensity peaks were prioritised. In addition, other peaks with fragment patterns resembling those of high intensity peaks were often selected. These could also be identified using SIM.

- Comparability: Linseed oil, animal glue, egg yolk and the paint sample IC21G were prioritised, as these were analysed both with and without derivatisation. These were investigated in more detail than others.
- Important markers reported in literature: Some of these were searched for by using simulated ion monitoring (SIM)[4].

Identities of possible markers were either confirmed, suspected or unknown. Peak notations depended on the degree of identification of the marker. Identities of markers were confirmed by comparison with a reference sample and/or by comparison with MS fragment patterns reported in the literature.

For markers of which the identity was suspected, this suspicion was based on literature reports and/or hits in NIST, which were more or less unambiguous, but not yet confirmed by comparisons with reference samples.

For confirmed and suspected markers, both were denoted by one or two letters, where at least the first letter is a capital letter, such as palmitic acid (P), stearic acid (S), phosphoric acid (PA) and styrene (St). Exceptions to this rule were 2,5-diketopiperazines (DKPs), which were denoted according to the two amino acids from which the compound was formed. An example was cyclo(Proline-Glycine) which was denoted Pro-Gly in the pyrogram. These cyclic compounds could give rise to two stereoisomers differentiated by numbers. A question mark (?) was added to the name of the compound in the table of selected markers, when the identity was suspected, but not confirmed.

The denotation of an identified peak was based on the source of that given compound: Pyrrole in samples without derivatisation is denoted “Py” and

[4]Simulated ion monitoring (SIM) is a chromatographic tool simulating scanning modes of certain mass spectrometers after recording mass spectra in full scan mode. The aim is to search for presence of specific fragments. An example: presence of the fragment *m/z* 186 is characteristic for diketodipyrrole (Fig, 3.8, tR = 31.02).

methylated pyrrole found in samples, where a derivatisation agent was added, was denoted "Py" as well.

Markers of unknown composition were denoted according to the analytical approach for that sample (with or without derivatisation, Table 3.1). When more than one suggested marker in a pyrogram had unknown identity, these were differentiated by numbers (look to Fig. 3.7 for examples). Unknowns of samples with derivatisation were represented by a small delta "δ"[5] and without derivatisation were represented by an "ø"[6].

Table 3.1: Peak notations for markers with unknown identities.

Sample	With derivatisation (δ)	Without derivatisation (ø)
Linseed oil + chalk	lδ	*No unknowns selected*
Beeswax	bδ*	*Not run*
Egg yolk	yδ	yø
Egg white	wδ	*Not run*
Animal glue	aδ	aø
Casein	*No unknowns selected*	*Not run*
IC21G	Iδ	iø

* Some unknowns of beeswax do not follow this rule (see explanation below table).

For beeswax, there were some exceptions to these notation rules, as it contained multiple long-chained fatty acids and saturated fatty acids had quite distinct fragment patterns (*1.1.3 Beeswax*). Shorter-chained saturated fatty acids had been analysed in this thesis as well, and unknown compounds with similar fragment

[5]As δ is the symbol mathematical derivatisation
[6]As ø is the mathematical symbol for "nothing" or "null"

patterns were denoted “fa”, for “unknown fatty acid”. Unknown compounds which were suspected to be hydrocarbons[7] were marked “hc”.

Pyrographic peaks caused by TMAH were marked with “x” and contaminations were marked with “*”. Contaminations were suspected to be related to septa softening compounds in the pyrolyser and/or the inlet, bleeding from different septa (teflon, phthalate) or dust (TDCPP[8]). The former two pyrographic peaks were strong after replacements of o-ring or septa in the pyrolysis unit (Fig. 4.4). These contaminants/system interferences appeared around t_R = 28-30 min and 40 min in Temperature programme 1 with these fragmentations patterns: (*m/*z 65, 77, 105, 121, 149, 176), (*m/*z 83, 99, 111, 127, 155, 173) and (*m/*z 75, 99, 191, 209). Base peak is underlined.

Regarding methylated compounds, names deviate from IUPAC as the non-derivatised compound present in the original sample was the compounds of interest. For example, “Palmitic acid, methyl ester” was regarded a more intuitive denotation than “methyl palmitate”, when representing presence of “palmitic acid”. This corresponds with nomenclature used in the scientific field.

2. Mass spectra

Due to different scanning ranges (*2.2.3 Mass Spectrometry (MS)*), some spectra began recording at *m/z* 45, while others at *m/z* 60. The scanning range will be indicated for each pyrogram.[9] Most mass spectra are presented in a shorter version than recorded. They were cut when insignificant or no peaks were present.

[7]Suspicion due to all top hits in NIST being hydrocarbons, and literature stating that hydrocarbons are present in beeswax.

[8]Tris(1,3-dichloroisopropyl)phosphate (TDCPP), a “Chlorinated tris”. A chlorinated organophosphate which have been used as flame retardants, pesticides, plasticizers, and nerve gases.

[9]The scanning range also affects the intensity of the main pyrographic peak of TMAH as well as the presence of additional peaks caused by TMAH. Scanning range A generates a high intensity TMAH peak around tR = 1 min and additional peaks of low intensity. Scanning range B generates a TMAH peak of lower intensity around tR = 1 min in addition to 1-2 additional significant peaks at around tR = 5 min and tR = 7 min (Appendix IV).

When a pyrographic peak of interest partially overlapped with other peaks, a function in the software to subtract interfering mass spectra was used. An asterisk (*) in a mass spectrum denotes a peak from an interfering spectrum, that should have been subtracted before the data was collected. This means that both pyrograms and mass spectra can contain asterisks. In both cases, these indicate interferences to be overlooked.

The mass spectra are presented chronologically with respect to retention times (t_R) of their matching pyrographic peaks. Retention times are reported after the compound name to help the reader identify the peak in the pyrogram. The retention time for a specific peak varied from pyrogram to pyrogram.

In addition to the pyrograms of mock samples reported in this chapter, more detailed versions of can be found in Appendix II. Additional pyrograms of paint samples can be found in Appendix III, and pyrograms and mass spectra of reference samples (the reference library) can be found in Appendix IV.

3.1 Lipid-based binding media

3.1.1 Linseed oil + chalk + TMAH

The pyrolytic profile of linseed oil mixed with chalk + TMAH (Fig. 3.1) contained few peaks. Seven peaks were selected: Four identified compounds and three unknowns.

The identified compounds were dimethylated azelaic acid (Az), dimethylated suberic acid (Su), methylated palmitic acid (P) and methylated stearic acid (S). The latter two were identified by comparisons with reference samples, the former two through NIST and partially through reference samples. Reference samples of dimethylated diacids were analysed as undiluted liquids, which overloaded the system, and mass spectra of these references were thus only partially representative for the compounds[10]. However, their identities were strongly suspected, since diacids (especially azelaic acid) are reported extensively as markers of drying oils.

Three unknown peaks (lδ1, lδ2 and lδ3) had no good matches in NIST or the literature. Compounds lδ2 and lδ3 showed similar fragment patterns (overlapping peaks: *m/z* 67, 91, 109, 119, 137, 169, 201) with consecutive 32 *m/z*-losses in the last part of the spectra and showed additional fragmental similarities. The fragmentation pattern of lδ1 (*m/z* 67, 79, 95, 123, 155, 187) also showed consecutive 32 *m/z*-losses in addition to having the 67 *m/z*-peak in common with lδ2 and lδ3. It is therefore possible that these compounds are structurally related.

All peaks except for dimethylated azelaic acid and methylated palmitic acid were below column bleeding.[11]

[10] In the mass spectra of the diacids the molecular ions were very intense (see Appendix IV). It is likely that the formation of dimers and other secondary reactions have occurred. Their mass spectra are thus not representative for low concentration samples but were used as a guide.

[11] Experiments lengthening the pyrolysis time (t2) from 2 sec to 5 or 10 sec resulted in pyrograms with more peaks above column bleeding. These are not reported here, but the information could be of interest for future investigations.

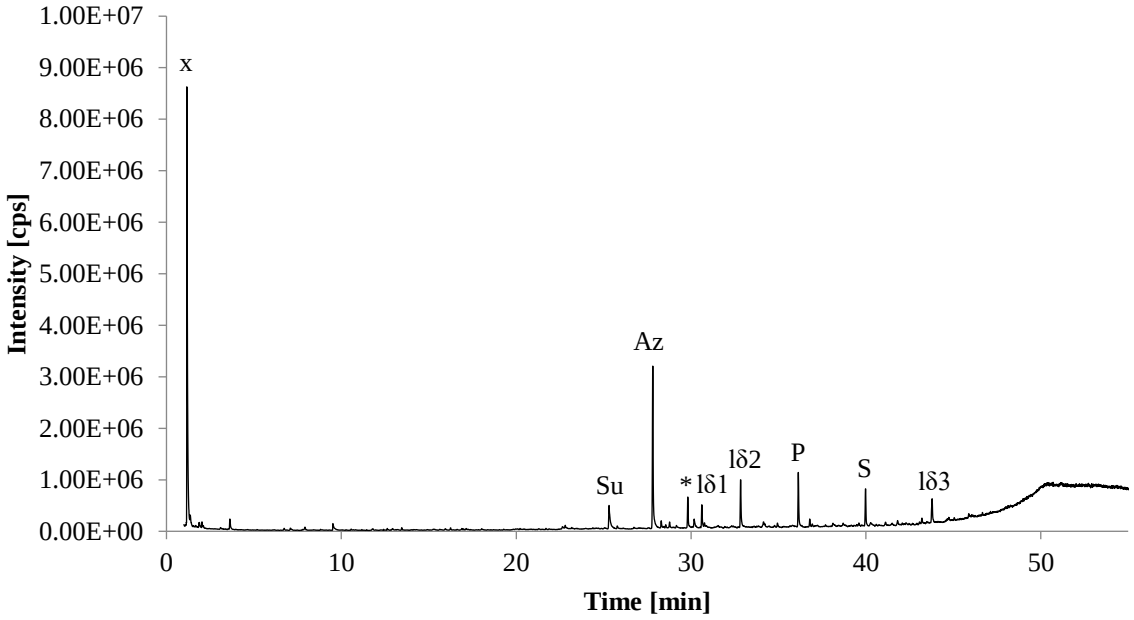
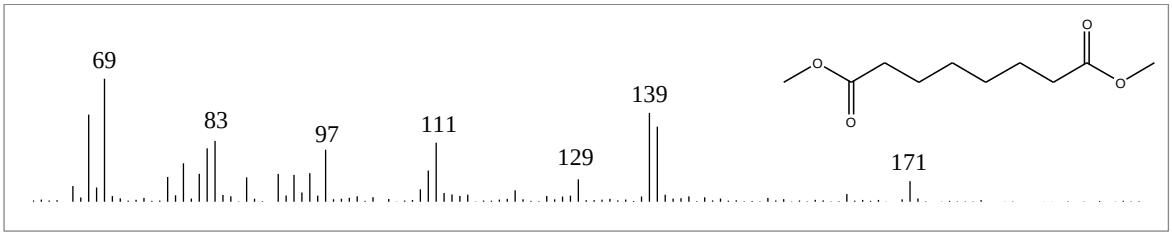


Figure 3.1: Pyrogram of dried linseed oil (mixed with chalk), pyrolysed in presence of TMAH. Analysed using Scanning range A.

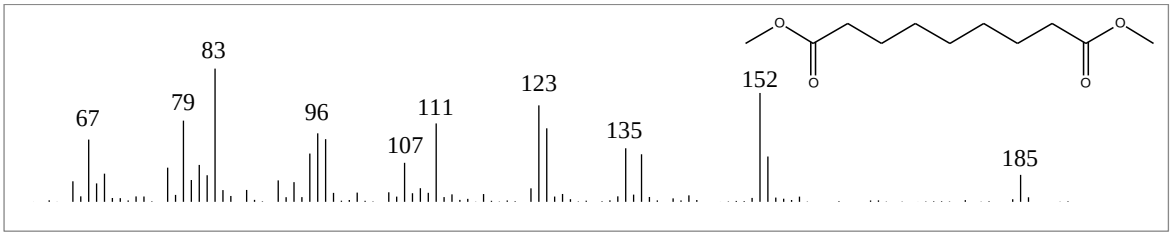
Table 3.2: Selected markers of Linseed oil + Chalk + TMAH.

Peak notation	Marker compound
Su	Suberic acid, dimethyl ester? (Me-diC8)
Az	Azelaic acid, dimethyl ester? (Me-diC9)
lδ1	Unknown (possible lδ2 homolog)
lδ2	Unknown (possible lδ1 homolog, similar fragment pattern to lδ3)
P	Palmitic acid, methyl ester (Me-C16:0)
S	Stearic acid, methyl ester (Me-C18:0)
lδ3	Unknown (similar fragment pattern to lδ2)
x	Tetramethyl ammonium hydroxide (TMAH)
*****	Contamination/system peak (source accounted for)

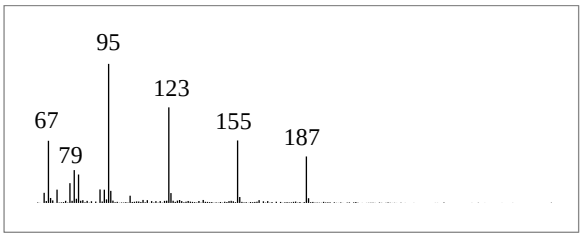
Su: Suberic acid, dimethyl ester? | t_R 25.31



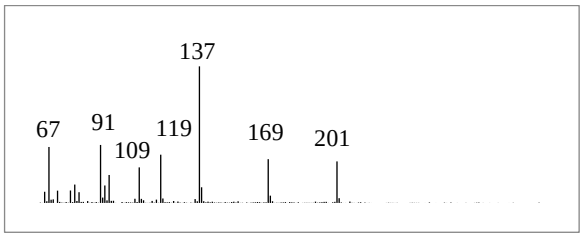
Az: Azelaic acid, dimethyl ester? | t_R 27.81



lδ1: Unknown | t_R 30.62



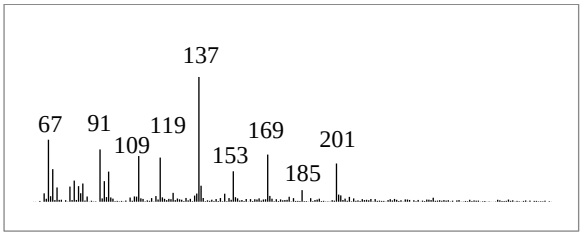
lδ2: Unknown | t_R 32.83



P: Palmitic acid, methyl ester | t_R 36.13

S: Stearic acid, methyl ester | t_R 39.98

lδ3: Unknown | t_R 43.79



3.1.2 Linseed oil + chalk (ø)

The pyrolytic profile of non-derivatised linseed oil + chalk (Fig. 3.2) was relatively simple. Three peaks were selected, all identified.

The identified peaks were azelaic acid (Az), palmitic acid (P) and stearic acid (S). These were identified through partial matches in NIST.

Palmitic acid was the main peak.

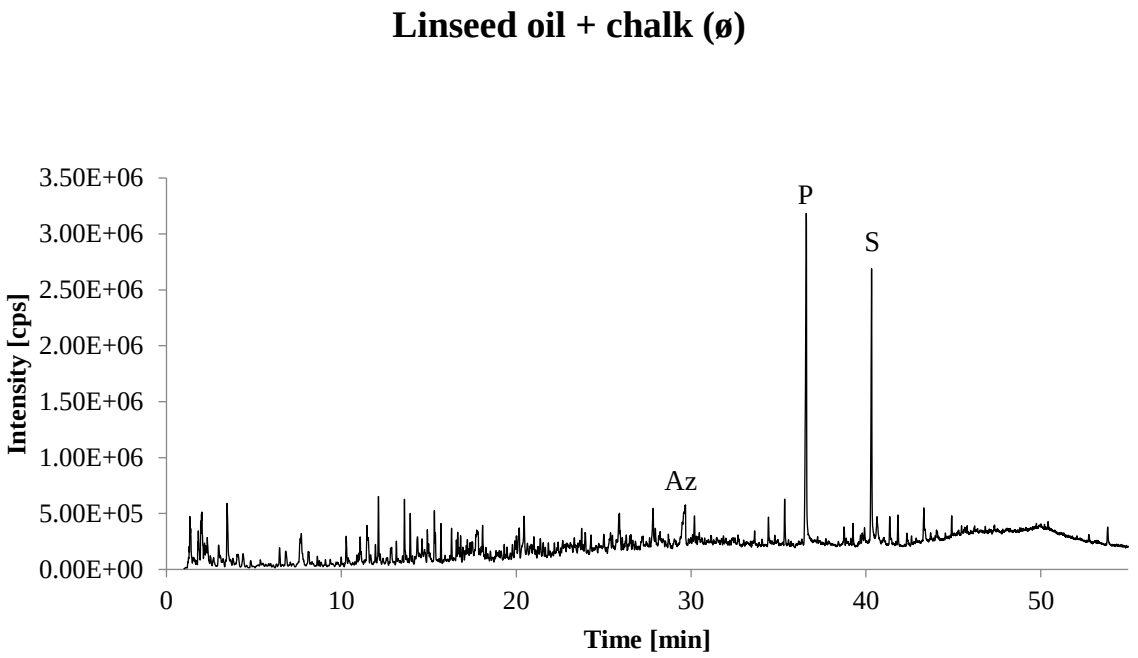
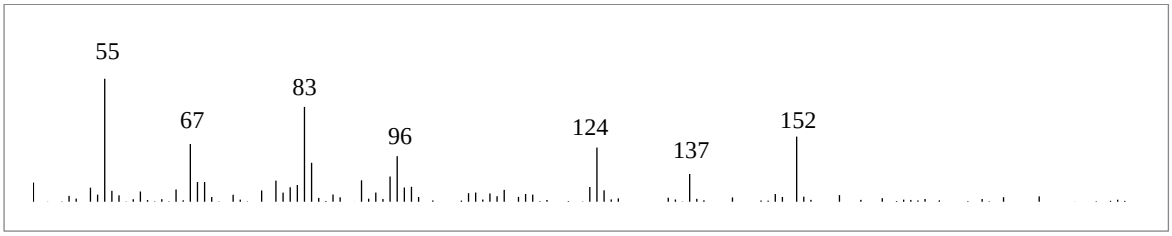


Figure 3.2: Pyrogram of dried linseed oil (mixed with chalk), without derivatisation. Analysed using Scanning range B.

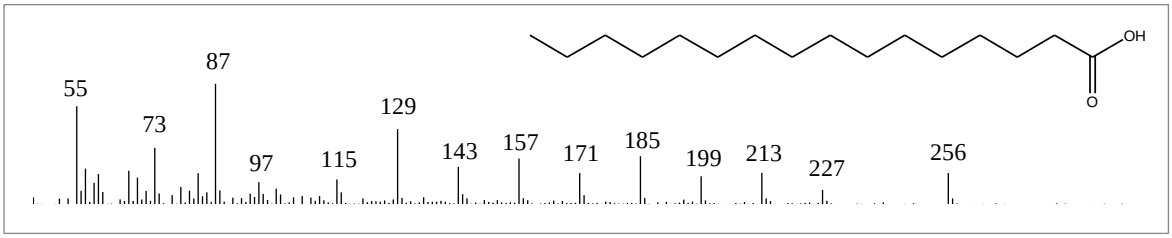
Table 3.3: Selected markers of Linseed oil + Chalk (no derivatisation).

Peak notation	Compound
Az	Azelaic acid? (C9)
P	Palmitic acid? (C16:0)
S	Stearic acid? (C18:0)

Az: Azelaic acid? | t_R



P: Palmitic acid? | t_R 36.59



S: Stearic acid? | t_R 40.32

3.1.3 Beeswax + TMAH

The pyrolytic profile of beeswax + TMAH (Fig. 3.3) was detailed, but relatively simple. Nine peaks were selected: Two identified compounds and seven unknowns.

The identified compounds were methylated palmitic acid (P) and methylated stearic acid (S).

The unidentified compounds were various longer-chained compounds. Some displayed MS fragment patterns similar to those of the methyl esters of shorter-chained saturated fatty acids analysed as reference samples (Appendix IV) and were thus denoted fa[12]. Others are marked as unidentified long-chained hydrocarbons (hc), based partially on hits in the NIST library and partially on the literature stating the presence of long-chained hydrocarbons. The two most intense peaks, bδ1 and bδ2, could also be other types of long-chained hydrocarbons, or methylated long-chained alcohols based on literature stating presence of these in wax. The identities of these compounds have been thoroughly investigated by others and was prioritised in this thesis.

[12] As explained in detail in the initial section of this chapter under 1. Pyrograms

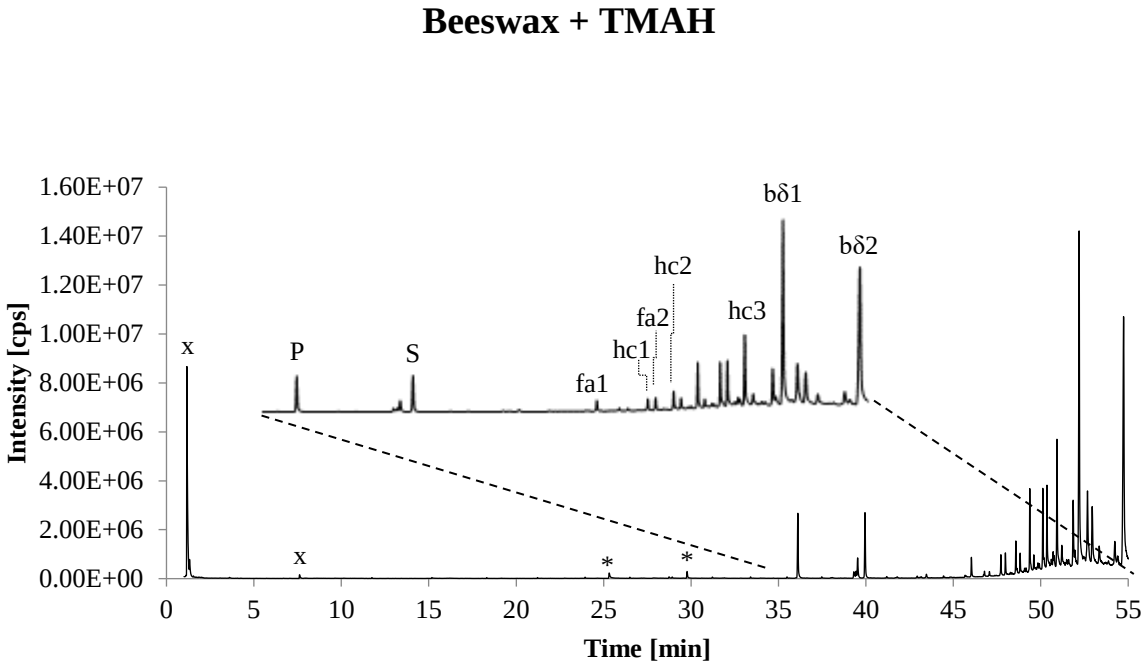
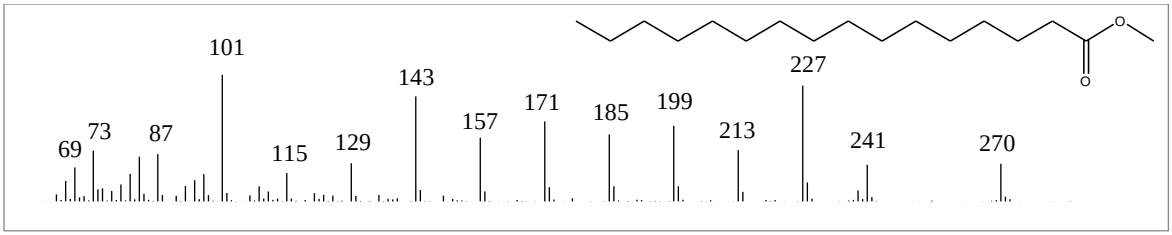


Figure 3.3: Pyrogram of beeswax pyrolysed with TMAH. Analysed using Scanning range A. The selected cross section spans from tR = 35 minutes to tR = 55 minutes.

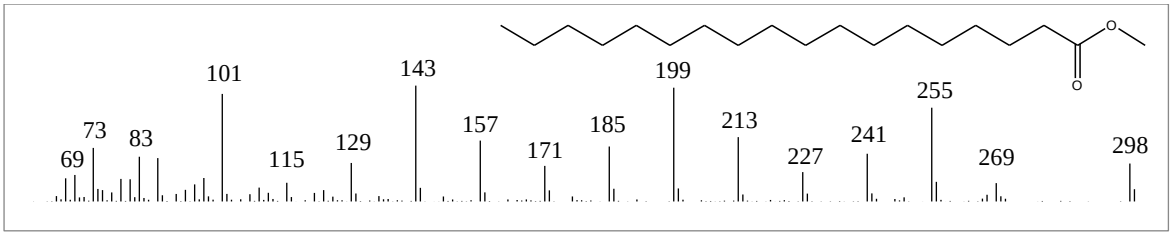
Table 3.4: Selected markers of beeswax+TMAH.

Peak notation	Compound
P	Palmitic acid, methyl ester (C16:0)
S	Stearic acid, methyl ester (18:0)
fa1	Unknown long-chained fatty acid, methyl ester
hc1	Unknown long-chained hydrocarbon
fa2	Unknown long-chained fatty acid, methyl ester
hc2	Unknown long-chained hydrocarbon
hc3	Unknown long-chained hydrocarbon
bδ1	Unknown
bδ2	Unknown
x	Tetramethyl ammonium hydroxide (TMAH)
*****	Contamination/system peak (source accounted for)

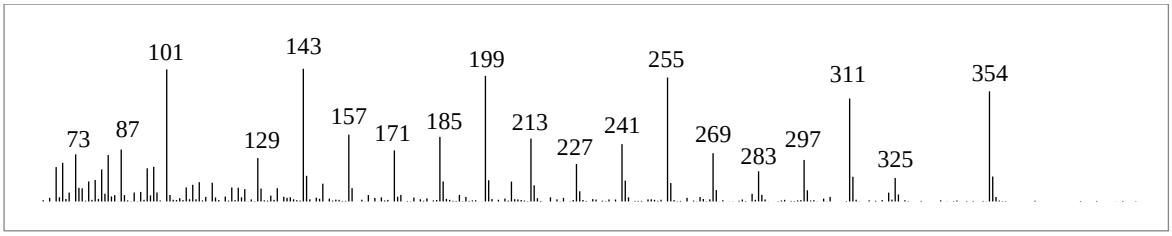
P: Palmitic acid, methyl ester | t_R 36.11



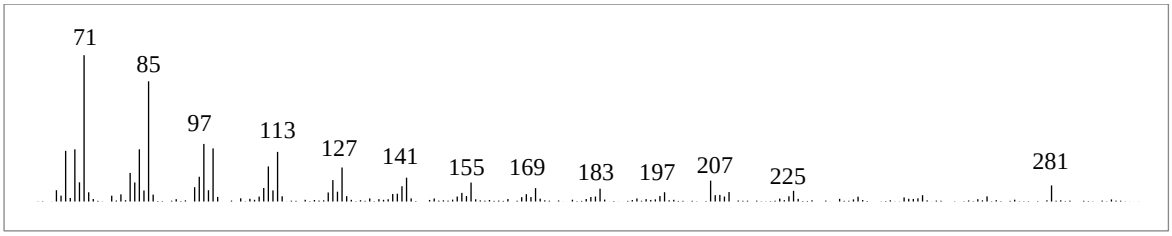
S: Stearic acid, methyl ester | t_R 39.95



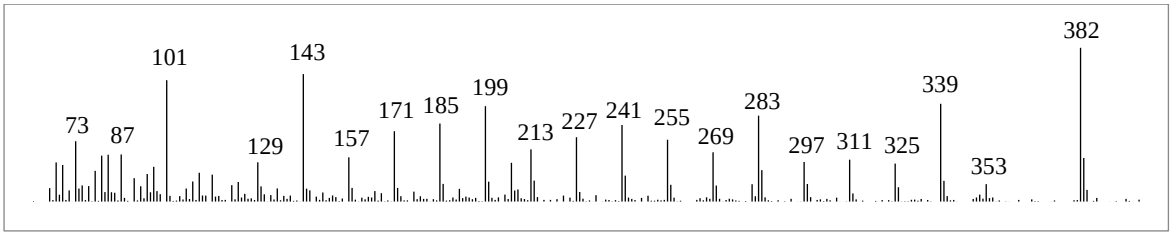
fa1: Unknown long-chained fatty acid, methyl ester | t_R 46.03



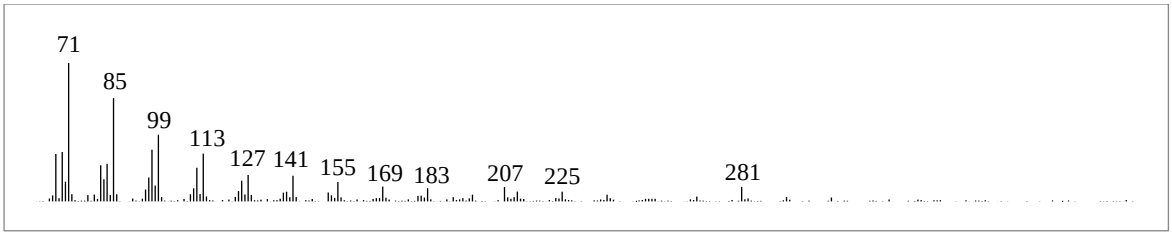
hc1: Unknown long-chained hydrocarbon | t_R 47.71



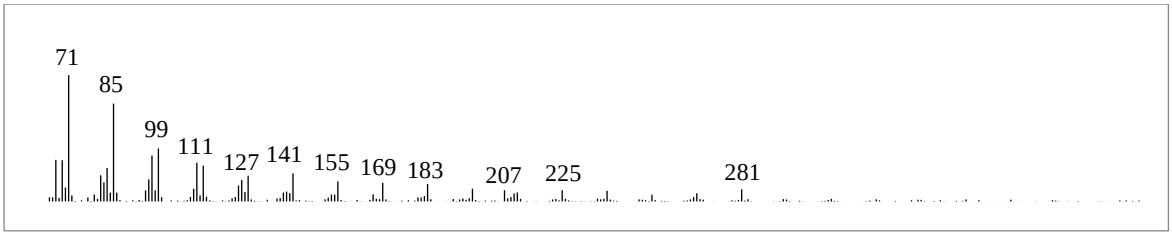
fa2: Unknown long-chained fatty acid, methyl ester | t_R 47.98



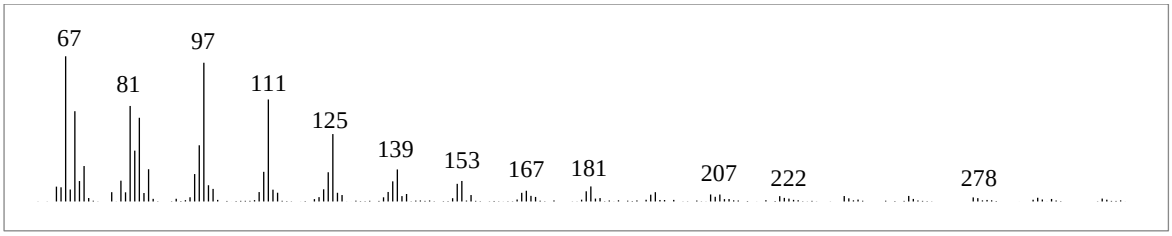
hc2: Unknown long-chained hydrocarbon | t_R 48.57



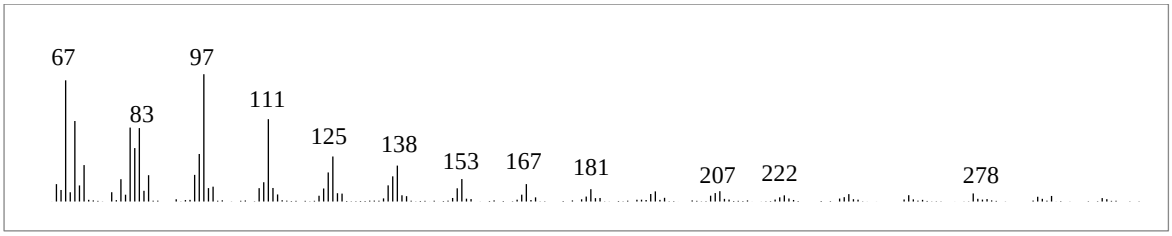
hc3: Unknown long-chained hydrocarbon | t_R 50.92



bδ1: Unknown | t_R 52.19



bδ2: Unknown | t_R 54.73



3.2 Proteinaceous binding media

3.2.1 Egg yolk + TMAH

The pyrolytic profile of egg yolk + TMAH (Fig. 3.4) was simple. Six peaks were selected: Five identified compounds and one unidentified.

The identified compounds were trimethylated phosphoric acid (PA), methylated palmitic acid, (P), methylated oleic acid (O), methylated stearic acid (S) and methylated cholesterol (C). All except phosphoric acid were identified by comparison with references. Trimethylated phosphoric acid was identified through NIST, supported by literature.

The unknown compound yδ1 was selected as possible marker, since it was clearly separated from other peaks in the pyrogram, and had a higher intensity compared to other unidentified peaks.

The pyrolytic profile was dominated by methyl esters of fatty acids and by methylated cholesterol. Methylated oleic acid was the main peak.

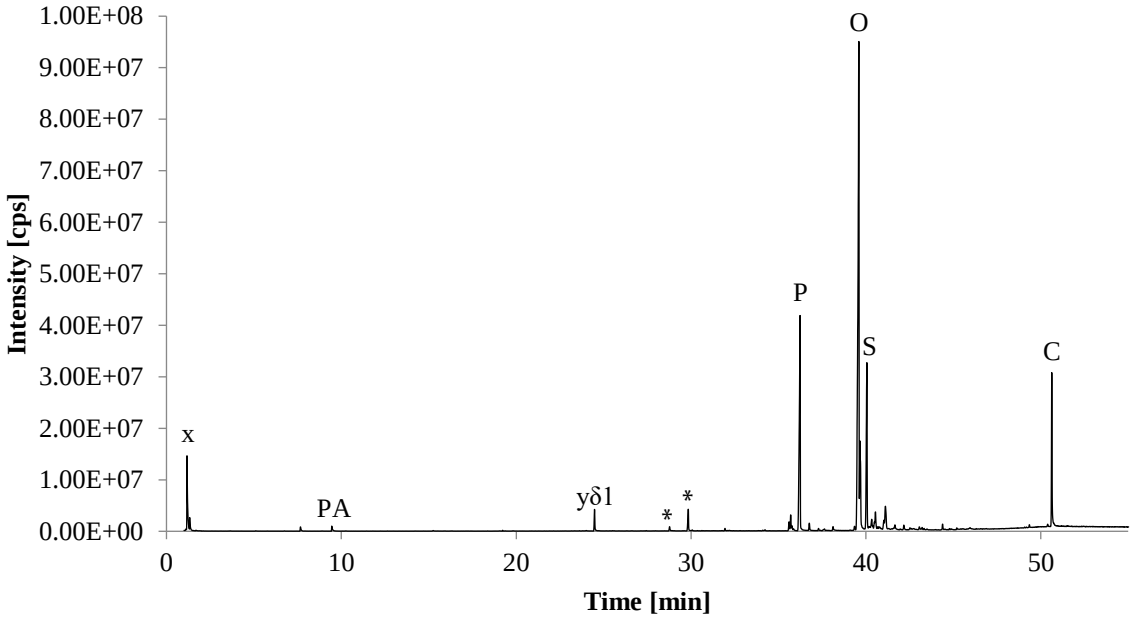
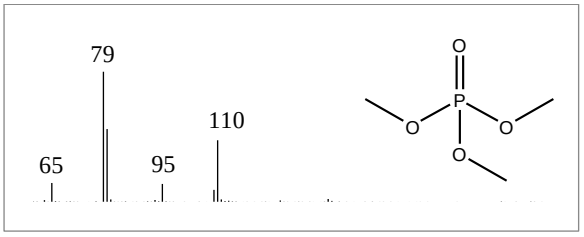


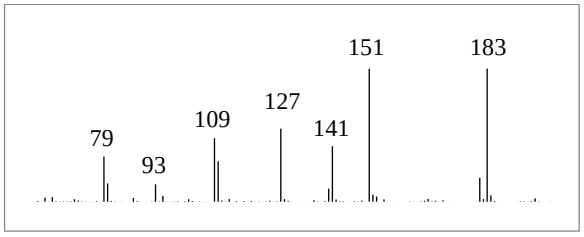
Figure 3.4: Pyrogram of dried egg yolk, pyrolysed with TMAH. Analysed using Scanning range A.

Table 3.5: Selected markers of egg yolk+TMAH.

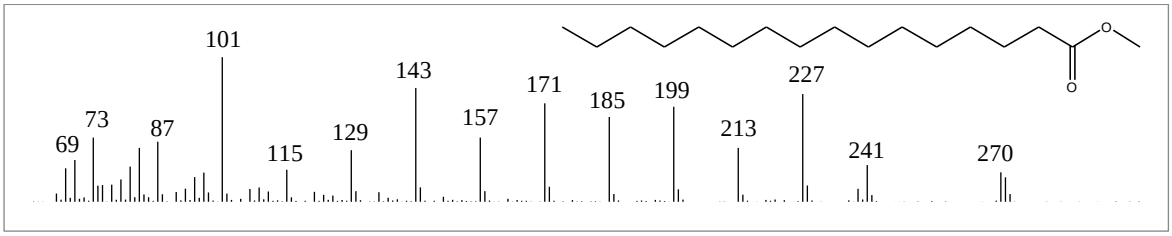
Peak notation	Compound
PA	Phosphoric acid, trimethyl ester?
yδ1	Unknown
P	Palmitic acid, methyl ester (Me-C16:0)
O	Oleic acid, methyl ester (Me-C18:1)
S	Stearic acid, methyl ester (Me-C18:0)
C	Cholesterol, methylated (Me-Chol)
x	Tetramethyl ammonium hydroxide (TMAH)
*****	Contamination/system peak (source accounted for)

PA: Phosphoric acid, trimethyl ester? | t_R 9.45 **yδ1**: Unknown | t_R 24.48

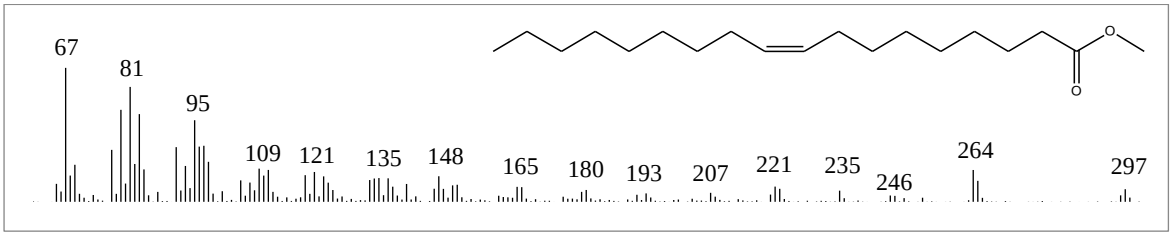




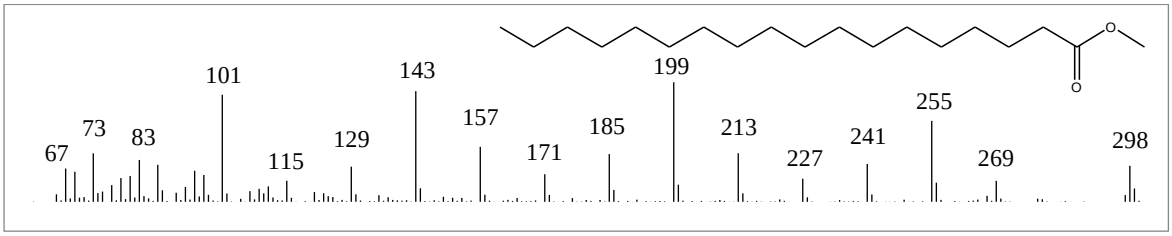
P: Palmitic acid, methyl ester | t_R 36.23



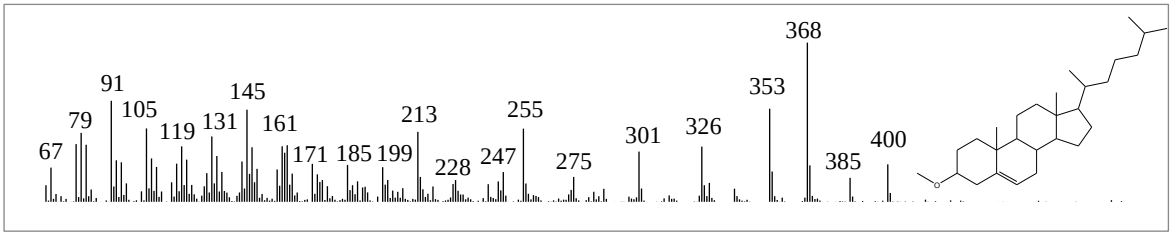
O: Oleic acid, methyl ester | t_R 39.06



S: Stearic acid, methyl ester | t_R 40.06



C: Cholesterol, methylated | t_R 50.63



3.2.2 Egg yolk (ø)

The pyrolytic profile of non-derivatised egg yolk (Fig. 3.5) was simple. Seven peaks were selected: Four identified compounds and three unidentified.

The identified compounds were palmitic acid (P), oleic acid (O), stearic acid (S) and cholesterol (C). These were identified through partial matches in NIST.

The unknown compounds, yø1, yø2 and yø3, had mass spectra resembling cholesterol, and NIST suggested several cholesterol derivatives as possible matches.

Cholesterol was the main peak.

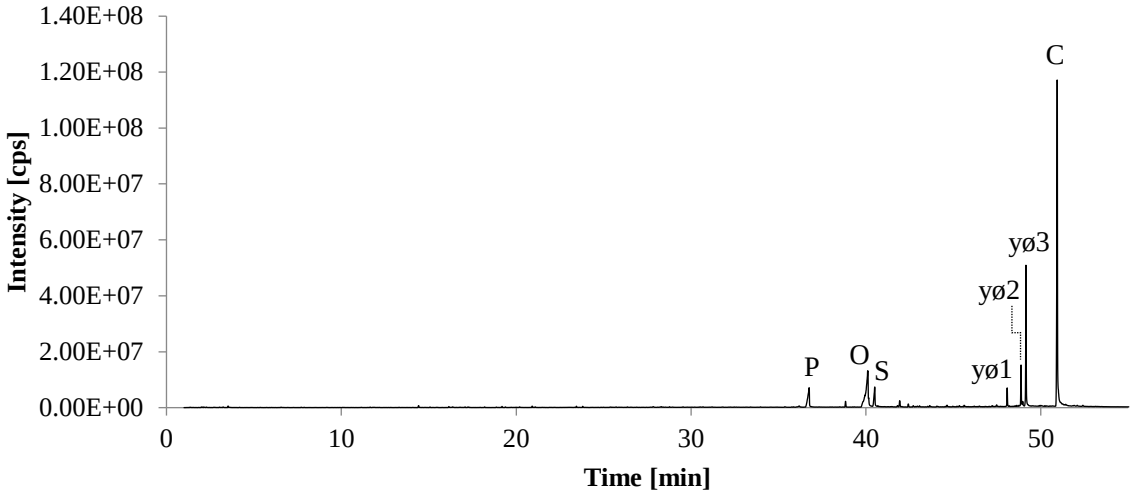


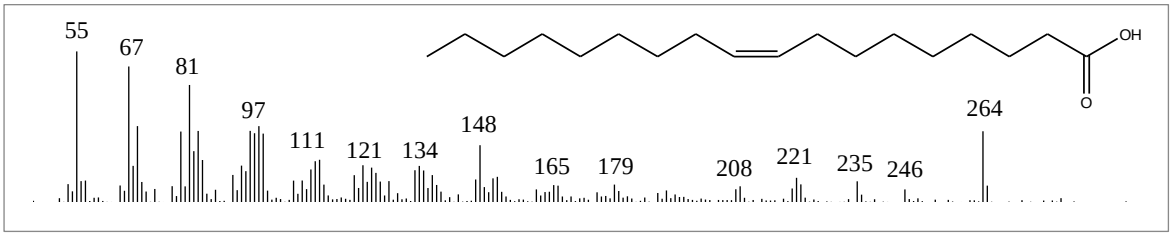
Figure 3.5: Pyrogram of dried egg yolk, without derivatisation. Analysed using Scanning range B.

Table 3.6: Selected markers of egg yolk (no derivatisation).

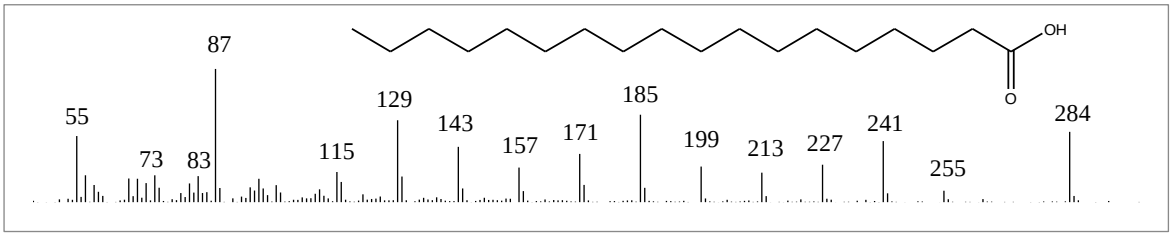
Peak notation	Compound
P	Palmitic acid (C16:0)
O	Oleic acid (C18:1)
S	Stearic acid (C18:0)
yø1	Unknown, cholesterol derivative?
yø2	Unknown, cholesterol derivative?
yø3	Unknown, cholesterol derivative?
C	Cholesterol

P: Palmitic acid? | t_R 36.76

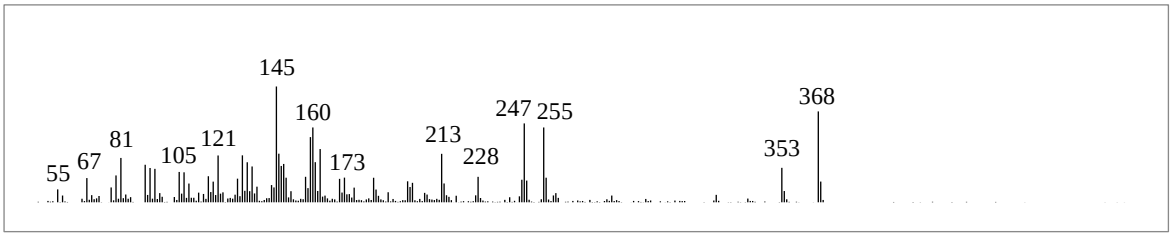
O: Oleic acid? | t_R 40.10



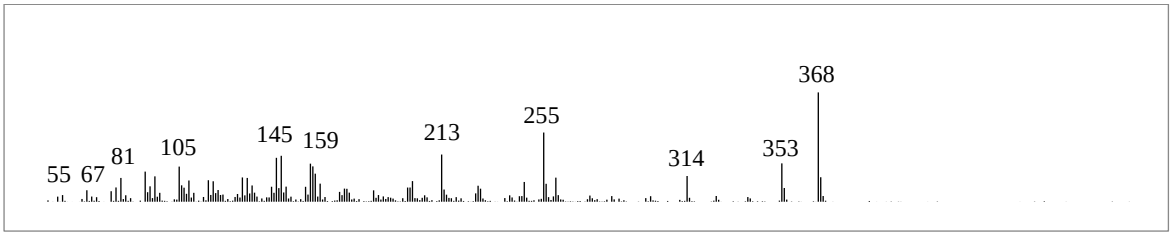
S: Stearic acid? | t_R 40.51



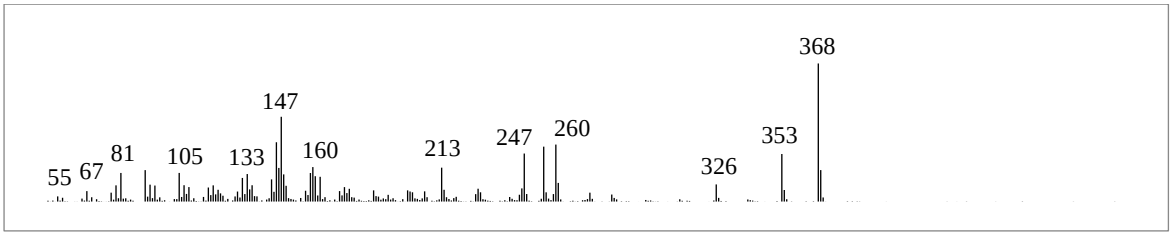
yø1: Cholesterol derivative? | t_R 48.08



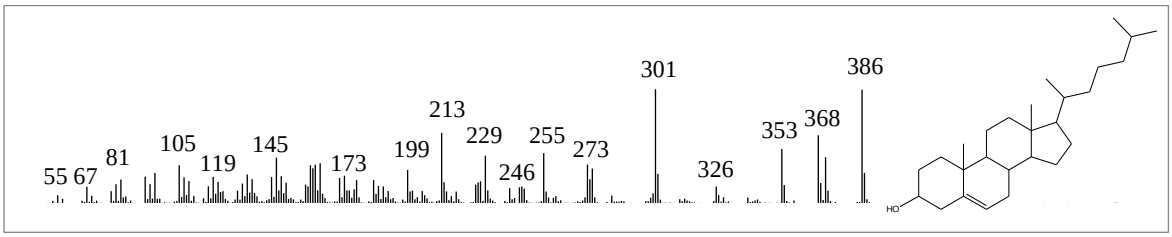
yø2: Cholesterol derivative? | t_R 48.87



yø3: Cholesterol derivative? | t_R 49.15



C: Cholesterol | t_R 50.63



3.2.3 Egg white + TMAH

The pyrolytic profile of egg white + TMAH (Fig. 3.6) was complex, with numerous peaks and few of them prominent. Nine peaks were selected: Four identified and five unknowns.

The identified compounds were all small aromatic compounds: Toluene (T), ethyl benzene (E), styrene (S) and indole (I). Toluene and indole were identified by reference matches, while ethyl benzene and styrene were identified by NIST-matches.

Two of the unidentified compounds, wδ3 and wδ4, had several possible hits in NIST of which both di- and monomethylated indole respectively seemed plausible. As there were other possible matches, this identification was too uncertain to be confirmed. The same is true for the unidentified compound wδ1, which resembles indole so much, that it was initially mistaken for indole. A suggestion in NIST, pyrindine, seemed likely based on the similarities in stereochemistry, but in literature, similar MS patterns have been reported for methylcyanobenzene and ethylcyanobenzene. Reference samples of suspected compounds would be needed to confirm or discredit these suspicions.

The pyrolytic profile of methylated egg white was dominated by small aromatic compounds and possibly derivatives thereof. Toluene was the main peak.

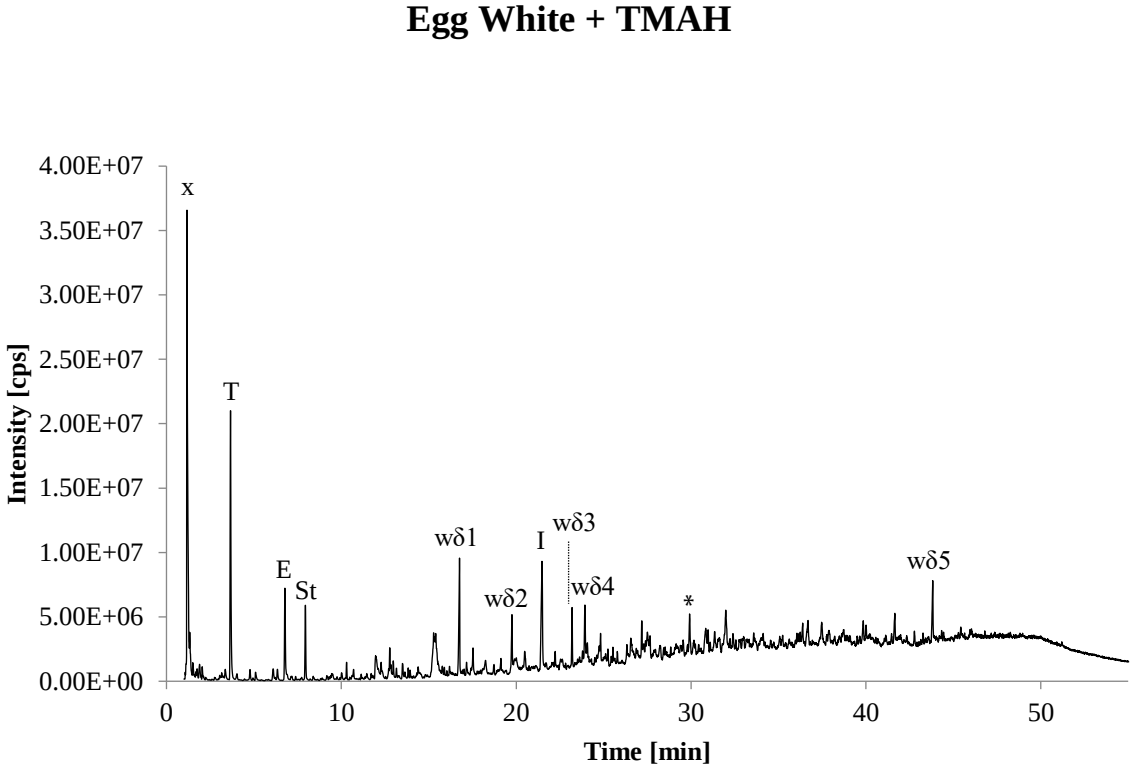


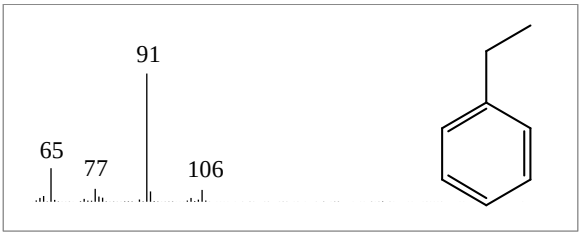
Figure 3.6: Pyrogram of dried egg white, pyrolysed with TMAH. Analysed using Scanning range A.

Table 3.7: Selected markers of egg white + TMAH.

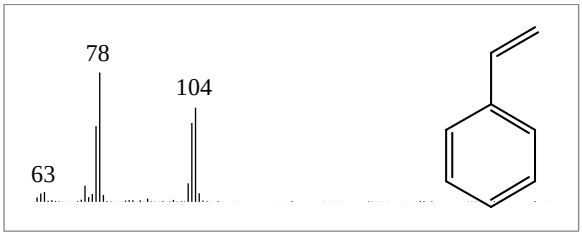
Peak notation	Compound
T	Toluene
E	Ethyl benzene?
St	Styrene?
wδ1	Unknown, structural isomer of indole? (Pyrindine? (m)ethylcyanobenzene?)
wδ2	Unknown, Benzene compound?
I	Indole
wδ3	Unknown, Indole homolog? (Dimethylated?)
wδ4	Unknown, Indole homolog? (Monomethylated?)
wδ5	Unknown
x	Tetramethyl ammonium hydroxide (TMAH)
*****	Contamination/system peak (source accounted for)

T: Toluene | t_R 3.67

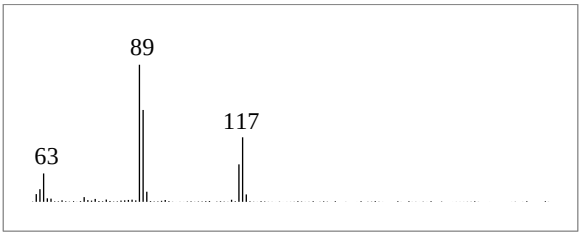
E: Ethyl benzene? | t_R 6.78



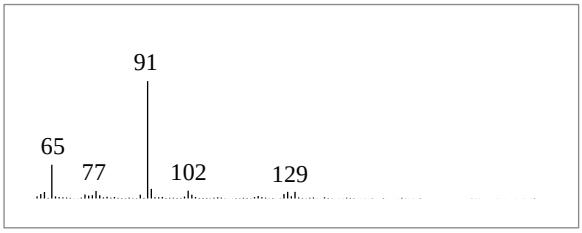
St: Styrene? | t_R 7.95



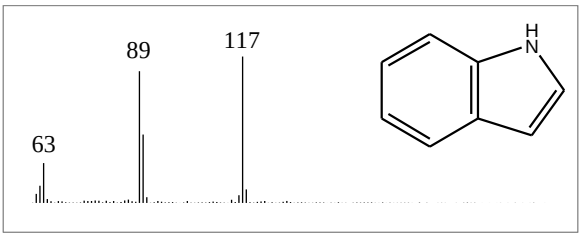
wδ1: Structural isomer of indole ? | t_R 16.76



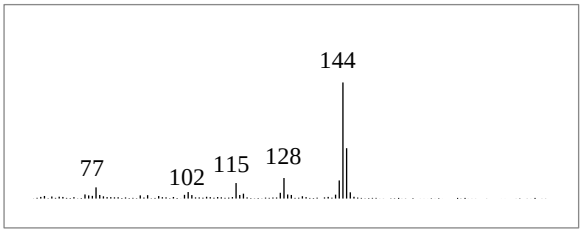
wδ2: Unknown | t_R 19.76



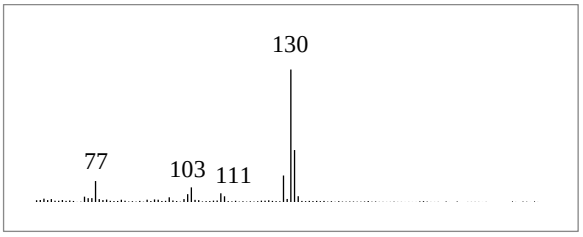
I: Indole | t_R 21.47



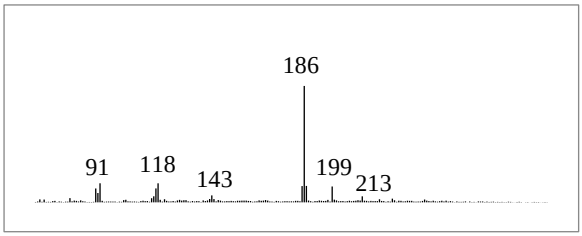
wδ3: Dimethylated indole? | t_R 23.19



wδ4: Monomethylated indole? | t_R 23.93



wδ5: Unknown | t_R 43.82



3.2.4 Animal glue + TMAH

The pyrolytic profile of animal glue + TMAH (Fig. 3.7) was complex due to the large number of high-intensity peaks. Fourteen peaks were selected: Seven identified and seven unidentified.

The identified compounds were methylated pyrrole (Py), isobarbituric acid (IA), methylated benzoic acid (B), methylated palmitic acid (P), methylated oleic acid (O), methylated stearic acid (S) and methylated cholesterol (C). Methylated pyrrole, isobarbituric acid and methylated benzoic acid were identified through NIST. The remaining were identified through reference sample matching.

The unidentified compounds were selected based primarily on their peak intensity. Some of the selected unknowns (aδ2, aδ3, aδ4, aδ5, aδ6 and aδ7) had mass spectra resembling those of 2,5-diketopiperazines (see mass spectra for non-derivatised animal glue Fig. 3.8) containing mass peaks characteristic for these compounds (e.g. *m/z* 55 together with *m/z* 68 or 70 and *m/z* 154, 168 or 194). These have not been investigated further.

Multiple peaks had higher intensity than the rest (aδ1, aδ5, P and O).

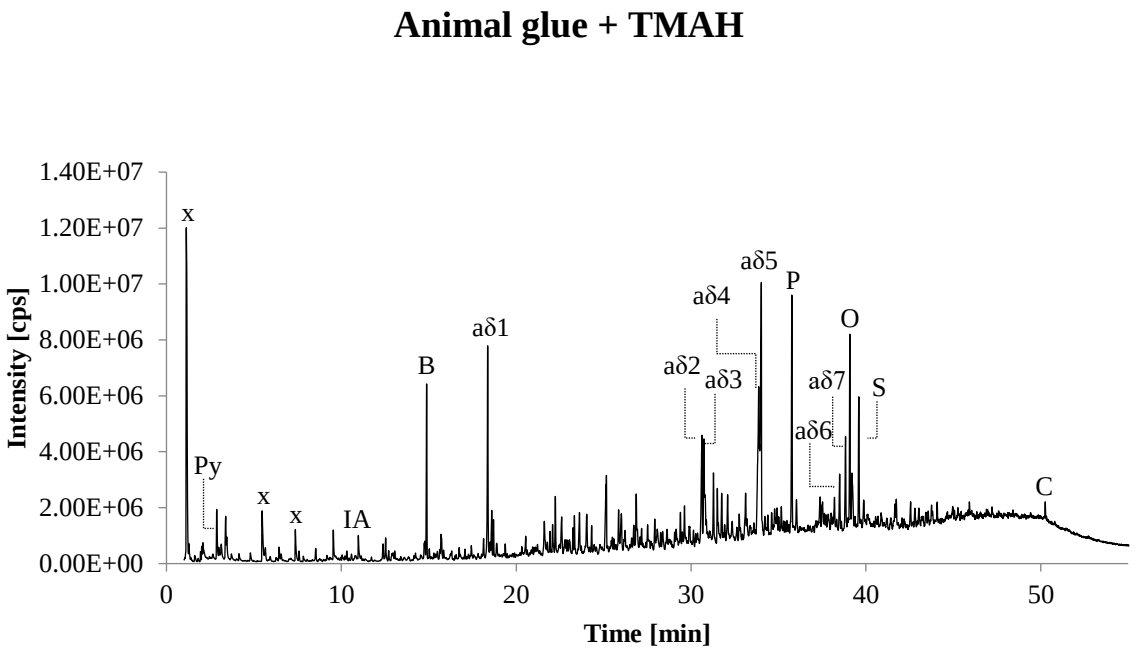
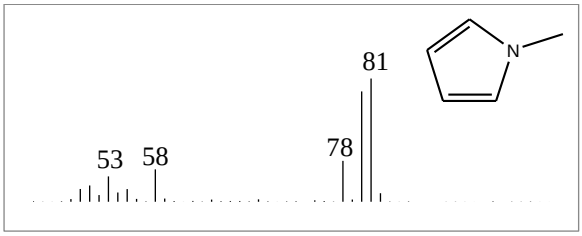


Figure 3.7: Pyrogram of rabbit glue (powder), pyrolysed with TMAH. Analysed using Scanning range B.

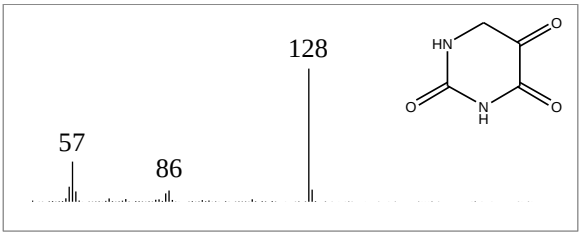
Table 3.8: Selected narkers of animal glue+TMAH.

Peak notation	Compound
Py	Pyrrole, methylated
IA	Isobarbituric acid?
B	Benzoic acid, methyl ester?
aδ1	Unknown
aδ2	Unknown, similar fragment pattern to aδ4 and aδ5
aδ3	Unknown
aδ4	Unknown, similar fragment pattern to aδ2 and aδ5
aδ5	Unknown, similar fragment pattern to aδ2 and aδ4
P	Palmitic acid, methyl ester (Me-C16:0)
aδ6	Unknown, similar fragment pattern to aδ7
aδ7	Unknown, similar fragment pattern to aδ6
O	Oleic acid, methyl ester (Me-C18:1)
S	Stearic acid, methyl ester (Me-C18:0)
C	Cholesterol, methylated
x	Tetramethyl ammonium hydroxide (TMAH)

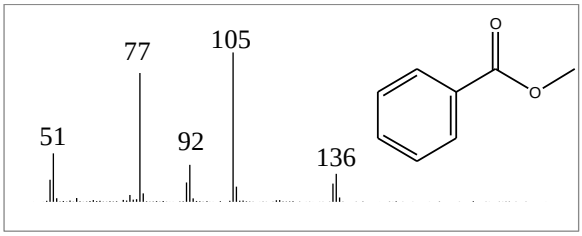
Py: 1-methyl-Pyrrole | t_R 2.88



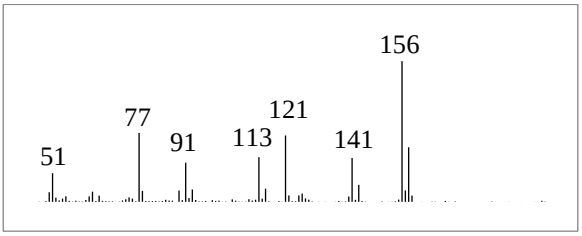
IA: Isobarbituric acid? | t_R 10.97



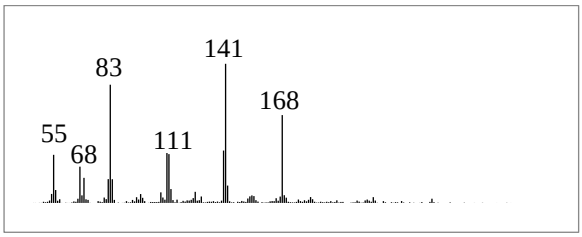
B: Benzoic acid, methyl ester? | t_R 14.87



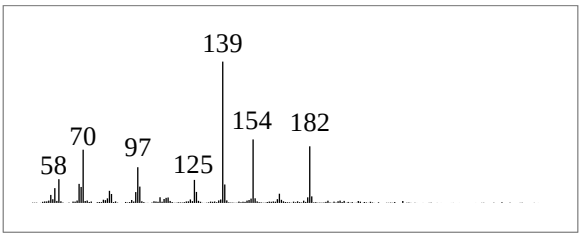
aδ1: Unknown | t_R 18.37



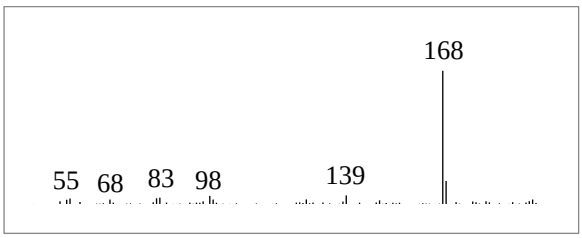
aδ2: Unknown (DKP?) | t_R 30.63



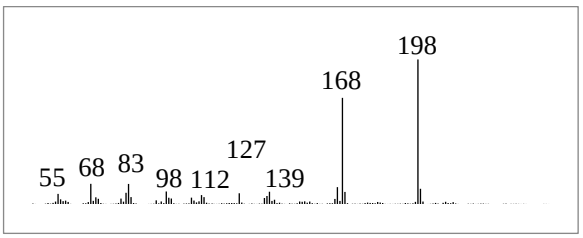
aδ3: Unknown (DKP?) | t_R 30.74



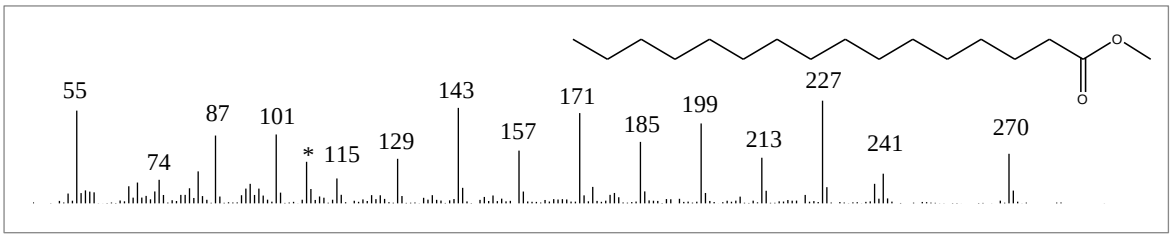
aδ4: Unknown (DKP?) | t_R 33.87



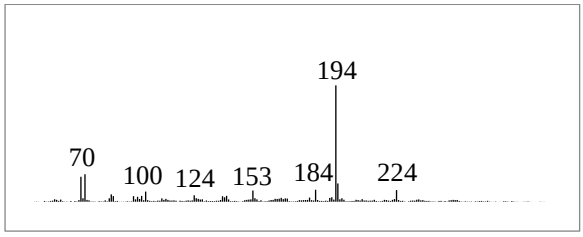
aδ5: Unknown (DKP?) | t_R 34.02



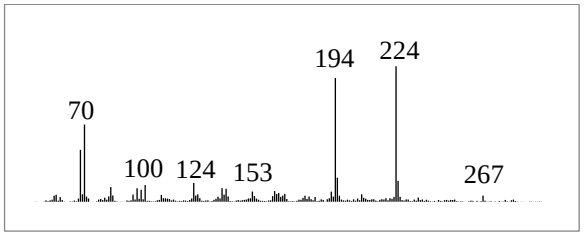
P: Palmitic acid, methyl ester | t_R 35.76



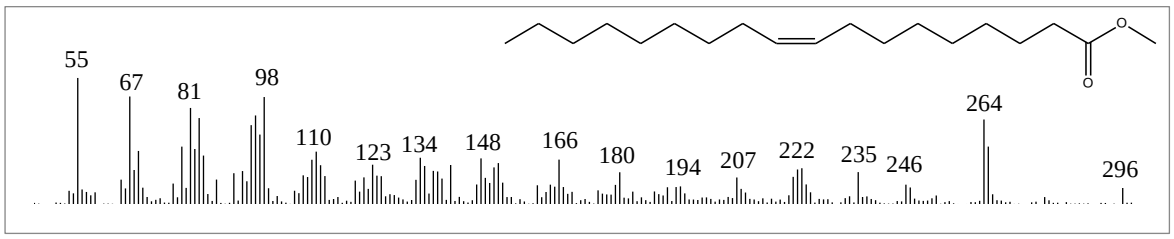
aδ6: Unknown (DKP?) | t_R 38.50



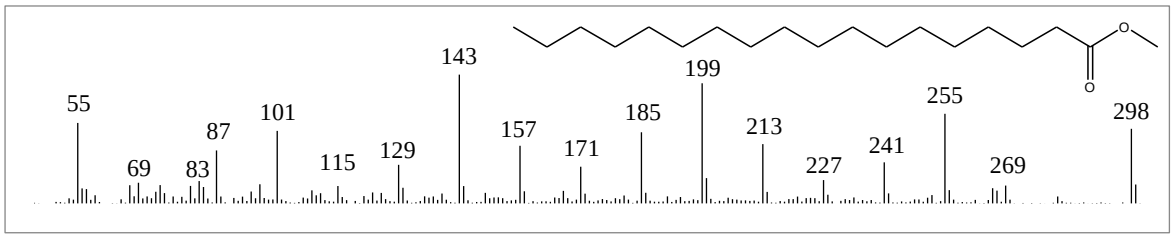
aδ7: Unknown (DKP?) | t_R 38.84



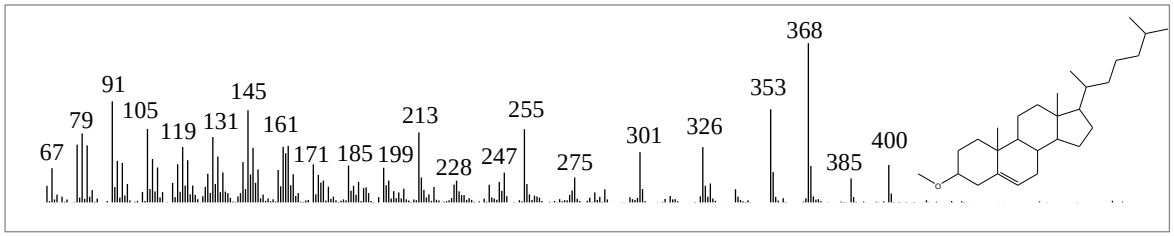
O: Oleic acid, methyl ester| t_R 39.08



S: Stearic acid, methyl ester | t_R 39.59



C: Cholesterol, methylated | t_R 50.63



3.2.5 Animal glue (ø)

The pyrolytic profile of non-derivatised animal glue (Fig. 3.8) was complex due to the large number of peaks with relatively high intensity. Seventeen peaks were selected: Ten identified compounds and seven unidentified.

The identified compounds were pyrrole (Py), toluene (T), diketodipyrrole (DK-Py), cyclo(proline-alanine)1 (Pro-Ala1), cyclo(proline-alanine)2 (Pro-Ala2), cyclo(proline-glycine) (Pro-Gly), cyclo(proline-proline) (Pro-Pro), palmitic acid (P), cyclo(proline-hydroxyproline)1 (Pro-Hyp1) and cyclo(proline-hydroxyproline)2 (Pro-Hyp2). Pyrrole and toluene were identified through reference sample matching. Palmitic acid was identified through NIST. Diketodipyrrole and the 2,5-diketopiperazines were identified through matches in literature.

The unidentified compounds were selected based primarily on their peak intensity.

Cyclo(proline-glycine) was the main peak.

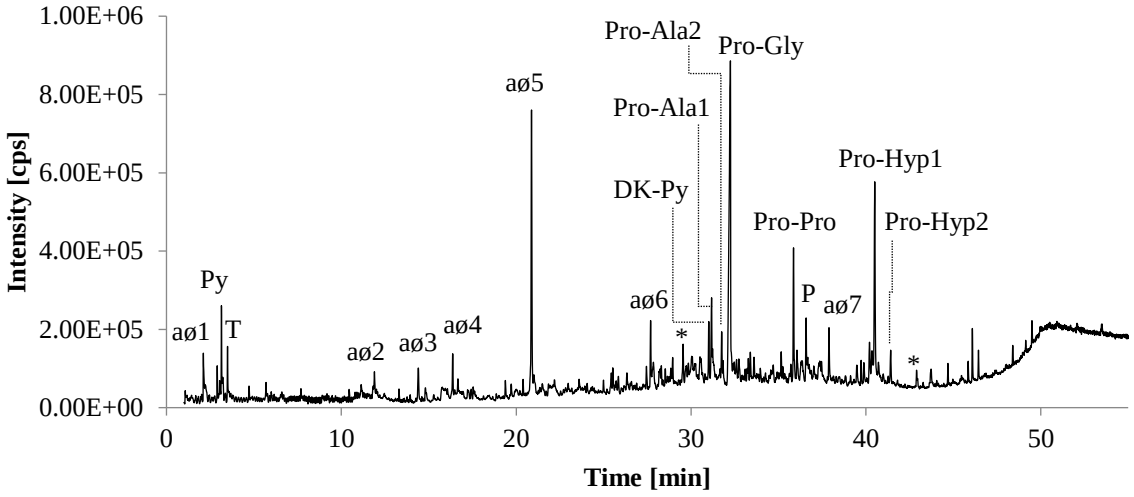
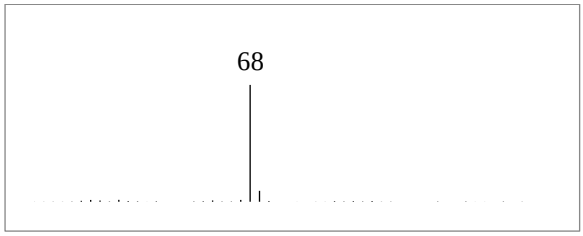


Figure 3.8: Pyrogram of rabbit glue (powder), without derivatisation. Analysed using Scanning range B.

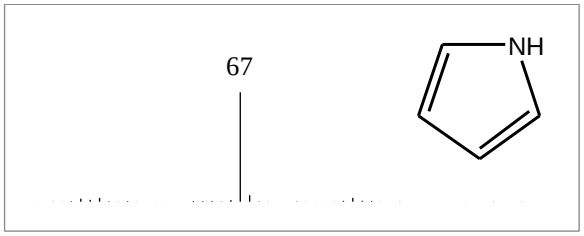
Table 3.9: Selected markers of animal glue (no derivatisation).

Peak notation	Compound
aø1	Unknown
Py	Pyrrole
T	Toluene
aø2	Unknown
aø3	Unknown
aø4	Unknown. Pyrindine? (m)ethylbenzene?
aø5	Unknown
aø6	Unknown
DK-Py	Diketodipyrrole
Pro-Ala1	Cyclo(Pro-Ala)1 (2,5-diketopiperazine, DKP)
Pro-Ala2	Cyclo(Pro-Ala)2 (2,5-diketopiperazine, DKP)
Pro-Gly	Cyclo(Pro-Gly) (2,5-diketopiperazine, DKP)
Pro-Pro	Cyclo(Pro-Pro) (2,5-diketopiperazine, DKP)
P	Palmitic acid?
aø7	Unknown
Pro-Hyp1	Cyclo(Pro-Hyp)1 (2,5-diketopiperazine, DKP)
Pro-Hyp2	Cyclo(Pro-Hyp)2? (2,5-diketopiperazine, DKP)
*****	Contamination/system peak (source accounted for)

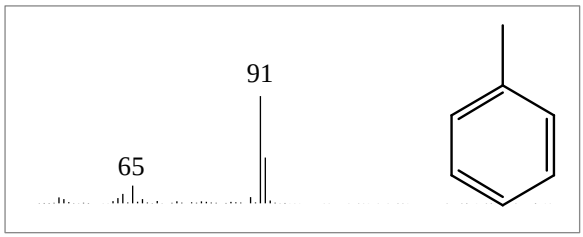
aø1: Unknown | t_R 2.11



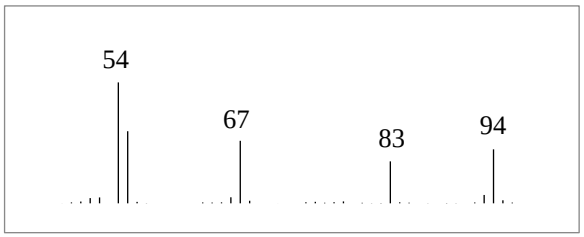
Py: Pyrrole | t_R 3.14



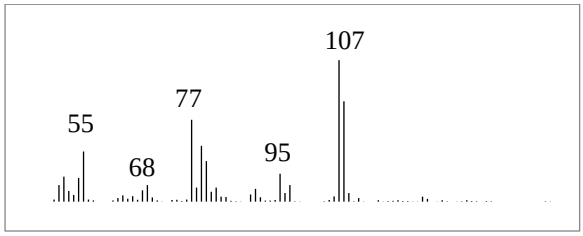
T: Toluene | t_R 3.49



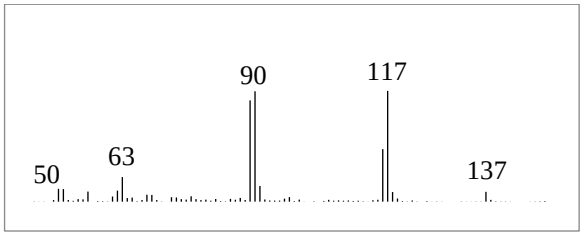
aø2: Unknown | t_R 11.89



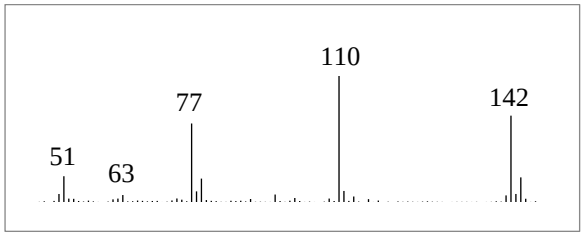
aø3: Unknown | t_R 14.40



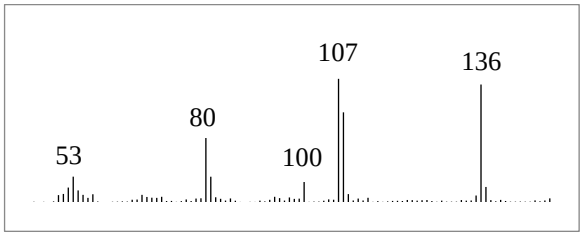
aø4: Unknown, indole structural isomer? | t_R 16.37



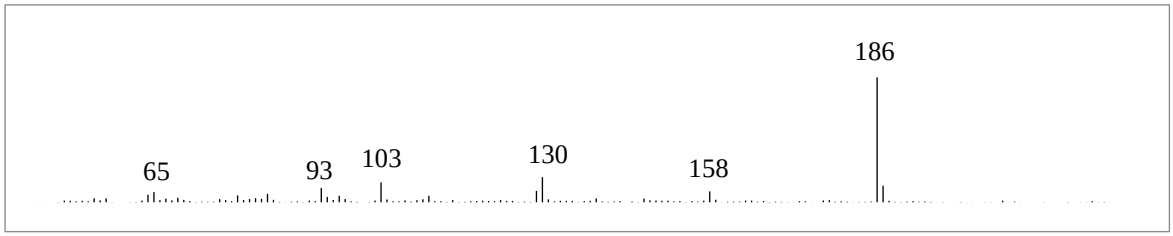
aø5: Unknown | t_R 20.88



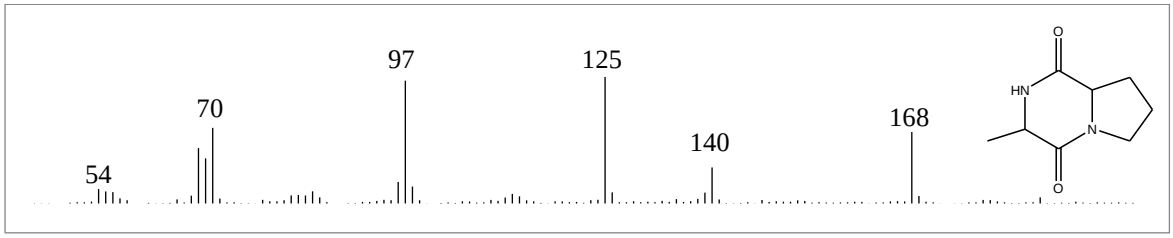
aø6: Unknown | t_R 27.69



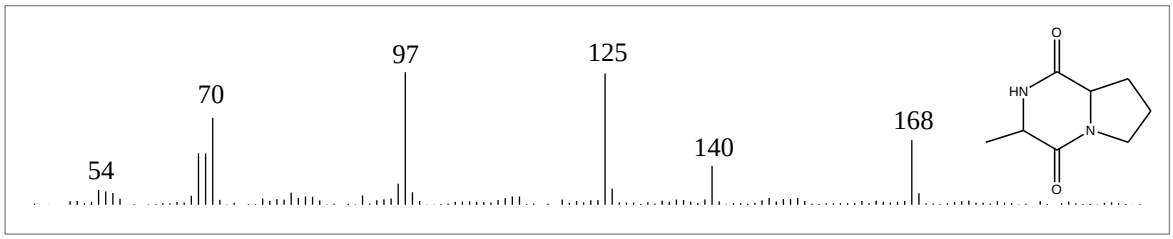
DK-Py: Diketodipyrrole | t_R 31.02



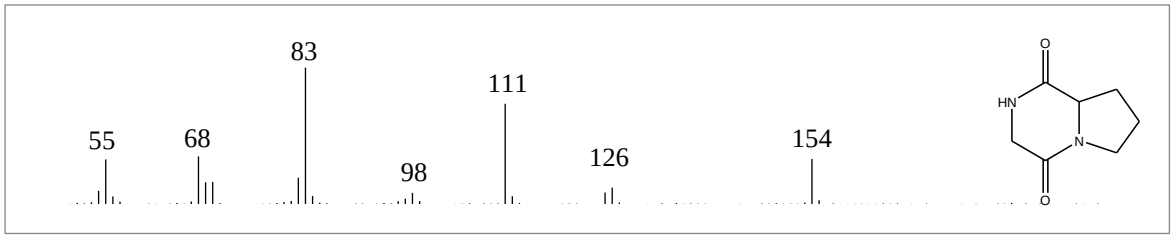
Pro-Ala1: Cyclo(proline-alanine), stereoisomer 1 (DKP) | t_R 31.18



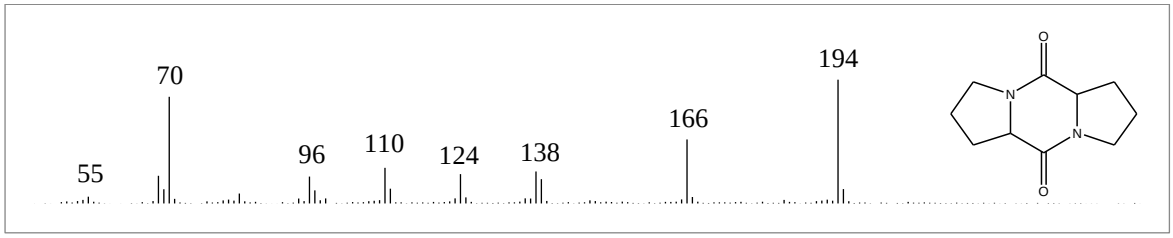
Pro-Ala2: Cyclo(proline-alanine), stereoisomer 2 (DKP) | t_R 31.75



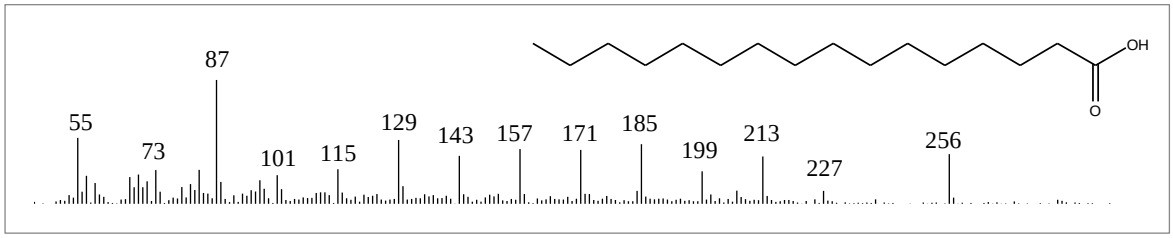
Pro-Gly: Cyclo(proline-glycine) (DKP) | t_R 32.25



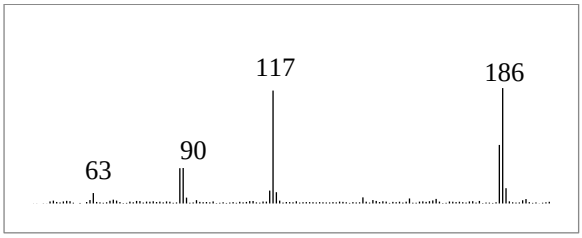
Pro-Pro: Cyclo(proline-proline) (DKP) | t_R 35.86



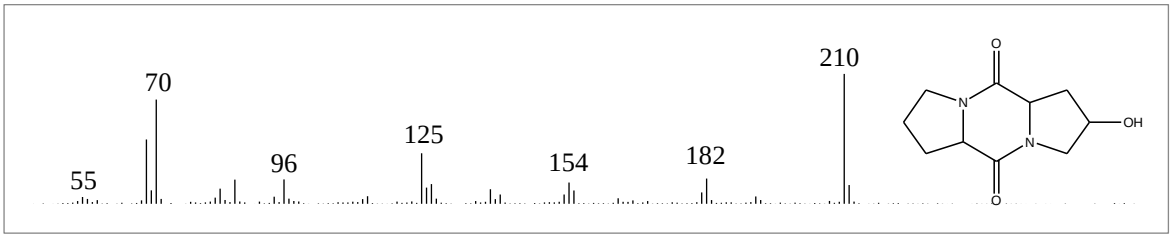
P: Palmitic acid? | t_R 36.58



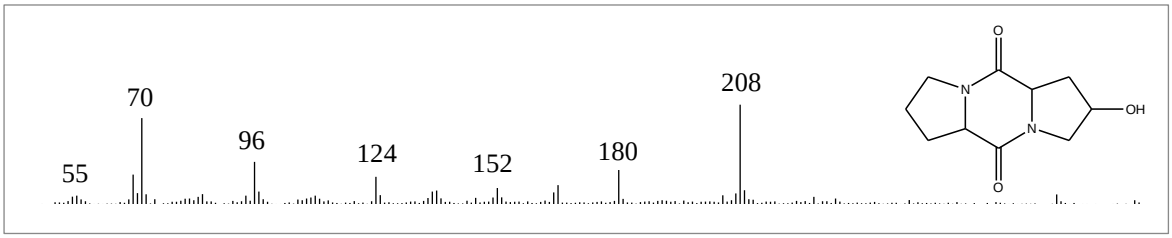
aø7: Unknown | t_R 37.89



Pro-Hyp1: Cyclo(proline-hydroxyproline), stereoisomer 1 (DKP) | t_R 40.51



Pro-Hyp2: Cyclo(proline-hydroxyproline), stereoisomer 2? (DKP) | t_R 41.42



3.2.6 Casein + TMAH

The pyrolytic profile of casein + TMAH (Fig. 3.9) was complex due to the large number of high-intensity peaks. Nine peaks were selected, and all of them were identified:

Toluene (T), styrene (St), trimethylated phosphoric acid (PA), methylated benzoic acid (B), indole (I), methylated palmitic acid (P), methylated oleic acid (O), methylated stearic acid (S) and methylated cholesterol (C). Styrene, trimethylated phosphoric acid and methylated benzoic acid were identified through NIST. The remaining were identified through reference sample matching.

Trimethylated phosphoric acid and methylated palmitic acid had higher intensity than the rest of the peaks.

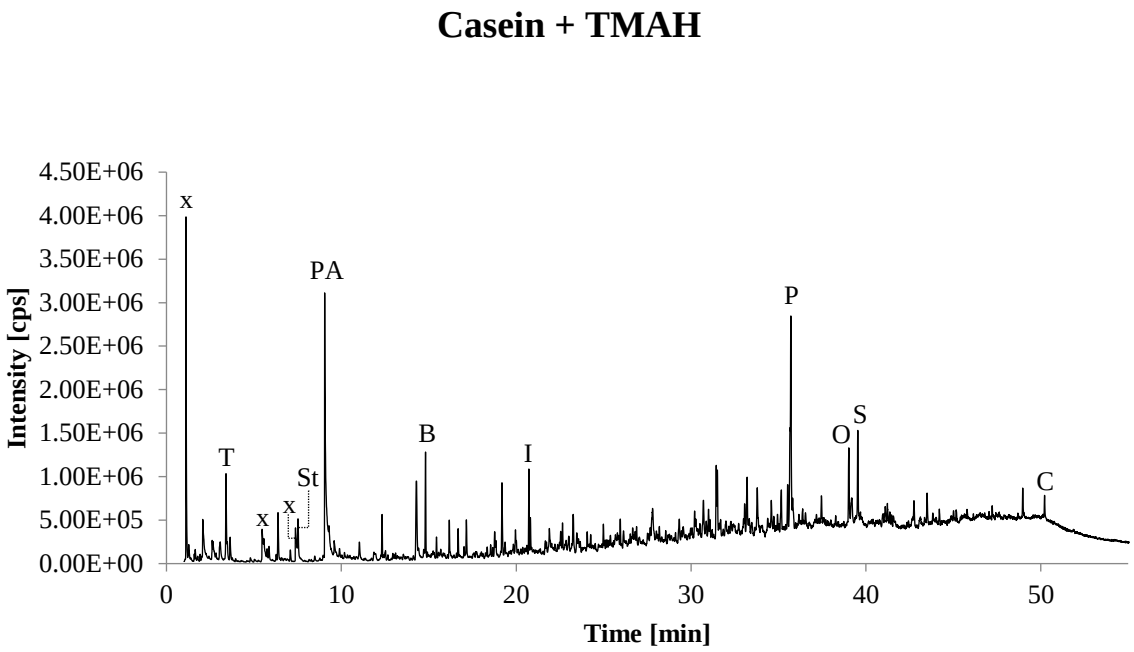
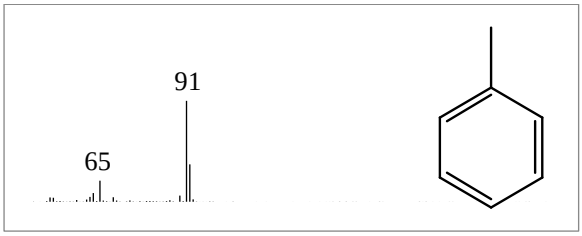


Figure 3.9: Pyrogram of casein (powder), pyrolysed with TMAH. Analysed using Scanning range B.

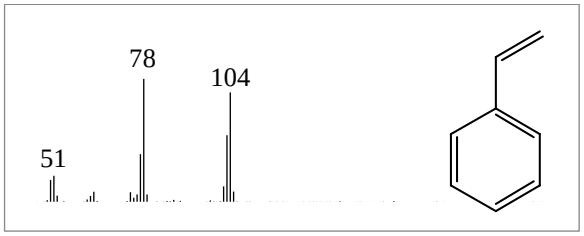
Table 3.10: Selected markers of casein+TMAH.

Peak notation	Compound
T	Toluene
St	Styrene?
PA	Phosphoric acid, trimethyl ester?
B	Benzoic acid, methyl ester?
I	Indole
P	Palmitic acid, methyl ester (Me-C16:0)
O	Oleic acid, methyl ester (Me-C18:1)
S	Stearic acid, methyl ester (Me-C18:0)
C	Cholesterol, methylated
x	Tetramethyl ammonium hydroxide (TMAH)

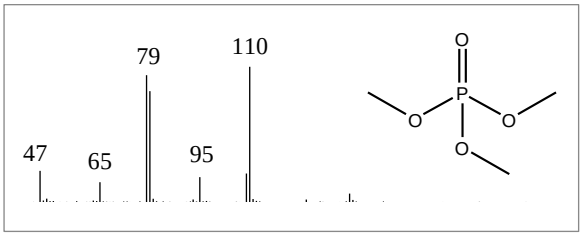
T: Toluene | t_R 3.41



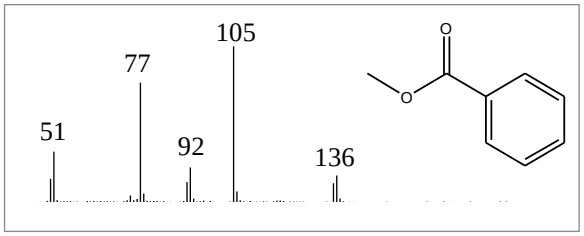
St: Styrene? | t_R 7.52



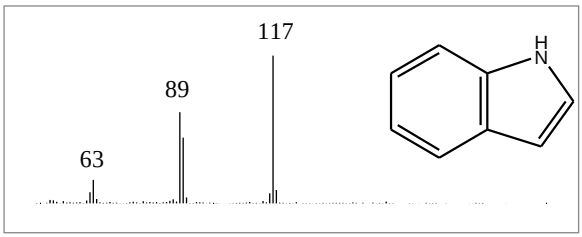
PA: Phosphoric acid, trimethyl ester? | t_R 9.05



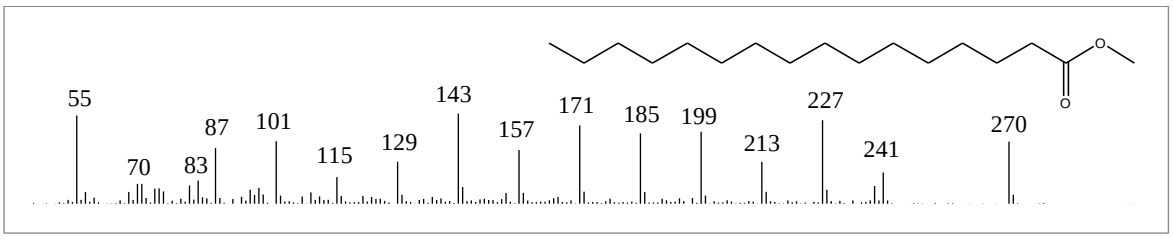
B: Benzoic acid, methyl ester? | t_R 14.81



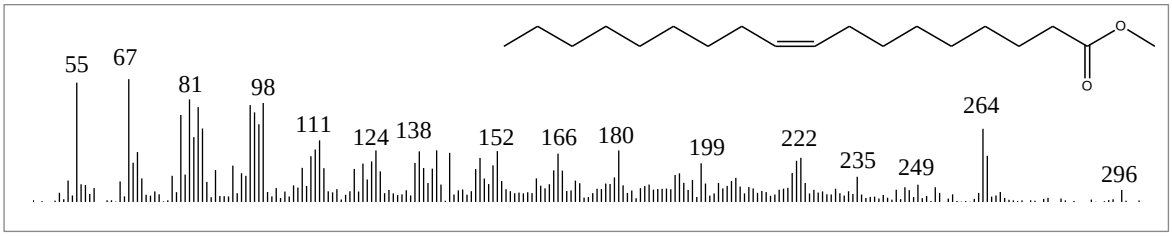
I: Indole | t_R 20.73



P: Palmitic acid, methyl ester | t_R 35.71

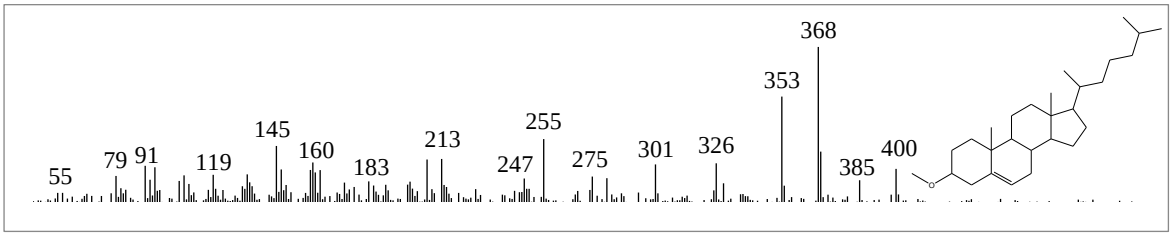


O: Oleic acid, methyl ester | t_R 39.02



S: Stearic acid, methyl ester | t_R 39.54

C: Cholesterol, methylated | t_R 50.22



3.3 Paint samples

Six paint samples were analysed (*2.1.4 Paint samples*). In the following, two analyses of green samples from Icon21 are presented, to illustrate identification of markers in detail with both pyrograms and mass spectra of selected peaks. Pyrograms of the remaining analyses are found in Appendix III.

3.3.1 Icon21 Green + TMAH

The pyrolytic profile of Icon21 Green + TMAH (Fig. 3.10) was relatively simple compared to those of proteinaceous mock samples + TMAH (Fig. 3.6, Fig. 3.7 and Fig. 3.9). Ten peaks were selected: Eight identified peaks and two unknowns.

The identified peaks were methylated pyrrole (Py), isobarbituric acid (IA), methylated benzoic acid (B), dimethylated suberic acid (Su), dimethylated azelaic acid (Az), dimethylated sebacic acid (Se), methylated palmitic acid (P) and methylated stearic acid (S). Methylated pyrrole, isobarbituric acid and methylated benzoic acid were identified through NIST (the latter in very low intensity, only detected using SIM). Methyl esters of palmitic acid and stearic acid were identified through reference sample matching. Dimethyl esters of suberic acid and azelaic acid matched corresponding MS fragment patterns of these compounds found in found in linseed oil+ chalk+TMAH (Fig. 3.1), and sebacic acid was identified partially through NIST, and had similar fragment patterns as dimethyl esters of suberic and azelaic acid (all contained *m/z* 55 together with *m/z* 67 or 69, *m/z* 82 or 83, *m/z* 110 or 111).

Dimethylated azelaic acid was the main peak.

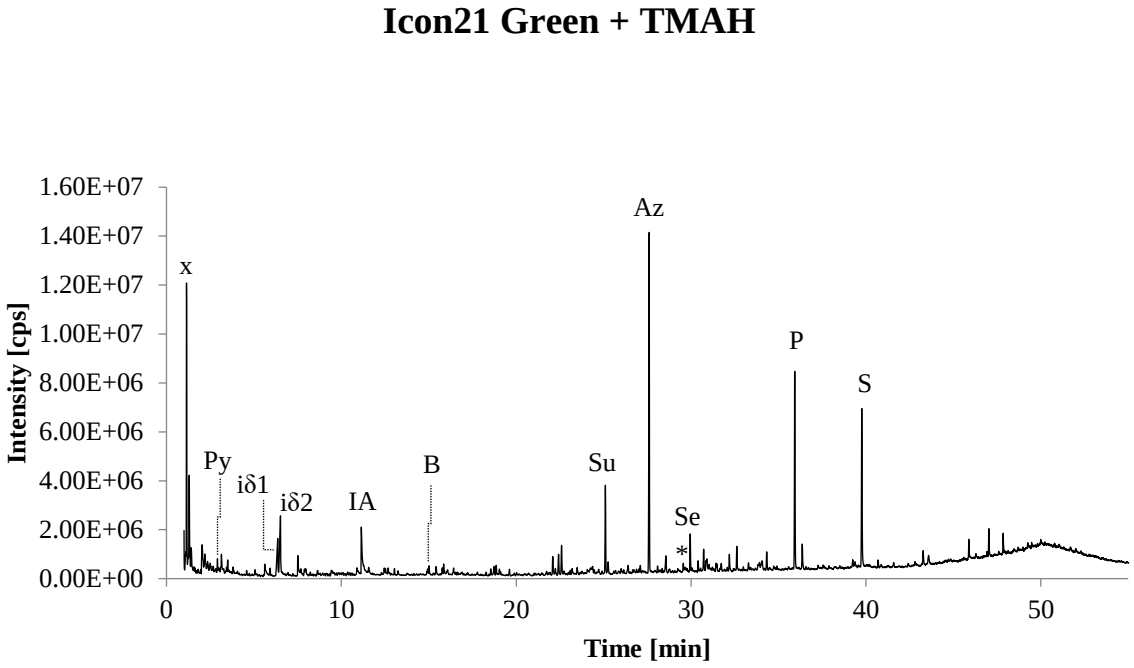
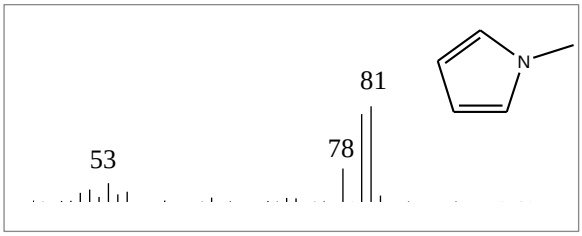


Figure 3.10: Pyrogram of green paint sample from Icon21, pyrolysed with TMAH (IC21G). Analysed using Scanning range B.

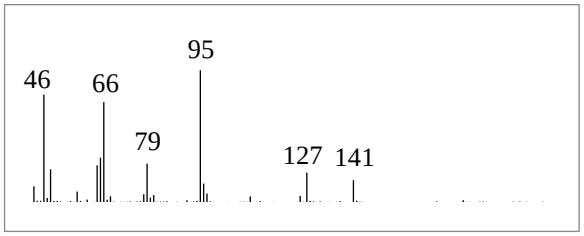
Table 3.11: Selected markers of the green paint sample from Icon21+TMAH.

Peak notation	Marker compound
Py	Pyrrole, methylated
iδ1	Unknown
iδ2	Unknown
IA	Isobarbituric acid?
B	Benzoic acid, methyl ester?
Su	Suberic acid, dimethyl ester? (Me-diC8)
Az	Azelaic acid, dimethyl ester? (Me-diC9)
Se	Sebacic acid, dimethyl ester? (Me-diC10)
P	Palmitic acid, methyl ester (Me-C16:0)
S	Stearic acid, methyl ester (Me-C18:0)
x	Tetramethyl ammonium hydroxide (TMAH)
*****	Contamination/system peak (source accounted for)

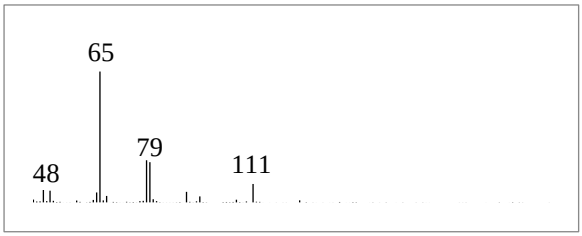
Py: 1-methyl-Pyrrole | t_R 2.92



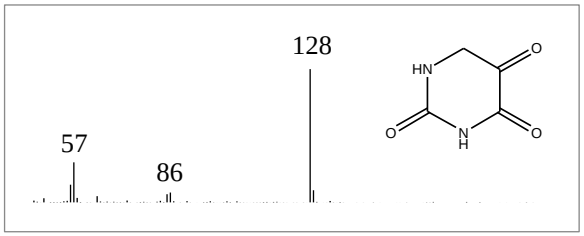
iδ1: Unknown | t_R 6.36



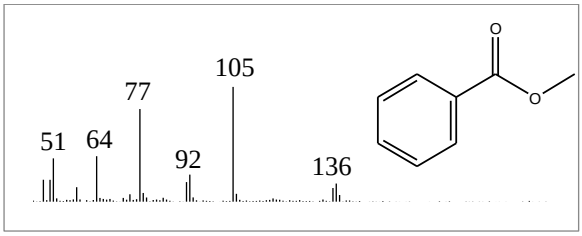
iδ2: Unknown | t_R 6.52



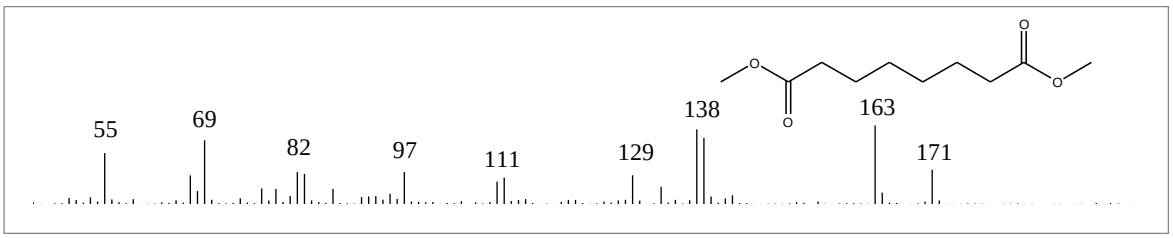
IA: Isobarbituric acid? | t_R 11.14



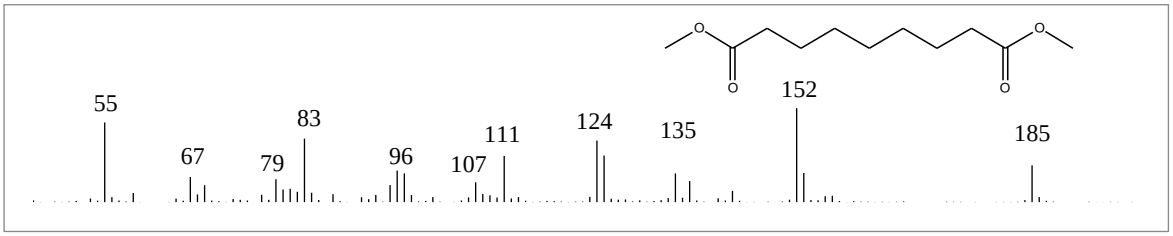
B: Benzoic acid, methyl ester? | t_R 15.02



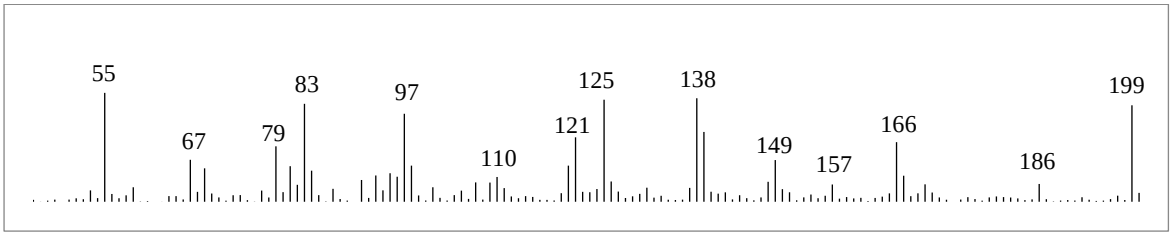
Su: Suberic acid, dimethyl ester | t_R 25.10



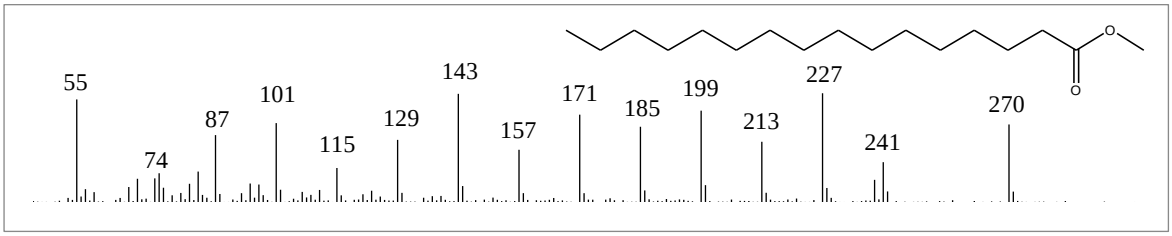
Az: Azelaic acid, methyl ester | t_R 27.61



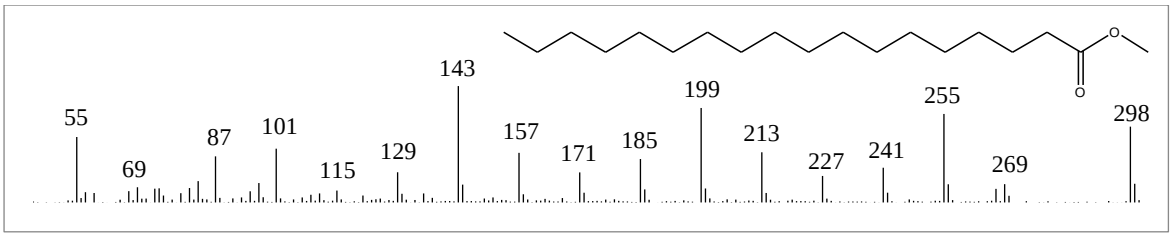
Se: Sebacic acid, methyl ester | t_R 29.95



P: Palmitic acid, methyl ester | t_R 35.93



S: Stearic acid, methyl ester | t_R 39.76



3.3.2 Icon21 Green (ø)

The pyrolytic profile of non-derivatised Icon21 green (Fig. 3.11) was moderate in complexity. Fifteen peaks were selected: Ten identified compounds and five unidentified.

The identified compounds were pyrrole (Py), toluene (T), As_4, diketodipyrrole (DK-Py), cyclo(proline-glycine) (Pro-Gly), cyclo(proline-proline) (Pro-Pro), palmitic acid (P); stearic acid (S), cyclo(proline-hydroxyproline)1 (Pro-Hyp1), cyclo(proline-hydroxyproline)2 (Pro-Hyp2). Pyrrole and toluene were identified through matches with reference samples. Palmitic acid and stearic acid were identified through NIST. As_4, which is a compounds generated from the inorganic pigment As_2S_3, was identified through comparison with literature (Chiavari and Prati, 2003). Diketodipyrrole and the 2,5-diketopiperazines were also identified through matches in literature and additionally compared with spectra in animal glue analysed without derivatisation.

The unidentified compounds were selected based primarily on peak intensity. Two of the unidentified compounds matched unidentified compounds of animal glue: aø3 and aø4.

The main peak, iø3, was unidentified.

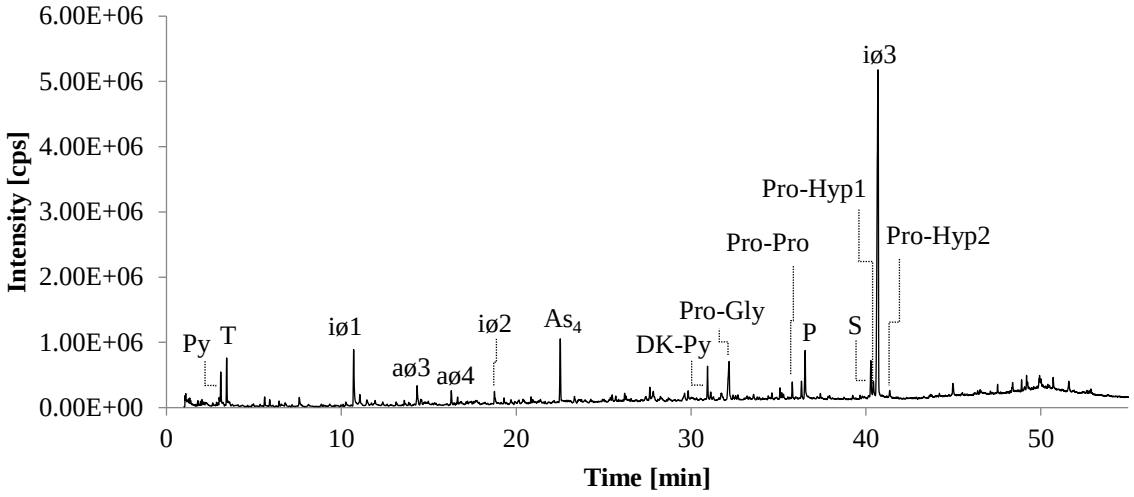
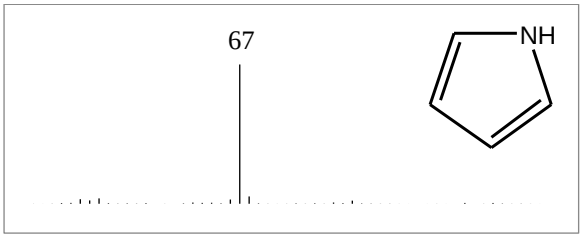


Figure 3.11: Pyrogram of green paint sample from Icon21, without derivatisation. Analysed using Scanning range B.

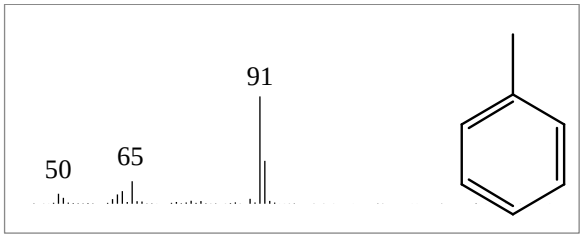
Table 3.12: Selected markers of the green sample from Icon21 (no derivatisation).

Peak notation	Compound
Py	Pyrrole
T	Toluene
iø1	Unknown
aø3	Unknown found in animal glue (ø)
aø4	Unknown found in animal glue (ø)
iø2	Unknown
As_4	As_4 (formed from the inorganic pigment As_2S_3)
DK-Py	Diketodipyrrole
Pro-Gly	Cyclo(Pro-Gly) (2,5-diketopiperazine, DKP)
Pro-Pro	Cyclo(Pro-Pro) (2,5-diketopiperazine, DKP)
P	Palmitic acid?
S	Stearic acid?
Pro-Hyp1	Cyclo(Pro-Hyp)1 (2,5-diketopiperazine, DKP)
iø3	Unknown
Pro-Hyp2	Cyclo(Pro-Hyp)2? (2,5-diketopiperazine, DKP)

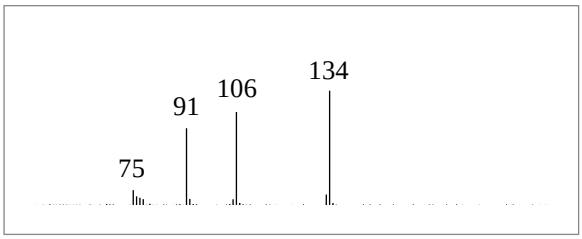
Py: Pyrrole | t_R 3.10



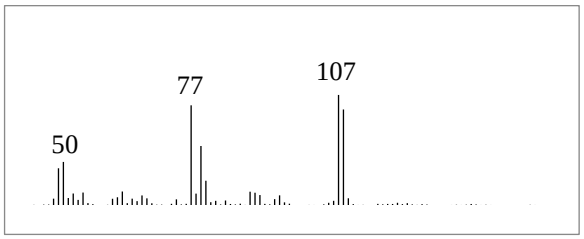
T: Toluene | t_R 3.44



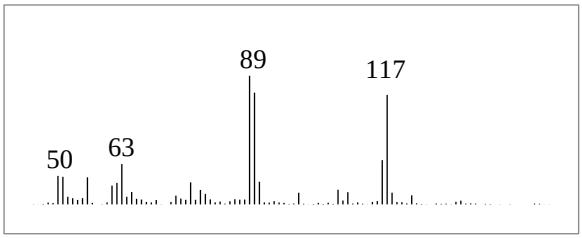
iø1: Unknown | t_R 10.70



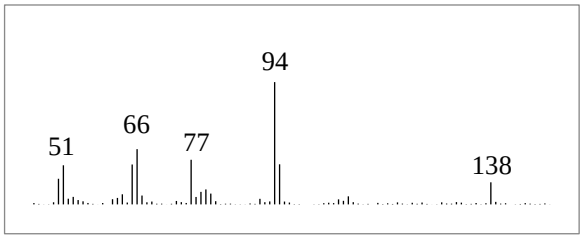
aø3: Unknown, animal glue | t_R 14.34



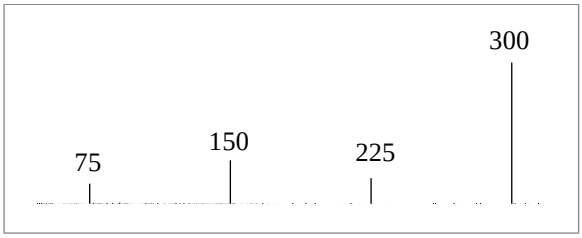
aø4: Unknown, animal glue? | t_R 16.29



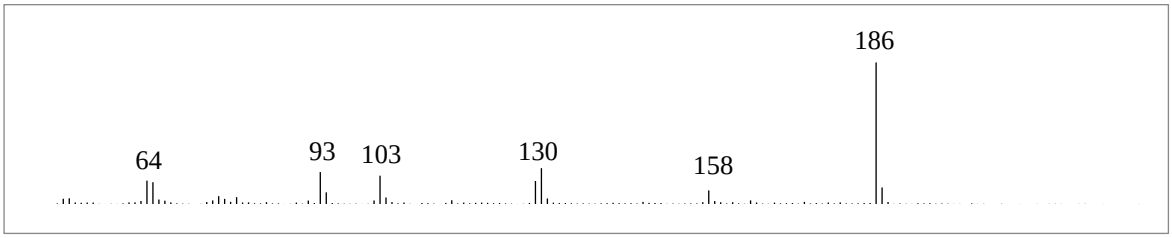
iø2: Unknown | t_R 18.75



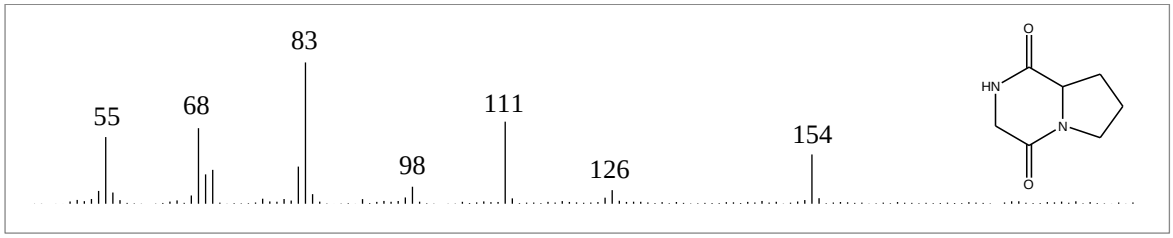
As$_4$: As$_4$ (formed from the inorganic pigment As_2S_3) | t_R 22.53



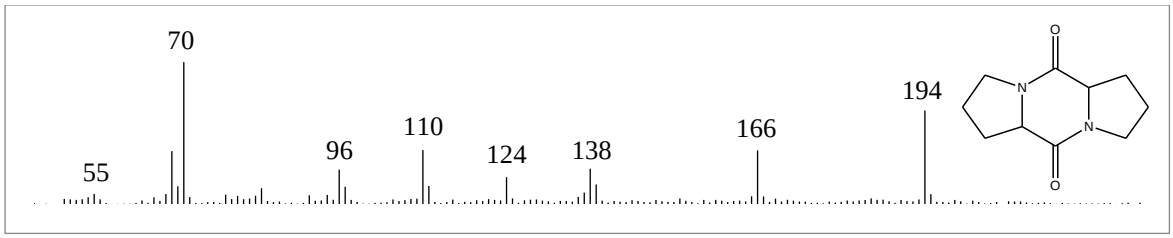
DK-Py: Diketodipyrrole | t_R 30.94



Pro-Gly: Cyclo(proline-glycine) (DKP) | t_R 32.18



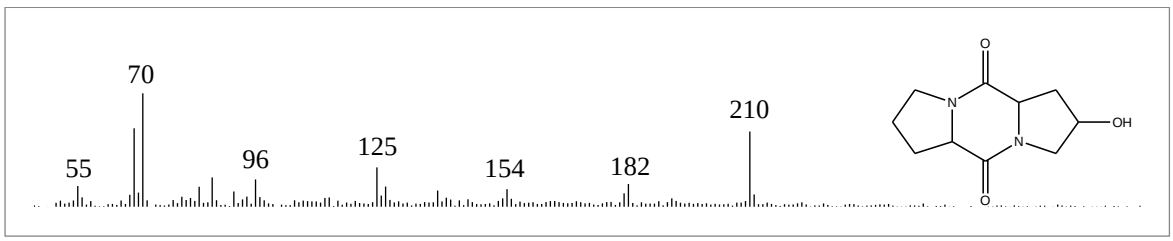
Pro-Pro: Cyclo(proline-proline) (DKP) | t_R 35.79



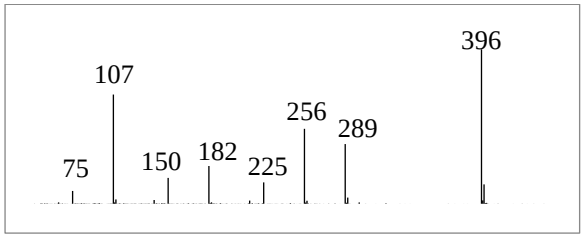
P: Palmitic acid? | t_R 36.53

S: Stearic acid? | t_R 40.28

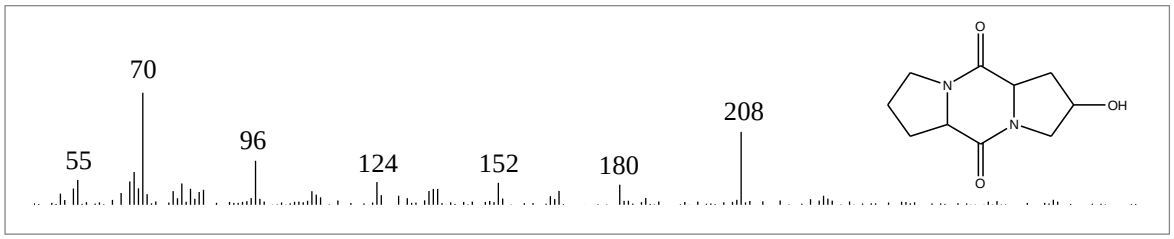
Pro-Hyp1: Cyclo(proline-hydroxyproline), stereoisomer 1 (DKP) | t_R 40.43



iø3: Unknown | t_R 40.69



Pro-Hyp2: Cyclo(proline-hydroxyproline) Stereoisomer 2? (DKP) | t_R 41.37



Chapter 4: Discussion

> *Out of clutter, find simplicity. From discord, find harmony. In the middle of difficulty, lies opportunity.*
>
> Albert Einstein

This thesis aimed to explore the limits and possibilities of characterising organic binding media in ancient paint using pyrolysis-GC/MS for two groups of organic materials: lipid-based materials and proteinaceous materials. As focus of this thesis was split between the characterisation process of binding media and method development for pyrolysis-GC/MS, the discussion is divided in two. Part I discuss the characterisation of each binding medium (linseed oil, beeswax, egg yolk, egg white, animal glue and casein), while Part II discusses challenges related to reproducibility and method improvements.

The characterisation process was divided into three stages:

1) Identifying marker compounds in mock samples.
2) Investigating simultaneous detection of more than one group of organic binding media.
3) Investigating detection of organic binding media in ancient paint.

The stages were investigated parallelly, and fragments from six paint samples (*2.1.4 Paint samples*) were analysed.

Early in the project, the necessity of analysing standards and creating a reference library specific for the samples, the pyrolyser and the particular GC/MS system at NTNU became apparent. The library is found in Appendix IV.

Part I

Characterisation of binding media in paint

4.1 General characterisation process

Mock samples were investigated first, aiming to identify both lipid and proteinaceous markers before analysing paint samples. The characterisation process of the analysed mock samples will be discussed in section *4.2 Binding media*. During the characterisation process, it was important to keep in mind, that classification of binding media is based on the group of organic material most prominent in the material (*1.1 Organic paint binders*).

Pyrograms of mock samples (linseed oil, beeswax, egg yolk, egg white, animal glue and casein) together with mass spectra of selected possible markers are presented in section *3.1 Lipid-based binding media* and *3.2 Proteinaceous binding media*. Expanded versions of pyrograms are found in Appendix II in order to show details better. Pyrograms of all paint samples (both from Mota Giyorgis, Icon9, both from Icon21 and both from Wukero Cherkos) are presented in Appendix III. In the following text, "Fig. AIII-#" refers to pyrograms of paint samples in Appendix III. The markers identified in mock and paint samples are summed up in Table 4.1 and 4.2 respectively.

To be able to detect lipid-based and proteinaceous binders in the same analysis (simultaneous detection), markers for both must be generated under the same pyrolysis conditions. As markers of lipids (e.g. fatty acids) generally require derivatisation to be detected in a gas chromatographic system (Husek, 2000), different derivatisation techniques (methylation and silylation reagents) were investigated in the Lauschke project. In this project, it was decided to move forward with only tetramethylammonium hydroxide (TMAH), as this proved to be the most promising derivatisation reagent in the Lauschke project (*1.3.3 Derivatisation*).

The initial hope for simultaneous detection of lipid-based and proteinaceous markers, was to identify both marker types in samples analysed in presence of TMAH. Identifying markers for lipid-based binders is relatively straightforward,

as their pyrolytic profiles are simple and therefore easy to interpret (e.g. Fig. 3.1). Identifying markers of proteinaceous binders, on the other hand, is challenging as proteinaceous materials generate complex pyrolytic profiles (e.g. Figs. 3.7, 3.8 or 3.9), due to the many reactions that can occur during pyrolysis (Chiavari and Galletti, 1992; Colombini *et al.*, 2010) (*1.1.4 Proteinaceous material*).

A dilemma arose, as lipid markers were only identified in analyses involving TMAH, but the most promising proteinaceous markers, 2,5-diketopiperazines (DKPs), could apparently not be identified when samples were pyrolysed in presence of TMAH (*1.1.4 Proteinaceous material*). It would therefore be difficult to identify both materials under the same pyrolytic conditions based on DKPs as markers for proteinaceous materials. Still, some small aromatic compounds (the other proteinaceous marker type) can be identified in samples pyrolysed in presence of TMAH and were found in all proteinaceous mock samples (Figs. 3.5, 3.6, 3.7, 3.8 and 3.9) and all paint samples (Figs. AIII-1-8).

Most samples were pyrolysed in presence of TMAH, but analyses without derivatisation were investigated as well, aiming to explore the possibility of characterising non-derivatised proteinaceous materials and to test the limits regarding non-derivatised lipid-based materials. By comparing the information gained from the different approaches one could better evaluate whether simultaneous detection was a possibility or not.

Fig. 4.1 and 4.2 together illustrates the importance of analysing samples in presence of a derivatising reagent. In Fig.4.1, pyrograms of linseed oil, the green sample of Icon21 (IC21G), and animal glue pyrolysed in presence of TMAH are presented, and, in Fig. 4.2, the same samples analysed without derivatisation is presented. Especially for the paint sample (IC21G), presence of drying oil is evident, as azelaic acid is the main peak, in the derivatised sample (Figs. 4.1 and 3.1). Had this sample only been analysed without derivatisation, drying oils would not have been suspected.

The two approaches were found to be complementary. Pyrolysis in presence of TMAH targeted compounds considered suitable for GC/MS analyses only after derivatisation, such as fatty acids and diacids (markers of lipids), while pyrolysis without derivatisation targeted compounds that can pass through the column non-derivatised, such as 2,5-diketopiperazines and other possible markers of proteins.

All mock and paint samples were analysed with the methylation approach, whilst linseed oil, egg yolk, animal glue and the green sample of Icon21, additionally, were analysed without derivatisation. The discussion in section *4.2 Binding media*, will therefore include a comparison of the approaches for these samples.

Six paint samples were analysed, ranging in age from the $18^{th}/19^{th}$ century to the 10^{th} century or even older, and eight analyses were run from these six paint samples. Pictures of the paintings and icons, from which the samples were collected, can be found in Appendix VI, and pictures of the collected microsamples are found in Fig. 2.2.

Analyses of ancient paint samples revealed complex pyrolytic profiles (Figs. AIII-1-8), although less complex than animal glue and casein (Figs. 3.7, 3.8 and 3.9), which was not surprising as materials lose complexity, due to degradation through aging (Orsini, Parlanti and Bonaduce, 2017). Most analyses of paint samples resulted in inconclusive pyrograms. The markers identified for one mock sample (either single markers, overall profiles or quantitative ratios, *1.3.2 Markers*) were often found in other mock samples as well (Table 4.1). Egg white and casein, in particular, had very similar pyrolytic profiles, and drying oils and egg yolk/whole egg could be difficult to distinguish as well. The possibility that the artist had mixed organic compounds, either in the paint layer(s) or in other layers of the artwork, complicated the interpretation of real paint samples further.

Quantitative evaluations of azelaic acid and palmitic acid, which were markers of drying oils and egg (*1.1 Organic paint binders*), were based on comparisons of peak intensities, which can be measured by peak areas or peak heights. Ratios

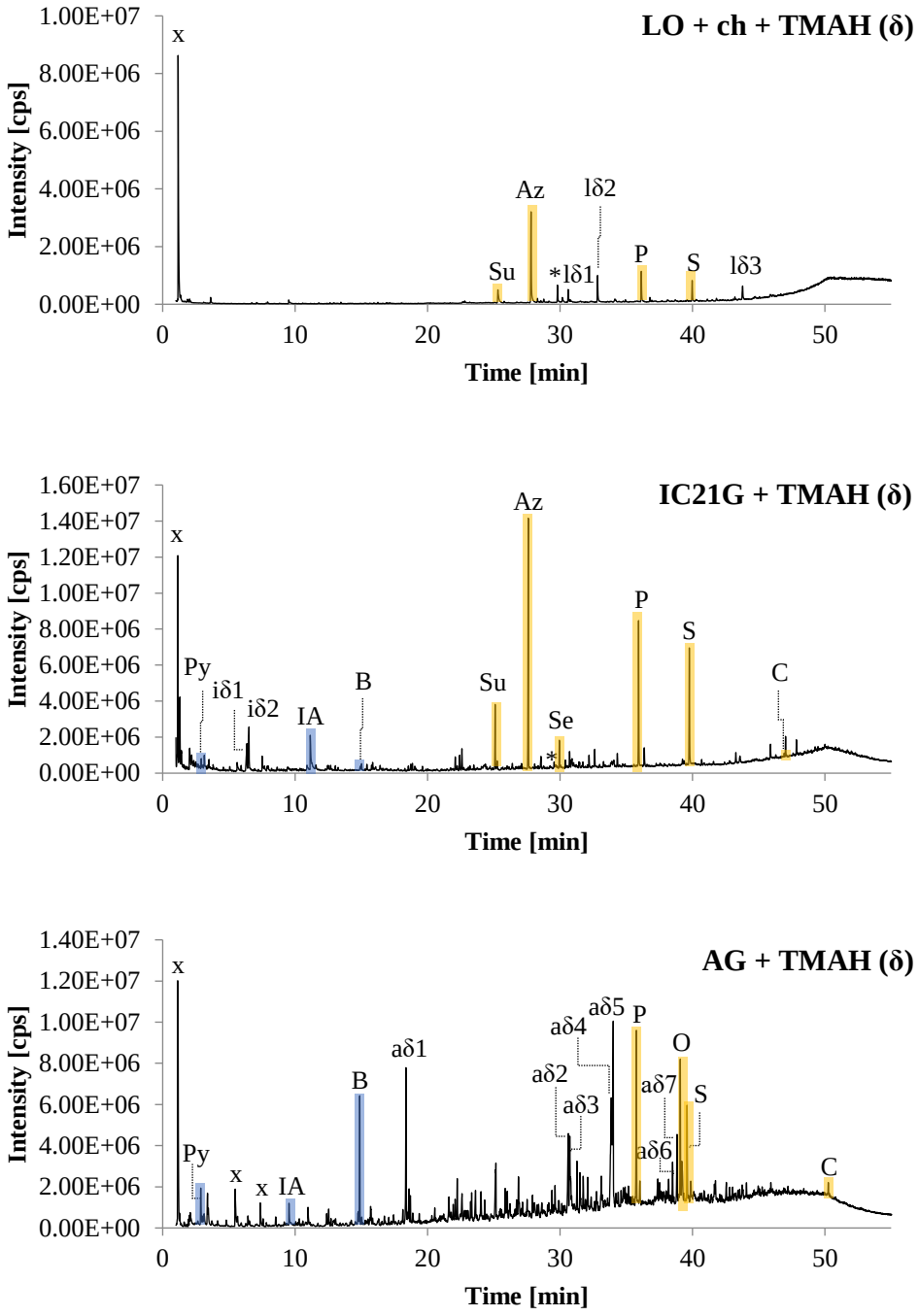


Figure 4.1: Comparison of pyrolytic profiles samples run with derivatisation (δ). Icon21 Green + TMAH (δ) (middle) compared to linseed oil + chalk + TMAH (δ) (top) and animal glue + TMAH (δ) (bottom). Yellow-coloured peaks are lipid markers and blue-coloured peaks are proteinaceous markers.

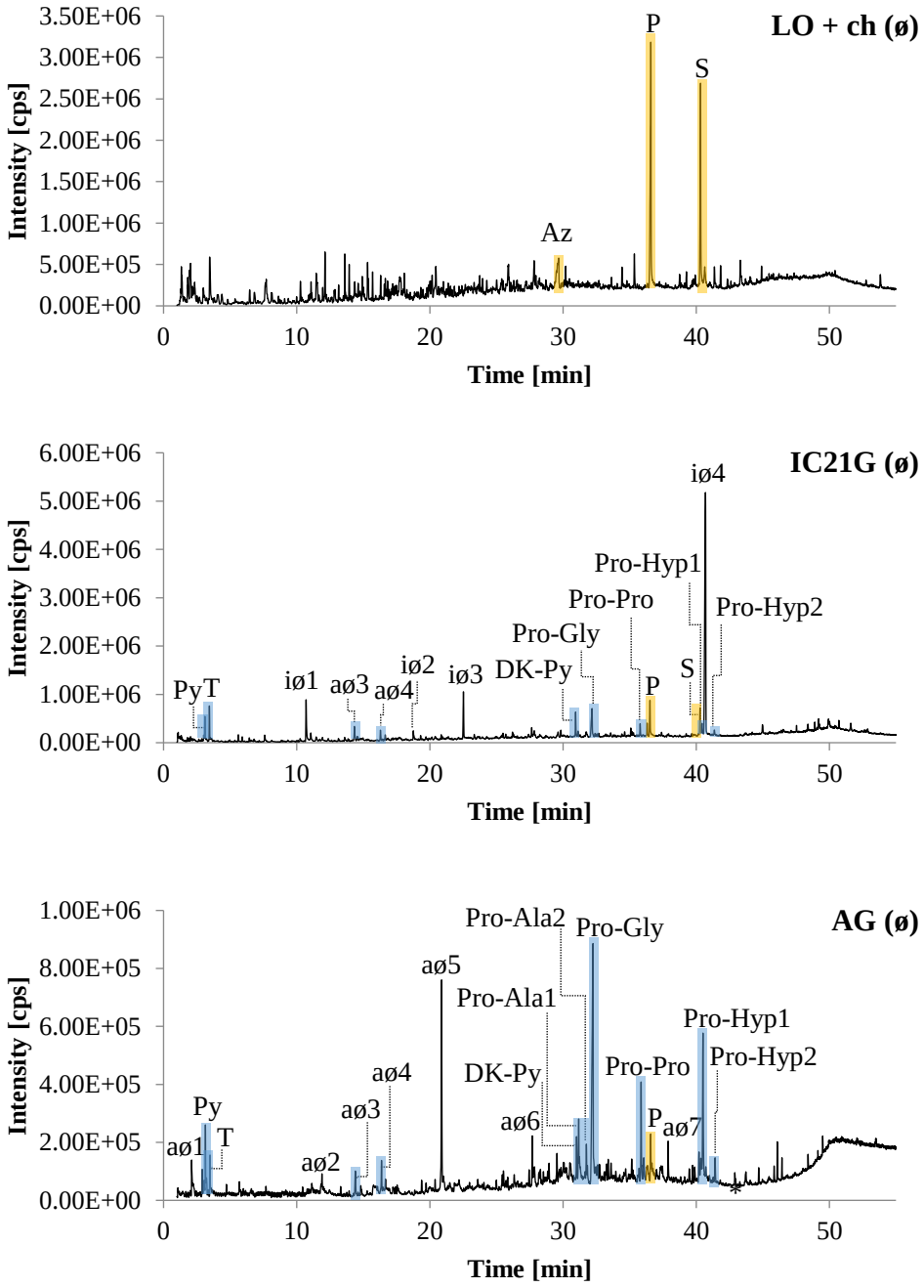


Figure 4.2: Comparison of pyrolytic profiles of samples run without derivatisation (ø). Icon21 Green (ø) (middle) compared to linseed oil + chalk (ø) (top) and animal glue (ø) (bottom). Yellow-coloured peaks are lipid markers and blue-coloured peaks are proteinaceous markers.

were tentatively assessed by comparing peak heights, which is less accurate than peak area but considered adequate for the purpose of this thesis.

Peak intensities are, therefore, based on peak heights, which, under ideal conditions, is assumed to be proportional to the concentration of the compound. A high intensity peak therefore represents a compound present in high concentration in the pyrolysate. In the following, pyrographic peaks are described as “high” (close to, or main peak), “low” (close to baseline), “moderately high” (between high and low intensities, closer to main peak than baseline) or “moderately low” (between high and low intensities, closer to baseline than main peak). When TMAH is present, the intensity of its peaks is ignored when describing the remaining peaks.

Table 4.1: Identified markers in mock samples. **Bold and underlined: Main peak**. Underlined: High intensity peak. *Italic*: *Low intensity peak*. Binding media: LO = Linseed oil + Chalk, EY = Egg Yolk, EW = Egg White, AG = Animal Glue, Cas = Casein, BW = Beeswax. δ = TMAH added, ø = without derivatisation. Lipid compounds: Su = Suberic acid, Az = Azelaic acid, Se = Sebacic acid, P = Palmitic acid, O = Oleic acid, S = Stearic acid, Wax = wax-related markers, PA = Phosphoric acid, C = Cholesterol. Small aromatic compounds: Py = Pyrrole, T = Toluene, E = Ethylbenzene, St = Styrene, IA = Isobarbituric acid, B = Benzoic acid, I = Indole. 2,5-diketopiperazines: DK-py = Diketodipyrrole, Pro-Ala = Cyclo(Proline-Alanine), Pro-Gly = Cyclo(Proline-Glycine), Pro-Pro = Cyclo(Proline-Proline), Pro-Hyp = Cyclo(Proline-Hydroxyproline).

Binding media/ markers	**Fatty acids and other lipid compounds**									**Small aromatic compounds**							**2,5-Diketopiperazines**				
	Su	Az	Se	P	O	S	Wax	PA	C	Py	T	E	St	IA	B	I	DK-py	Pro-Ala 1+2	Pro-Gly	Pro-Pro	Pro-Hyp 1+2
LO(δ)	Su	**Az**		P		S															
LO(ø)		Az		**P**		S															
BW(δ)				P		S	FA,HC														
EY(δ)				P	**O**	S		*PA*	C												
EY(ø)				P	O	S			**C**												
EW(δ)											**T**	E	St			I					
AG(δ)				P	O	S			C	Py	T			IA	B						
AG(ø)				P						Py	T						DK-py	Pro-Ala1+2	**Pro-Gly**	Pro-Pro	Pro-Hyp1+2
Cas(δ)				P	O	S		**PA**	C		T		St		B	I					

Lipid associated

Protein associated

Table 4.2: Identified markers in paint samples (youngest first, oldest last). **Bold and underlined: Main peak**. Underlined: High intensity peak. *Italic*: *Low intensity peak*. Paint samples: MGS/L = Mota Giyorgis small/large, IC9 = Icon9, IC21B/G = Icon21 brown/green, WCW/Y = Wukero Cherkos white/yellow. δ = TMAH added, ø = without derivatisation. Lipid compounds: Su = Suberic acid, Az = Azelaic acid, Se = Sebacic acid, P = Palmitic acid, O = Oleic acid, S = Stearic acid, Wax = wax-related markers, PA = Phosphoric acid, C = Cholesterol. Small aromatic compounds: Py = Pyrrole, T = Toluene, E = Ethylbenzene, St = Styrene, IA = Isobarbituric acid, B = Benzoic acid, I = Indole. 2,5-diketopiperazines: DK-py = Diketodipyrrole, Pro-Ala = Cyclo(Proline-Alanine), Pro-Gly = Cyclo(Proline-Glycine), Pro-Pro = Cyclo(Proline-Proline), Pro-Hyp = Cyclo(Proline-Hydroxyproline).

Paint sample/ markers	Fatty acids and ther lipid compounds									Small aromatic compounds							2,5-Diketopiperazines				
	Su	Az	Se	P	O	S	Wax	PA	C	Py	T	E	St	IA	B	I	DK-py	Pro-Ala 1+2	Pro-Gly	Pro-Pro	Pro-Hyp 1+2
MGS(δ)		Az		P		S									B						
MGL(δ)		Az		**P**		S		PA							B						
IC9(δ)		*Az*		**P**		S	FA HC	*PA*							*B*						
IC21B(δ)	Su	Az		**P**		S									*B*						
IC21G(δ)	Su	**Az**	Se	P		S			*C*	*Py*				IA	*B*						
IC21G(ø)				P		S				Py	T						DK-py		Pro-Gly	Pro-Pro	Pro-Hyp 1+*2*
WCW(δ)				P		S		PA		*Py*				IA	B						
WCY(δ)				P		S				*Py*				**IA**	*B*						

Lipid associated

Protein associated

4.2 Binding media

In this section, each of the six analysed binding media (linseed oil, beeswax, egg yolk, egg white, animal glue and casein) will be discussed one by one. For each binder, the diagnostic potential of selected markers was evaluated based on all the paint samples, in which the specific binder was suspected. Drying oil (linseed oil), for instance, was suspected in four paint samples: Mota Giyorgis (Figs. AIII-1 and AIII-2), Icon9 (Fig. AIII-3) and both the brown and green samples of Icon21 (Figs. AIII-4 and AIII-5).

As the same marker compounds are present in several binding media, repetition will occur. With this approach, all aspects relevant for one binding media then will be described together in the same place. The markers identified in mock and paint samples are, as mentioned, summed up in Table 4.1 and 4.2 respectively. Both tables contain two sections: One for markers associated with lipids (yellow) and one for markers associated with proteins (blue).

Markers associated with lipids (fatty acids, diacids, phosphoric acid and cholesterol) were easily identified in samples pyrolysed in presence of TMAH. The most common markers, fatty acids, were found in all mock samples (Figs. 3.1, 3.3, 3.4, 3.7, 3.9), except for egg white (Fig. 3.6), as well as in all paint samples (Figs. AIII-1-8). When pyrolysing mock and paint samples in presence of TMAH, the main peak was most often a lipid-based marker, such as dimethylated azelaic acid for linseed oil (Fig. 3.1), methylated oleic acid for egg yolk (Fig. 3.4), trimethylated phosphoric acids for casein (Fig. 3.9) and methylated palmitic acid for Mota Giyorgis (Fig. AIII-2).

Markers associated with proteins were divided into the two categories: Small aromatic compounds and 2,5-diketopiperazines (DKPs) (overview in Table 4.1, description of categorisation in *1.1.4 Proteinaceous material*).

Small aromatic compounds were identified in proteinaceous samples pyrolysed in presence of TMAH: Egg white (Fig. 3.6), animal glue (Fig. 3.7) and casein (Fig. 3.9), as well as in all the analysed ancient paint samples (Figs. AIII-1-8). The aromatic compounds were all small, containing maximum two rings. They were either aromatic hydrocarbons, such as styrene (Figs. 3.6 and 3.9), or heteroaromatics, such as pyrrole (Figs. 3.7 and 3.8) and were found in the first half of pyrolygrams. They appeared in both non-derivatised samples and samples pyrolysed in presence of TMAH.

DKPs were identified in the non-derivatised sample of animal glue (Fig. 3.8) and the non-derivatised green sample of Icon21 (Figs. 3.11 or AIII-6). They were found in the latter half of pyrograms, but have also been reported in the first half (Orsini, Parlanti and Bonaduce, 2017). DKPs have also been reported for casein and egg white (Orsini, Parlanti and Bonaduce, 2017), but animal glue was the only proteinaceous material analysed without derivatisation in this project.

4.2.1 Linseed oil – a drying oil

Linseed oil was analysed both with and without derivatisation. When pyrolysed in presence of TMAH, the pyrolytic profile (Fig. 3.1) was clear and simple. The identified peaks corresponded to those reported in the literature (*1.1.2 Linseed oil – a drying oil*): Dimethylated diesters of suberic acid and azelaic acid, and methylated esters of palmitic acid and stearic acid (Bonaduce and Andreotti, 2009). Dimethylated azelaic acid was the main peak, and the ratio between dimethylated azelaic acid and methylated palmitic acid (the Az/P ratio) was assessed to be far above 1[13].

[13] A valid Az/P ratio is determined by peak areas. Peak areas for of azelaic acid and palmitic acid have not been calculated in this project, but ratios have been tentatively assessed by comparing peak heights. Peak heights determined on "well-behaving" pyrographic peaks recorded within a linear temperature programme section are close to proportional to peak areas.

The non-derivatised sample was not expected to generate a diagnostic profile, as the paint film exclusively should contain fatty acids and diacids, which generally require derivatisation before being analysed with GC (Husek, 2000). Still, the sample was run to investigate the possibility of identifying both lipids and proteins in a non-derivatised sample. Two narrow peaks of high intensities, palmitic and stearic acid, appeared, and azelaic acid was detected as well, but as a broad and low intensity peak (Fig. 3.2). The Az/P ratio was, therefore, unsuitable as marker for drying oils in analyses without derivatisation, and it was, as expected, not possible to identify linseed oil in non-derivatised samples.

Of the paint samples pyroysed in presence of TMAH, dimethylated azelaic acid was detected in the samples from Mota Giyorgis (Figs. AIII-1 and AIII-2), Icon9 (Figs. AIII-3) and Icon21 (Figs. AIII-4 and AIII-5). Thus, all the analysed paint samples contained azelaic acid, except the two from Wukero Cherkos (the oldest samples, Figs. AIII-7 and AIII-8).

For both the samples from Mota Giyorgis (Fig. AIII-1 and AIII-2), the Az/P ratio was assessed to be between 0.3 and 1 (around 0.5 for both). No other markers pointed towards drying oils, and presence of egg was, thus, suspected. However, the Az/P ratio is high compared to that of Az/P ratios reported for egg in the literature (Chiavari, Fabbri and Prati, 2005). Therefore, the origin of azelaic acid in this sample is difficult to interpret.

For the sample from Icon9 (Fig. AIII-3), the Az/P ratio was far below 0.3, which indicates presence of egg and will therefore be discussed in the corresponding section (*4.2.3 Egg yolk*).

For the brown sample of Icon21 (AIII-4) the Az/P ratio was assessed to be around 0.3. The low Az/P ratio could indicate whole egg/egg yolk, but suberic acid was present as well, indicating drying oils. Theoretically, one can imagine that an egg sample generating azelaic acid could generate suberic acid as well, but it was not

reported as marker for egg in the reviewed literature. Either oil or egg were, therefore, suspected.

The green sample from Icon21 (Fig. 3.10 and Fig. AIII-5) had an Az/P ratio above 1, clearly indicating drying oils. Suberic and sebacic acid were present as well, strengthening this interpretation.

To sum up, Az/P ratios above 1 and presence of other diacids (markers of drying oil) resulted in a confident identification of drying oils. When azelaic acid was present, but the Az/P ratio below 1, the origin of azelaic acid was inconclusive. Presence of other diacids strengthened the suspicion of drying oils, but additional markers for distinction between egg and drying oils are needed, when Az/P ratios are below 1. Such additional markers are yet to be found.

4.2.2 Beeswax – a natural wax

Beeswax was only pyrolysed in presence of TMAH, as non-derivatised linseed oil (the other lipid-based material) did not give a diagnostic pyrolytic profile. The necessity of derivatisation when analysing lipid-based material had already been established.

Methylated esters of palmitic and stearic acids were the only compounds fully identified in the pyrogram (Fig. 3.3). Methylated palmitic acid was the main peak reported by Asperger, Engewald and Fabian (1999) (*1.1.3 Beeswax*), but, in this project, methylated palmitic acid and stearic acids had moderate intensities compared to the main peak, an unidentified compound, bδ1. The discrepancies in peak intensities between the literature reports and the ones recorded in this project could be due to different instrumental setups[14] (*4.3.2 Deviating markers*).

[14] Interestingly, Asperger and his collaborators also used a Pyrola pyrolyser, a Pyrola 9p. This was the only article in the reviewed literature related to binding media characterisation in which a Pyrola pyrolyser was mentioned.

Beeswax was added to the binding media library since one paint sample, Icon9, displayed a characteristic overall profile in the last part of the pyrogram (Fig. AIII-3). Waxes were suspected, and it had many diagnostic peaks in common with beeswax – or at least a waxy substance, such as methylated palmitic acid, methylated stearic acid, unknown long-chained fatty acids, unknown long-chained hydrocarbons, and the unknown compounds bδ1 and bδ2.

Many of the unknown compounds had similar/almost identical MS patterns, eluting at different retention times, e.g. hc1, hc2 and hc3, which all have the MS pattern *m/z* 71, 85, 97, 113, 127, 141, 155, 169, 207, (281) and were eluting at t_R = 47.71, 48.57 and 50.92 respectively. Further identification of the remaining peaks would require reference samples of suspected methyl esters of fatty acids, methyl ethers of alcohols, hydrocarbons and other reported markers by Asperger, Engewald and Fabian (Asperger, Engewald and Fabian, 1999). This have provided an overall pyrolytic profile, as basis for the identification of beeswax. The reference library for the pyrolysis-GC/MS setup at IKJ/NTNU is yet to be updated with these possible markers.

4.2.3 Egg yolk

Egg yolk was was analysed both with TMAH (Fig. 3.4) and without derivatisation (Fig. 3.5), and was a representative for whole egg as well, as the pyrolytic profiles were found to be very similar in the Lauschke project (*1.1.5 Egg*). It contains 16.5% proteins (Mills and White, 1994b, p. 42), and is regarded a proteinaceous material, although proteinaceous markers are generally not reported for egg yolk or whole egg. Only lipid markers were detected in this project as well: Fatty acids (Figs. 3.4 and 3.5), cholesterol (Figs. 3.4 and 3.5), possible derivatives of cholesterol (Fig. 3.5) and phosphoric acid (Fig. 3.4). An unidentified compound, yδ1, was also selected as possible marker (Fig. 3.4). Cholesterol and phosphoric acid have been reported as markers for egg yolk/whole egg (Mills and White,

1994c; Chiavari and Prati, 2003; Melucci *et al.*, 2011) but are not exclusively associated with egg yolk. Cholesterol was detected in animal glue (Fig. 3.7) and casein (Fig 3.9) and phosphoric acid was detected in casein (Fig. 3.9) as well, but in much lower amounts.

Egg yolk is the only binding media wherein cholesterol is present in significant amounts (5.2 % of the total lipid content) (Mills and White, 1994c, p. 88). In In the non-derivatised sample of egg yolk (Fig. 3.5), cholesterol was the main peak. Although researchers have suggested cholesterol as a possible marker in early research, it is mostly considered too unstable with aging to be detected in ancient samples (Chiavari and Prati, 2003).

Phosphoric acid, which was detected in small amounts egg yolk (Fig. 3.4), was reported as marker in the literature as well (Chiavari and Prati, 2003; Melucci *et al.*, 2011). It is derived from a specific egg yolk protein, lecithin, and was, previously, not detected in samples of casein (Chiavari and Prati, 2003). In this project, however, phosphoric acid was detected as the main peak in casein (Fig. 3.9). It could, therefore, be a marker for both egg (yolk) and casein.

As mentioned in *4.2.1 Linseed oil – a drying oil*, azelaic acid is occasionally reported as marker of egg yolk or whole egg, but in much lower intensities than the fatty acids palmitic acid (Chiavari, Fabbri and Prati, 2001, 2005). An Az/P ratio lower than 0.3 suggests presence of egg yolk/whole egg or non-drying fats (Mills and White, 1994a, p. 171; Bonaduce *et al.*, 2016, p. 305).

In accordance with literature reports (Chiavari *et al.*, 1993; Chiavari, Fabbri and Prati, 2001, 2005; Melucci *et al.*, 2011), azelaic acid was not found in egg yolk (Figs. 3.4 and 3.5). As a result, it is difficult to interpret pyrograms of paint samples, where azelaic acid *is* present, but where the Az/P ratio was below 1.

Alkylnitriles (hexa-, hepta- and octadecanenitrile), formed in reactions between fatty acids and amines in the aromatic amino acids (Chiavari *et al.*, 1998; Orsini,

Parlanti and Bonaduce, 2017), have been suggested as markers of egg yolk/whole egg but were, likewise, not detected in the mock samples (Figs. 3.4 and 3.5). Reference samples of alkyl nitriles were run, proving that these compounds *are* possible to detect in the pyrolysis-GC/MS setup at IKJ/NTNU.

Presence of egg yolk/whole egg was, however, suspected in the larger sample from Mota Gyiorgis (Fig. AIII-2), the sample from Icon9 (Fig. AIII-3), the green sample from Icon21 (Figs. 3.10 or AIII-5) and the white sample from Wukero Cherkos (Fig, AIII-7).

In the larger sample from Mota Giyorgis (Fig. AIII-2), an Az/P ratio of approximately 0.5 together with presence of phosphoric acid in relatively high abundance could indicate presence of egg, although the Az/P ratio is too high. Mixtures of egg and drying oil (*tempera grassa*) is also a possibility (Bonaduce and Andreotti, 2009). Phosphoric acid could also stem from another binding medium, e.g casein, as casein glue in the grounding layer (Fig. 1.1) or as casein tempera. Mixtures are difficult to characterise, and whether egg yolk/whole egg is present in the sample of Mota Giyorgis is inconclusive.

Icon9 (Fig. AIII-3) showed an Az/P ratio much lower than 0.3. Since factors like pigments can influence the formation of azelaic acid (Chiavari, Fabbri and Prati, 2005), a low Az/P ratio should be accompanied by other markers before concluding on the presence of egg yolk/whole egg. In this paint sample, phosphoric acid was present as well, but in low abundance. As with the sample of Mota Giyorgis, this indicates presence of egg yolk/whole egg, drying oils, casein or a mixture of these.

Despite being considered unstable through aging, cholesterol was detected in the green sample of Icon21 (Fig. 3.10 or AIII-5). This was a sample where the azelaic acid content strongly indicated presence of drying oil, as the Az/P ratio was above 1. The peak intensity of cholesterol was quite low. If egg yolk/whole egg was the source of the cholesterol, then *tempera grassa* could be a painting technique used.

Other possible sources could be animal glue and casein either in the grounding layer (Fig. 1.1) or added to the paint as binding medium.

In the white sample from Wukero Cherkos (Fig. AIII-7), phosphoric acid was present in a moderately high amount. There were no azelaic acid or any other markers associated with egg yolk/whole egg present, and phosphoric acid was therefore thought to originate from something else than egg yolk/whole egg, e.g. casein.

For all these four paint samples (Mota Giyorgis, Icon9, the green sample from Icon21 and the white sample from Wukero Cherkos), the presence of egg yolk/whole egg was, therefore, inconclusive, as no markers unambiguously indicating presence of egg yolk/whole egg.

4.2.4 Egg white

Egg white contains 10 % proteins (Mills and White, 1994b, p. 42) and, in contrast to egg yolk, animal glue and casein, it does not contain any lipids. Egg white was only pyrolysed in presence of TMAH, in this project, and the small aromatic compounds, toluene, ethyl benzene, styrene and indole (Fig. 3.6), were identified as markers.

In addition, five unknown compounds were selected as possible markers. These were suspected to be small aromatic compounds as well, as the top hits in NIST were aromatic compounds, and their fragments patterns were similar to those of other aromatic compounds detected. Examples of the latter are wδ1 with a fragment pattern, *m/z* 63, 89, 117, almost identical to indole (Appendix IV) and wδ2 with the fragment pattern *m/z* 65, 77, **91**, 102, 129 (bold being the base peak), where the fragments *m/z* 65 and 91 together are characteristic for toluene (Appendix IV).

These aromatic compounds were not detected in paint samples, with the exception of toluene, which was detected in the non-derivatised green sample of Icon21 (Fig. AIII-6), probably due to presence of animal glue (*4.2.5 Animal glue*).

Concludingly, egg white has proven to be one of the most difficult binding media to characterise, but it was only investigated with TMAH in this project. Researchers have started detailed investigations of specific proteins present in egg white pyrolysed without derivatisation (Orsini, Duce and Bonaduce, 2018), so new advances in this field might be developed in the (nearby) future.

4.2.5 Animal glue

Identification of markers in pyrograms of animal glue was reported as particularly difficult by preceding investigators (Lauschke, 2016). It was, therefore, investigated in more detail than the remaining binding media in this project. In the Lauschke project it was analysed with different derivatisation reagents (*1.3.3 Derivatisation*), while, in this project, it analysed both in presence of TMAH and without derivatisation.

The sample pyrolysed in presence of TMAH was specifically difficult to interpret, due to the large number of peaks with varied intensities (Fig. 3.7). None of the high intensity peaks were identified as pyrrole or methylated pyrrole, which was, reported, extensively, as the main marker of animal glue[15] (Chiavari and Galletti, 1992; Stankiewicz *et al.*, 1997; Chiavari *et al.*, 1998; Bonaduce and Andreotti, 2009; Dallongeville *et al.*, 2016; Orsini, Parlanti and Bonaduce, 2017). As the intensity of pyrrole was much lower than anticipated, it was initially overlooked. Even in a pyrrole reference sample (Appendix IV), the pyrrole peak was so tiny,

[15] Interestingly, methyl pyrrole is also formed in non-derivatised samples, although it seems that methyl pyrrole is more abundant than pyrrole in samples run with TMAH, and that pyrrole is more abundant than methyl pyrrole in non-derivatised samples. This assumption is based on the pyrograms reported in literature and on own experiments.

that it was overlooked then as well. Not before pyrrole was analysed without pyrolysis (gas injected), an unambiguous identification of pyrrole was achieved (Appendix IV). The same was true for toluene, which also was detected in low intensities.

Identified peaks, with high intensities, were either methylated benzoic acid or methylated fatty acids (palmitic, oleic and stearic acid). That the fatty acids were so prominent in the pyrolytic profile was surprising, as these compounds were not mentioned in the reviewed literature. Apparently rabbit skin glue, the glue type analysed in this project, has a higher fat content (5%) than other animal glues or gelatines (1%) (Schellmann, 2007).

In the pyrogram of animal glue pyrolysed in presence of TMAH, most of the high-intensity peaks were not identified. Methylated benzoic acid was the only identified high intensity peak, which is not associated directly with lipids and it was only identified in methylated samples. It is not unlikely, that the non-derivatised form is too polar for the gas chromatographic column. This could be clarified by running a reference sample of non-derivatised benzoic acid, but a pyrogram of both methylated and non-derivatised benzoic acid are yet to be added to the reference library (Appendix IV). Methylated benzoic acid was identified through NIST as well as through comparison with a list of fragments found in the literature (Chiavari, Montalbani and Otero, 2008)[16]. Benzoic acid was also present in high intensity in casein pyrolysed in presence of TMAH (Fig. 3.9) and was therefore not specific for animal glue, but seemingly protein-specific.

For animal glue pyrolysed in presence of TMAH, the only other identified compounds were methylated cholesterol and isobarbituric acid. Like methylated

[16] In a study by Chiavari, Montalbani and Otero (2008), benzoic acid is mentioned as a marker for benzoin (a resin) used in varnishes for violins. Resins is a lipid-soluble group of binding medium, and were also used as varnishes and coatings (Colombini and Modugno, 2009). Resins were not specifically investigated in this project, but it is highly unlikely that benzoic acid originates from the resin benzoin in all the six paint samples investigated.

pyrrole and toluene, these were present in low intensities and had, to this author's knowledge, not been reported in pyrolytic profiles of animal glue. Cholesterol has instead been suggested as marker for egg (Chiavari and Prati, 2003).

Isobarbituric acid is worthwhile noticing here, as it showed up in three of the paint samples analysed with TMAH (Fig. AIII-5, AIII-7 and AIII-8). In the yellow sample of Wukero Cherkos (Fig. AIII-8), it was, in fact, the main peak. Taking into account that isobarbituric acid, to this author's knowledge, not previously has been mentioned in the literature, this was an interesting result.

DKPs were, as expected, not detected in the animal glue pyrolysed in presence of TMAH (Fig. 3.7). Still, some of the unidentified compounds, such as aδ3, aδ6 and aδ7, were suspected to be DKP-related compounds, as their fragment patterns contained DKP-related fragments, e.g. *m/z* 70 together with *m/z* 154. These eluted in the pyrographic zone in which diketopiperazines are usually detected (around t_R = 20-40 min).

It was not possible to verify these suspicions, as the reference library (Appendix IV) lacks entries of relevant dipeptides pyrolysed in presence of TMAH and there were no reports of these in the reviewed literature. Focus for the reference library was, initially, other marker compounds, e.g. pyrrole and fatty acids. One dipeptide sample, proline-glycine[17], was pyrolysed in presence of TMAH to identify diagnostic peaks. The resulting pyrolytic profile was peak-rich (Appendix IV) and the main peaks of the methylated dipeptide sample were only present in animal glue (3.7) in very low intensities and only detected using simulated ion monitoring. The low intensities implied that DKPs in methylated samples were easily overlooked and complex and time consuming to interpret.

[17] Proline-glycine was chosen since cyclo(proline-glycine) was the most prominent peak in the pyrogram of animal glue pyrolysed without derivatisation, and this dipeptide was therefore considered the most likely to generate peaks that could be identified in animal glue.

The pyrolytic profile of animal glue without derivatisation (Fig. 3.8) resembled the pyrograms reported by Orsini, Parlanti and Bonaduce (2017). Many of the main peaks were DKPs and DKP-related compounds corresponding to the compounds reported as specific for animal glue: Cyclo(proline-glycine), cyclo(proline-hydroxyproline), cyclo(proline-proline) and diketodipyrrole.

Pyrrole and toluene were identified in this pyrolytic profile as well, but in much lower intensities than reported in literature (Chiavari and Galletti, 1992; Stankiewicz *et al.*, 1997; Orsini, Parlanti and Bonaduce, 2017), where pyrrole and toluene were the main peaks. Still, animal glue pyrolysed without derivatisation gave rise to a diagnostic pyrolytic profile, especially due to the DKPs and DKP-related compounds.

Animal glue was identified in the green sample of Icon21, pyrolysed in presence of and without derivatisation (Figs. 3.10 and 3.11), and suspected in the two samples from Wukero Cherkos, both pyrolysed in presence of TMAH (Figs. AIII-7 and AIII-8).

In the green sample of Icon21, animal glue was identified mainly based on the non-derivatised sample (Fig. 3.11). DKPs and DKP-related compounds, characteristic for animal glue, were found (cyclo(proline-glycine), diketodipyrrole, cyclo(proline-proline) and cyclo(proline-hydroxyproline)). In addition, small aromatic compounds (pyrrole and toluene), and unidentified compounds found in the non-derivatised mock sample of animal glue (aø3 and aø4, Fig. 3.8) were also found, strengthening the identification.

Further evidence of animal glue was apparent in the green sample of Icon21 was pyrolysed in presence of TMAH, as it contained methylated pyrrole, methylated benzoic acid and methylated cholesterol (all three in low intensities) and isobarbituric acid (in moderately low intensity). Interestingly, these markers, associated with proteins, were initially overlooked, as the sample clearly indicated drying oils, due to presence of diacids and fatty acids.

In the samples from Wukero Cherkos (Fig. AIII-7 and AIII-8), animal glue was suspected based on the presence of methylated pyrrole, isobarbituric acid, and methylated benzoic acid. It is noteworthy, that these exact compounds corresponded with the compounds identified in the green sample of Icon21 pyrolysed in presence of TMAH (in that marker category). As the non-derivatised green sample of Icon21 (Fig. 3.11) showed presence of animal glue due to presence of the diagnostic DKPs, this could be true for non-derivatised analyses of the samples of Wukero Cherkos as well.

For further work, it would therefore be interesting to analyse the samples from Wukero Cherkos without derivatisation. If these analyses clearly show presence of animal glue, then the combination of methylated pyrrole, isobarbituric acid and methylated benzoic acid could be clear markers of animal glue in a paint sample. It would also be interesting to see if such old samples would generate diagnostic DKPs in general, as highly degraded samples of proteinaceous material tend to have similar pyrolytic profiles regardless of protein type (Orsini, Parlanti and Bonaduce, 2017).

Another noteworthy point was, that methylated benzoic acid was found in all paint samples pyrolysed in presence of TMAH (Fig. AIII-1-5, AII7 and AIII8, summed up in Table 4.2). The compound was recorded in various intensities, and has, interestingly, not previously been reported as associated with proteins. It was identified in the mock samples of both animal glue and casein pyrolysed in presence of TMAH. Here, it was detected in intensities much closer to the main peak of the pyrolytic profile, unlike the paint samples where it is present in lower intensities, indicating possible degradation over time.

Isobarbituric acid could, potentially, be a strong marker for animal glue, which possibly grow in concentration through aging, in contrast to methylated benzoic acid. In the sample of animal glue pyrolysed in presence of TMAH (Fig. 3.7), the isobarbituric acid peak had a moderately low intensity. For the green sample of

Icon21, a 15th century icon, (Fig. AIII-5 in Appendix III), the intensity was likewise moderately low (although the intensity relative to methylated benzoic acid was larger), and in both samples from Wukero Cherkos, 10th century church, (Fig. AIII-7 and AIII-8 in Appendix III), the peak of isobarbituric acid was either the main peak or close to the main peak.

Methylated benzoic acid and isobarbituric acid could be specifical for pyrograms here at NTNU, possibly related to the pyrolysate formation specifically for Pyrola®2000. This pyrolyser is an uncommon type, which, in some way, might favour the formation of methylated benzoic acid and isobarbituric acid during pyrolysis (*4.3.2 Deviating markers*). Differences in reported markers of proteins is addressed by Bonaduce & Andreotti (2009), who encourage researchers to create a reference library specific for their own laboratory (as mentioned in *1.1.4 Proteinaceous material*).

4.2.6 Casein

Casein and milk are described as the binding medium most difficult to detect by pyrolysis (Chiavari and Prati, 2003). Casein powder was added to the mock sample collection during this project and had not been investigated in the Lauschke project. It is yet to be investigated in detail, as animal glue was the main representative of proteinaceous materials in this thesis.

The casein sample was pyrolysed win presence ofTMAH (Fig. 3.9), and markers associated both with lipids and proteins were identified. The former (in methylated form) resembled the lipids identified in egg yolk: Phosphoric acid (main peak in casein), palmitic acid, oleic acid, stearic acid and cholesterol. The latter were small aromatic compounds overlapping with those found in egg white: Toluene, styrene and indole. The last identified compound, methylated benzoic

acid, was present in animal glue. Therefore, no specific diagnostic peaks were identified for casein.

However, methylated phosphoric acid was present in so high amounts, that the combination of this compound and methylated benzoic acid was suspected to be an indicator of casein.

Polysaccharides, which had been reported as possible markers, were not identified, as it was beyond the scope of this thesis to target yet another class of compounds. However, this could possibly be the key to identifying casein in future experiments.

Of the analysed ancient paint samples, casein was suspected in the larger sample from Mota Giyorgis (Fig. AIII-2), the sample from Icon9 (AIII-3) and the white sample of Wukero Cherkos (AIII-7), based on the simultaneous presence of methylated phosphoric acid and methylated benzoic acid in relatively high intensities. Further investigations are needed to eventually confirm casein in these paint samples. It would be interesting to analyse these samples without derivatisation with the pyrolysis-GC/MS setup at IKJ/NTNU.

4.2.7 Inorganic pigment

Interestingly, certain inorganic pigments such as cinnabar (HgS) and arsenic compounds (e.g. yellow orpiment, As_2S_3) can be identified with analytical pyrolysis, due to characteristic fragment patterns of compounds formed in the pyrolysate (Chiavari and Prati, 2003).

Although not specifically searched for, an inorganic pigment was identified in these experiments. One peak of relatively high abundance, in the non-derivatised green sample of Icon21, had a characteristic pattern (Fig. 4.3), and was identified as As_4, a compound formed when heating arsenic compounds. The main peak, *m/z* 300, corresponds to the molecular weight of As_4, which is followed by losses of *m/z* 75 (As) (Chiavari and Prati, 2003). The fragment pattern recorded for the pyrographic peak in the non-derivatised green sample of Icon21 corresponded with the pattern for As_4 illustrated by Chiavari and Prati (2003).

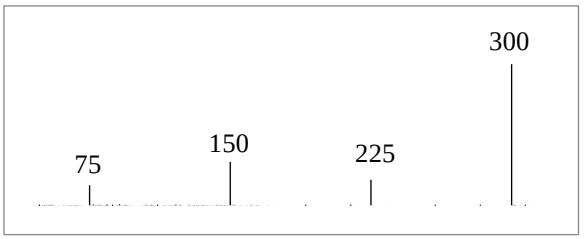


Fig. 4.3: Fragment pattern of As_4 detected in the non-derivatised green sample of Icon21

Part II

Method development and reproducibility

4.3 Reproducibility and method improvements

As the Pyrola®2000 pyrolyser recently had been purchased and was used for the first time in the Lauschke project, experimental difficulties were anticipated. However, the amount was larger than (somewhat naïvely) expected, and the timeframe of this thesis did not allow a desired optimisation. To avoid some challenges related to reproducibility in the future, a systematic investigation of the reproducibility of the pyrolysis-GC/MS setup at IKJ/NTNU would be beneficial to perform.

The additional objective of this thesis (*Chapter 1: Introduction*) was to explore analytical pyrolysis here at NTNU. This required insight into both pyrolysis in general and into the specific design of the Pyrola®2000 filament pyrolyser.

A typical pyrolytic analysis involves (Wampler, 2007b) (*1.2.1 Reproducibility*):

1) Sample preparation and sample handling before pyrolysis
2) Pyrolysate formation during the actual pyrolysis
3) Pyrolysate transfer from the pyrolyzer to the chromatographic system after pyrolysis
4) Analyses of the pyrolysate in the chromatographic system

As the quality of the results is no better than that permitted by any parts of the process, all steps must be considered (Wampler and Levy, 1987; Wampler, 2007b). The implemented adjustments are summed up in Table 2.1 and the adjusted experimental procedure illustrated in Fig. 2.4 (compared to Fig. 2.3). In addition, an overview of some important instrumental parameters in the three consecutive projects (Gebremariam, Lauschke, this thesis) are presented in Appendix V. In Appendix VII, a manual for future operators of the Pyrola®2000 can be found.

Three issues are considered especially significant for future operators: Mock sample preparation, and deviations related to both markers and mass spectra. These are discussed in *4.3.1 Contaminations and sample considerations*, *4.3.2 Deviating markers* and *4.3.3 Deviating mass spectra* respectively.

4.3.1 Contaminations and sample considerations

Regarding sample preparation, four factors are considered important for the reproducibility: Contaminations and sample size, shape and homogeneity (Wampler and Levy, 1987; Wampler, 2007b).

Sample manipulation can cause contaminations and cleaning procedures before and after every analysis was therefore important. Much of the method development was devoted to standardising precautionary steps (Table 2.1): Physical contact with samples was avoided and all equipment in touch with samples were carefully cleaned with soap, demineralised water and acetone before use. After every sample run, the filament and the quarts cell in the pyrolysis chamber were flame cleaned and subsequently an empty filament was analysed ("blank run"), before every new sample run (filament and quarts cell illustrated in Fig. 4.4).

The sample size and shape were continuously optimised. For reference samples, attempts were made to add as little as possible (the tip of a spatula or micro syringe plunger, see Appendix VIII), in order to avoid memory effect (compounds continuously present in pyrograms of following analyses). This often occurred for undiluted sample. Even a touch from the tip of a micro syringe plunger was "too much", resulting in problematic peak intensities far above 10^7 cps. In future analyses, it is thus strongly advised to dilute reference samples to avoid extensive, time-consuming cleaning procedures following the overload of the system. When a pyrogram contained peaks with an intensity above 10^7 cps, both the column and the pyrolysis chamber were conditioned: The GC column at 320°C for 5 minutes and the pyrolysis chamber overnight at 200°C. Before the first following "blank run" (empty filament), the column was conditioned again (320°C for 5 minutes) (see Fig. 2.4).

In addition to the contaminations from "lingering" reference compounds, which were somewhat self-inflicted and avoidable, the pyrolyser and the

chromatographic system produced undesirable peaks as well. The two most prominent peaks appeared around t_R = 28-30 minutes in temperature programme 1 (TP1), originating either from septa or the o-ring in the pyrolyser (Fig. 4.4), or possibly from septa or septa softeners in the inlet of the chromatograph. The compounds were identified as some sorts of "phtalat" and "Teflon", and their intensities greatly increased when a septum or o-ring in the pyrolyser were replaced. Another somewhat unavoidable contamination was the occasional occurrence of "chlorinated tris", which appeared around t_R = 42-44 minutes in TP1. "Chlorinated tris" is present in most dust, and probably appeared when dust from the air adhered to the pyroprobe or "flew" into in the pyrolysis chamber during sample application.

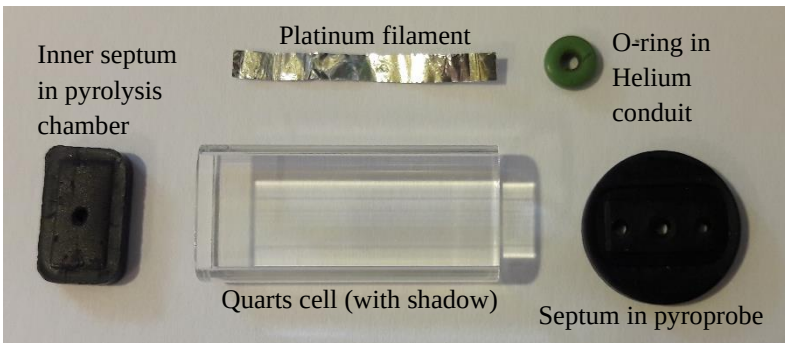


Figure 4.4: "Mischievous" parts in the Pyrola®2000. Quarts cell and filament (and filament holder, not in picture) must be clean before analysis. Septa and O-ring possibly produced interferences.

Sample size, shape and homogeneity should be optimised for a rapid and consistent thermal degradation. A sample of around 10-50 µg is optimal, as it is likely to be in close contact with the heating source (here; the filament) and therefore is experiencing the same temperature at the same time (Wampler and Levy, 1987). In this project, masses were too small to be measured in the laboratory, the solid sample sizes were tentatively described according to their area (in mm^2). Obviously, grains of samples are not two dimensional, but, as samples were preferred to be flat, their third dimension was disregarded.

A film or a slice was prefered. The former is easily obtained with liquids but is difficult with solid paint samples. It has been suggested to melt the sample or to dissolve it in a convenient solvent (Wampler and Levy, 1987; Wampler, 2007b), but this was neither attempted for mock nor paint samples, as the risks of losing any material was considered too high. Shape alterations of samples were, to this author's knowledge, not reported in the reviewed literature.

In this project, sample sizes were measured using millimetre paper, and the preferred sample size were 0.5-2mm^2 for mock samples and 1-2.5mm^2 for paint samples (Fig. 4.5 illustrates preferred sample size of egg yolk where two fragments were selected). Mock samples were, naturally, preferred smaller than paint samples, as these were made from organic material alone.

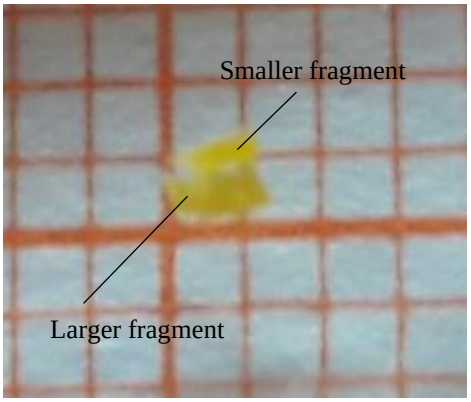


Figure 4.5: Example of a preferred sample size. The mock sample egg yolk. A smaller and a larger fragment were combined and evaluated to be approximately 1 mm^2.

For paint samples, the amount of organic material was evaluated. When samples contained a large grounding layer (Fig. 1.1) or much support material (e.g. inorganic wall material), the fragments, with little grounding/support attached, but still as much paint as possible, were selected. The samples from Wukero Cherkos were in this category (Fig. 2.2). Generally, the larger grounding layer or more support, such as wall material, the thicker the selected fragment was. During application, the aim was to place the samples with the paint "face down" on the filament, to ensure heating of the sample in the most reproducible way.

Mock samples were either rougher mixed size grains (beeswax, linseed oil, egg yolk and egg white) or fine homogenous grains/powders (animal glue and casein) (Fig. 2.2). The sample application of the latter was generally challenging, as they

often adhered to the sample handling tool (spatula) resulting in these being spread over a wide area of the filament. Adding the sample to a more restricted area would be better, for example by using a specialised powder handling tool designed by Pyrol AB, which was recently introduced in the laboratory. Sample application was, however, not the only issue related to powdery samples.

Occasionally, the fine grains were found after analysis as residue in the quarts cell in the pyrolysis chamber and on septa. They had fallen, blown or bounced off the filament during insertion of the probe into the pyrolysis chamber or actual pyrolysis. Consequently, extra cleaning procedures were often needed after powdery samples. This is a known problem related to pyroprobes, and it has been suggested to insert the sample into capillary quarts tubes and placing these in a coiled filament during pyrolysis (Wampler, 2007b, pp. 37–38). Sample application in capillary quarts tubes would not be an optimal solution for a Pyrola®2000, because the "high precision" of this pyrolyser relies on the close contact between the filament and the sample (Tydén-Ericsson, 1973; Wampler and Levy, 1987; Pyrol AB, 2019b).

In this project, a rather heterogenous group of materials was analysed, both regarding the state of matter (solids and liquids) and homogeneity (pure compounds, organic materials, mixtures of organic and inorganic materials). This gave rise to a range of considerations and resulted in optimisation of the sample types (reference, mock and paint samples) individually. Wampler (2007b), however, recommends consistency in sample size and shape for all sample types, which could be useful to keep in mind in future investigations.

To ensure that a sample is representable for the whole, samples should be as homogenous as possible, but few have this "luxury", when selecting a few micrograms of a solid material. This is a "constant problem", and one should be aware of the possible representability issues, both for paint and mock samples (Wampler, 2007b, pp. 44–45).

For mock samples, one must consider how representable they are for the paint, they try to mimic? The mock samples were not exposed to artificial aging, and were, to this author's knowledge, not prepared in accordance to historical recipes. Standardised aging protocols and correct use of "model paint replicas" (i.e. mock samples) have been advocated by Colombini group (Colombini *et al.*, 2010). In recent studies, standardised mock samples ("paint replicas/ reconstructions") recipes and artificial aging protocols were developed (Duce *et al.*, 2012, 2013; Ghezzi *et al.*, 2015; Pellegrini *et al.*, 2016). In these, powders were prepared as paint films before analysis.

For real paint samples, one must consider what the sample fragment actually represents? How large an area of the given colour? How much of the painting? Answering these types of questions requires systematic investigations far beyond the scope of this thesis.

In future work, it might be constructive to follow these standardisations (*1.3.1 Mock samples*) and produce new mock samples. One could also consider contacting the Colombini group as they explicitly encourage inter-laboratory collaborations.

4.3.2 Deviating markers

Some identified markers, considered important in the characterisation of proteinaceous material (toluene, pyrrole, isobarbituric acid and methylated benzoic acid), deviated from markers reported in literature. Inter-laboratory variations in protein markers have been discussed and explained as a result of differences in pyrolysers and instrumental setups (Bonaduce and Andreotti, 2009, pp. 306–308). As formation of markers depends on the physical design of the pyrolyser (Wampler and Levy, 1987), results obtained here that deviate from literature reports could be influenced by the particular design of the Pyrola®2000 pyrolyser, which is different from most other pyrolysers (*1.3.2 The pyrolyser*).

Isobarbituric acid and methylated benzoic acid were selected as potentially important markers (*4.2.5 Animal glue* and *4.2.6 Casein*) but had not been reported in literature. At this point, it can only be speculated why the Pyrola-pyrolyser would produce these compounds while other pyrolysers do not, if the issue in fact is pyrolyser-specific. It would therefore be interesting to investigate whether these compounds are formed in other pyrolysers as well, and if they are formed in mock samples of binding media prepared using the paint reconstruction recipes suggested by Colombini Group (*1.4.1 Sample types* and *4.3.1 Contaminations and sample considerations*)

Pyrrole and toluene, on the other hand, were present in much lower amounts than reported (*4.2.5 Animal Glue*). Pyrrole is often identified as the main peak in the pyrolytic profile of animal glue or other collagen-based material (Chiavari et al., 1993, 1998; Stankiewicz et al., 1997; Bocchini and Traldi, 1998; Bonaduce and Andreotti, 2009; Dallongeville et al., 2016; Orsini, Parlanti and Bonaduce, 2017). The low detection of volatile compounds could be due to the nature of pyroprobes. The heated chamber in pyroprobe pyrolysers, in which the sample is inserted before pyrolysation, “may produce volatization or denaturation, altering the nature of the sample before it is actually degraded” (Wampler, 2007b, p. 40).

However, high intensities of pyrrole have been reported using CDS pyroprobes (Stankiewicz et al., 1997; Chiavari et al., 1998). The chamber and sample heating still differ between the two types of pyroprobes, which could be the cause of lower intensities of volatile compounds in Pyrola compared to CDS pyroprobes.

4.3.3 Deviating mass spectra

Early in this project a difference between the mass spectra reported in the NIST library and those recorded with the pyrolysis-GC/MS setup here at IKJ/NTNU was recognised.

Some markers in linseed oil pyrolysed in presence of TMAH (Fig. 3.1) were suspected to be methylated fatty acids, but did not match the expected mass spectra in NIST (see Appendix X for an example of mass spectra for the same compound, recorded with two different mass analysers).

The mass spectra for methylated fatty acids, recorded for NIST, displayed fragment patterns with *m/z* 74 as base peak, while the mass spectra, recorded in this thesis, displayed fragments patterns containing *m/z* 73 instead (see mass spectra of e.g. methylated palmitic acid and methylated stearic acid in e.g. Fig. 3.1 and 3.4). With the mass analyser used for the NIST-library, *m/z* 73 is specific for non-methylated fatty acids (Gross, 2004, pp. 267–270), and it was, therefore, initially speculated that the fatty acids in linseed oil had not been methylated by TMAH. Consequently, fatty acid references, pyrolysed in presence of TMAH, and methylated fatty acid references were analysed. Also these had mass spectra with *m/z* 73 fragments instead of *m/z* 74 (Appendix IV). The deviation from the NIST library was therefore consistent.

Additionally, the fragment patterns recorded in this thesis were more detailed. Later, other deviations were discovered between those recorded in this project and reported in literature, e.g. for some 2,5-diketopiperazines and alkyl nitriles.

It is a known feature of 3D ion traps, such as the ITQ1100 ion trap (Thermo Scientific), that fragmentation patterns are more detailed and display spectral differences compared to standard quadrupole mass analysers. The NIST library was recorded using the latter, as these are more common (Sparkman, Penton and Kitson, 2011) (*1.2.2 Pyrolysis-GC/MS setup at IKJ/NTNU*).

The differences further enhanced the need for a library specific for the particular pyrolysis-GC/MS setup at IKJ/NTNU and is important to keep in mind when searching for markers using simulated ion monitoring (SIM). This was used to identify e.g. 2,5-diketopiperazines (Fig. 3.8 and 3.11). Although SIM of markers reported in literature was a valuable tool, it is preferable to identify markers through analyses of standard materials.

Chapter 5: Concluding remarks

Sensitivity and trouble are proportional

Primo Levi, 1985

The aim of this thesis was to review and further develop a characterisation method for binding media in paint using pyrolysis-GC/MS established by preceding investigators. As the project advanced, this analytical approach proved to be time-consuming and challenging, due to the introductory nature of the investigations and the sensitivity of the instrumental setup. Focus was, therefore, split between the characterisation process and method adjustments to improve the reproducibility.

Three stages were outlined to evaluate and finetune this method (*Chapter 1: Introduction*). The first was *"to repeat analyses of mock samples of binding media in paint and identify possible markers in these"*, the second *"to analyse paint samples and identify possible markers in these"* and the third *"to investigate the possibility of simultaneously detecting more than one group of organic material in the same sample run"*.

The first and second stages were closely connected, as the markers identified in mock samples had to be identified in paint samples as well, in order to be regarded as valid markers.

Linseed oil/drying oils was detected by presence of azelaic acid, if the ratio between this acid and palmitic acid (the Az/P ratio) was higher than 1. Linseed oil/drying oils were identified in paint samples pyrolysed in presence of TMAH, (but not in non-derivatised samples). Beeswax pyrolysed in presence of TMAH displayed a characteristic overall profile, similar to that described in literature, but identities of specific compounds are yet to be confirmed.

Egg yolk was analysed both with and without TMAH and showed presence of lipid markers in both samples, but the identified compounds (fatty acids, phosphoric acid and cholesterol) were found in other binding media as well.

The pyrograms of the remaining proteinaceous samples (egg white, animal glue and casein) were very complex and difficult to interpret. For egg white, none of the identified markers were specific, as the small aromatic compounds found (toluene, styrene and indole) coincided with those in casein. In addition to the coinciding aromatic compounds, casein showed presence of methylated benzoic acid together with phosphoric acid. These compounds were detected together in paint samples and could possibly indicate presence of casein therein. Non-derivatised samples of egg white and casein were not investigated.

Animal glue was pyrolysed both with and without presence of TMAH. For the former, identification could be based on methylated pyrrole, isobarbituric acid and methylated benzoic acid. For the latter, both small aromatic compounds (mainly pyrrole), and 2,5-diketopiperazines (DKPs) and related compounds (diketodipyrrole, cyclo(proline-glycine) and cyclo(proline-hydroxyproline)) were possible markers.

Isobarbituric acid and methylated benzoic acid have not been reported as markers of proteinaceous materials. In animal glue, isobarbituric acid was detected together with pyrrole, and these two compounds were found together in paint samples as well. This finding indicates isobarbituric acid as a possible marker for animal glue, when samples are pyrolysed in presence of TMAH. Methylated

benzoic acid was found in animal glue, casein and all six paint samples. As these two compounds have not been reported previously, it would be interesting to investigate their origin in such samples further.

The pursuit of *"simultaneously detecting more than one group of organic material in the same sample run"* (the third stage of this project), had been a particular focus for the scientific community. In retrospect, this aim, together with others, proved to somewhat naïve given the time frame of a master thesis. Lipid-based materials were clearly identified in samples pyrolysed in presence of TMAH but not in those run without. For proteinaceous materials it was to some extent the opposite. Without TMAH, interpretation of the pyrolytic profile was possible despite the complexity, but with TMAH, it became too challenging.

On a final note, it is interesting that derivatisation with TMAH accentuate lipid markers, whilst making proteinaceous markers less pronounced and, therefore, easily overlooked. The proteinaceous markers, especially DKPs and related compounds, which are currently investigated thoroughly in the scientific community, are more prominent when pyrolysed without derivatisation. When comparing pyrograms from the same paint sample, Icon21, pyrolysed both with and without TMAH, it is apparent that the two approaches complement each other. The complementary nature of these approaches has not previously been reported, and would therefore be interesting to explore further, to see if it can aid the pursuit of simultaneous detection in the future.

5.1 Further work

This projects could be continued in various directions, such as

- To produce mock samples (paint reconstructions) using the recipes of the heritage science group in Pisa (Colombini group) aiming to further standardise production and possibly artificial aging of these. One could consider contacting the group, as they specifically encourage inter-laboratory collaborations.

- To analyse the mock samples (animal glue in particular) and paint samples with another pyrolyser to investigate possible differences in presence of e.g. pyrrole, toluene, isobarbituric acid and methylated benzoic acid (deviating markers).

- To analyse the white and yellow paint sample from Wukero Cherkos without derivatisation to investigate proteinaceous markers in these, as animal glue was suspected due to strong presence of isobarbituric acid but not confidently confirmed.

- To further develop the reference library by adding e.g. non-derivatised 2,5-diketopiperazines/dipeptides, isobarbituric acid, methylated benzoic acid and the new marker types investigated by Orsini, Duce and Bonaduce (2018).

- To systematically investigate the reproducibility by e.g. investigating standard deviations of specific marker peaks and optimising sample size and application.

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