Analyses of volatile organic compounds in archaeological artifacts.

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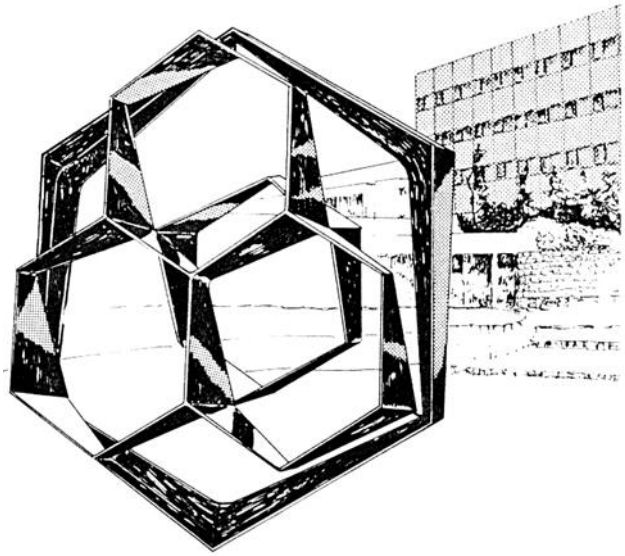

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Abbreviations

F_{crit}: Fisher ratio cut-off

F-ratio: Fisher ratio

GC: Gas chromatography

GC×GC: Comprehensive two-dimensional gas chromatography

GC×GC-MS: Comprehensive two-dimensional gas chromatography coupled mass spectrometry

GC×GC-TOFMS: Comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry

HCA: Hierarchical cluster analysis

HRTOF-MS: High resolution time-of-flight mass spectrometry

HS-SPME: Headspace solid-phase microextraction

MS: Mass spectrometry

P_M: Modulation period

PCA: Principal component analysis

TOFMS: Time-of-flight mass spectrometry

VOCs: Volatile organic compounds

Chapter 1: Introduction

During life cycle, most of the organic materials undergo natural biochemical decay processes leaving no evidence of their existence. However, some residues can be preserved for thousand years [1]. The analysis of those residues, their conservation and fossilization processes are essential to understand life in ancient times. Several types of organic residues have been recovered from archaeological objects, one of the most frequent have been adhesives [2, 3]. Adhesives are natural substances prepared from resins, gums, waxes or tars. Their ubiquitous role in ancient times as glues, coating, protecting and sealing agents explains their presence in numerous findings on archaeological sites [4]. It has been proven that they have been used for hafting tools, repairing ceramic vessels and in burial rituals [2, 3, 5, 6].

These organic residues have been widely studied using gas chromatography coupled to mass spectrometry (GC-MS) [7, 8, 9]. Often, sample preparation techniques such as extraction, purification and derivatization of the samples were performed to enhance detection and to increase the volatility of analytes. In this work, a different approach was followed. In order to preserve the sample if further analysis is required, the organic residues were directly studied by the non-destructive headspace solid-phase microextraction (HS-SPME) comprehensive two-dimensional gas chromatography (GC×GC) coupled to high resolution time-of-flight mass spectrometry (HRTOFMS) technique. In addition to allow the analysis of minimal amount of organic residues, this method minimized sample preparation.

In this work, archaeological residues recovered from the Royal Tomb in Qatna, Syria, were analyzed to confirm previous hypothesis regarding their nature i.e. bones, textiles, wood or soft tissues. In addition, their different states of preservation were investigated. Such analysis of organic residues requires knowledge of the degradation pathway involved. Therefore, the analysis of natural non-treated and heated substances, comprising resins, waxes, tars and glues, was investigated to get insight into thermally induced degradation. A comparison of the two sets of samples will reveal the different states of preservation of the samples recovered in Qatna. Moreover, the presence of Royal purple dye within the textiles was investigated. Since previous analyses have proved the presence of Royal Purple dyestuff, it was expected to identify specific analytes of this dyestuff within the textile remains.

1.1 Adhesives

In ancient times, adhesives were produced by the mixture of a wide range of natural substances. These complex molecular mixtures have already been produced in prehistoric periods, 45 000 years ago [8]. In archaeological studies, one objective was to investigate the origin and constitution of resins in order to understand the way those adhesives were produced. Therefore, analytical studies mainly focused on the chemical identification of the natural substances used in adhesives fabrication [2]. Analytical studies of adhesives by GC-MS contributed to the establishment of fingerprints and molecular biomarkers of several substances. The principal goal of these analyses being to make relationship between the molecular composition and the natural substances characterizing adhesives, in order to identify the different type of adhesives based on the materials used to create them [2, 3, 4, 8].

Among natural materials used to produce adhesives, the most commonly used were resins, waxes and tars. These substances are described in the following section.

1.1.1 Natural plant resins

Through times, resins have been used in multiple contexts in the lives of humans which make them remarkable and interesting substances. Resins can be defined as a mixture of volatile and non-volatile terpenoid or phenolic compounds that are secreted in specialized structures of the plant [10]. This definition allows the distinction between resins and related substances such as gums, waxes, oils and fats. It has to be emphasized that fossilized resins such as amber also played an essential role as natural dyes in ancient times [11, 12].

The synthesis of plant resins is based on the breakdown of carbohydrates, produced by photosynthesis, into simpler molecules. Different metabolic pathways are known yielding in a wide range of terpenoids and phenolic compounds. Terpenoid resins are generally produced by all kinds of plants while phenolic resins are produced by flowering plants [10]. In the majority of cases, terpenoid based resins have been used. However, some phenolic resins were also found to be important [10].

1.1.1.1 Terpenoid resins

As previously said, terpenoids constitute the largest class of plant compounds. More to 30.000 structures of terpenoids have been highlighted thanks to the development of chemical technology such as GC-MS. Although they present huge structural diversity, they can be

grouped in different categories by the association of isoprene (C_5H_8) structural elements (Figure 1).

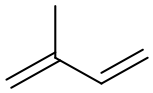

***Figure 1**: Molecular structure of an isoprene unit (C_5H_8)* [10].

From chemical reactions, isoprene units are connected together to form different precursors. Terpenoid resins are made of both volatile and non-volatile compounds. The combination of two or three isoprene units, leads respectively to the formation of monoterpenes (C_{10} compounds) and sesquiterpenes (C_{15} compounds) which constitute the volatile fraction of the resin [10]. In addition, GC-MS analysis highlighted the presence of oxygenated mono- and sesquiterpenes as well as diterpenes hydrocarbons within this volatile fraction of resins [12].

The non-volatile fraction of the terpenoid resin is characterized by diterpenes (C_{20} compounds) and triterpenes (C_{30} compounds) which can be functionalized by alcohols, aldehydes and/or esters [10]. Moreover, the composition of the resin determines its viscosity. A high concentration of di- and triterpenes will contribute to a more viscous resin. Therefore, the mixture of compounds constituting a resin is important for human use. Usually, 50 or more compounds are encountered in natural resins and only a few occur in high concentration in each kind of resin [12].

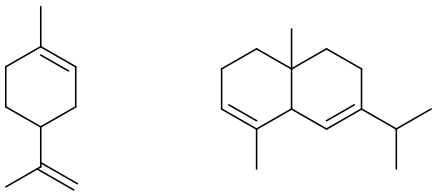

***Figure 2**: Molecular structure of (a) limonene (monoterpene) and (b) δ-selinene (sesquiterpenes)* [10].

1.1.1.2 Phenolic resins

Phenolic compounds are another diverse chemical class and are commonly found in plant products. In plants, they have numerous functions such as structural support, pigmentation of flowers, protection of the plant against antioxidants and signaling agents against microbes [10, 12]. These compounds are usually substituted by hydroxyl groups which are themselves substituted to form esters, ethers or glycosylic derivatives [10].

Many components of phenolic resin are synthesized by the enzymatic conversion of phenylalanine into cinnamic acid. Cinnamic acid, benzoic acid and benzaldehyde occur in the phenolic resins that are produced by several different plants such as *Liquidambar*, an American deciduous tree, *Dracaena*, an African tropical tree or *Myroxylon*, a leguminous tree [10].

1.1.2 Waxes

Since ancient times waxes were used for diverse purposes. Besides being used as adhesive, waxes were used as symbolic, sealing or lightening agents and for medicinal purposes [8]. The first waxy materials used by men were beeswax produced by honey bees. However, waxy materials also occur in plants where they play a main role as protecting agent to resist pests attack or dehydration [2].

The common chemical composition of waxes, even if they are made of complex molecular mixtures, is characterized by alcoholic esters, fatty acids and straight chain alkanes. Long-chain ketones and aldehydes were found is waxes as minor components [10].

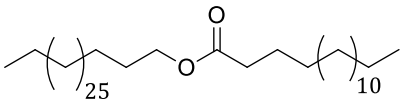

***Figure 3**: Molecular structure of alcoholic acid ($C_{45}H_{92}O_2$) prevalent in beeswaxes* [13].

1.1.3 Tars

Tars are black oily mixtures that have been used for several purposes such as adhesives [2], handle fixatives [14], hydro-repellents [3] or coating [9] in ancient times. They were naturally obtained by distillation of numerous organic materials and more particularly of coniferous trees.

Among the numerous tars, birch bark tar is produced by a controlled heating of the bark of the birch conifer. This tar has been commonly used as adhesive since the Paleolithic period [9]. It was used to seal, repair or coat ceramic vessels, to haft lithic tools, and also for medicinal purposes [15].

***Figure 4**: Birch bark tar made from the bark of the birch tree* [16].

Birch bark tar is characterized by triterpenoid markers presenting a lupane skeleton such as betulin and lupeol. Low amounts of lupenone, betulone and betulinic acid were determined to be markers of birch bark tar [15]. Suberin, a lipid material, and suberan, an aliphatic macromolecule, are also considered to be important components of birch bark tar. Using pyrolytic condition and sample derivatization, suberin produces monocarboxylic fatty acids, while the suberan gives rise to straight chain alkenes and alkanes [9].

1.2 The use of adhesives

Rare knowledges, such as the culture of prehistoric men, can be provided by the analysis of archaeological artifacts and their residues. For this reason, it is also important to develop non-destructive or non-invasive analytical techniques to study the recovered residues on archaeological artifact [17].

Regarding this work, two applications were of great interest: the use of resins as adhesives for tool hafting and in context of burial rituals.

1.2.1 Tool hafting

Hafting describes the process of attaching an artifact e.g. a stone to a handle, increasing their efficiency. To do so, bindings or adhesives can be used depending on the expected function of the tool. Bindings are more resistant to shocks than adhesives but less adapted for the hafting of knives [5]. This process points out the planned manufacture of tools and can be seen as a milestone in the history of technology.

***Figure 5**: Tool hafting using (a) adhesives and (b) bindings* [18].

Numerous hafting adhesives, among which birch bark tar and conifer resins are well-known, have been used as fixating agents worldwide since the Stone Age [14]. They were recovered on various tools, such as points, knives, hunting and/or domestic tools [12, 16]. Analysis of these residues can be used to (i) identify the origin of the adhesive, (ii) its preparation and (iii) to investigate the specific usage of the tool [17]. Therefore, the study of hafting adhesives and modifications of tools can improve our understanding of past human behavior [19].

Although small quantities of adhesives are usually recovered because of their degradation, radiocarbon dating was successfully performed [14, 20]. The oldest hafting fixatives found on archaeological artifacts, in Europe, was birch bark tar dating back to Middle Paleolithic period, between 70 000 and 35 000 years BC [12, 17]. The identification of such residues discovered in archaeological context can be challenging due to the very low amount available for analysis. Visual or even microscopic distinction between the different kinds of organic residues may not be possible. For this reason, it has been proven by K. Perrault et al. that HS-SPME-GC×GC-TOFMS is a valuable tool since it enables a total screening of unknown residues and it provides chemical information about sample types [22].

1.2.2 Funerary rituals

The reconstruction of funerary rituals has been of great interest to gain insight in ancestral cults. Burials consist of different activities presenting a specific function and taking part in the rites of passage [23]. Three different phases can be distinguished: a separation phase, a

transformation phase and an incorporation phase [23]. One task of archaeology is to define which activity is linked to which phase and to gain insight within the different steps of the rites of passage.

Adhesives found in funeral context can provide understandings in these ritual behaviors [20, 21]. For example, ceramic or stone vessels have usually contained a variety of products such as ointments, perfumes or incense composed of volatile compounds in combination with resinous materials. It has been suggested that these products have been used to treat the bodies in context of funeral rituals and avoid cadaveric odor [19, 22]. Therefore, residues analysis could provide information on the specific preparation during burial ritual [26].

1.3 Degradation pathway of adhesives

The identification of natural resins is often challenging due to the fact that their chemical composition can be significantly altered through time by degradation processes. In most cases, altered resins are recovered from archaeological sites due to oxidation or heat treatment. Numerous environmental factors can influence organic residues conservation such as temperature, light exposition, oxidation or reduction conditions and humidity [27]. Therefore, the analysis of such residues recovered from archaeological artifacts requires knowledge of the degradation pathways involved. For example, the modified structure of heated terpenoid structures found in resins can be related to the original non-modified biomarker. For this reason, non-heated and heated resins were analyzed in this work and compared to the organic residues recovered from the archaeological site of Qatna.

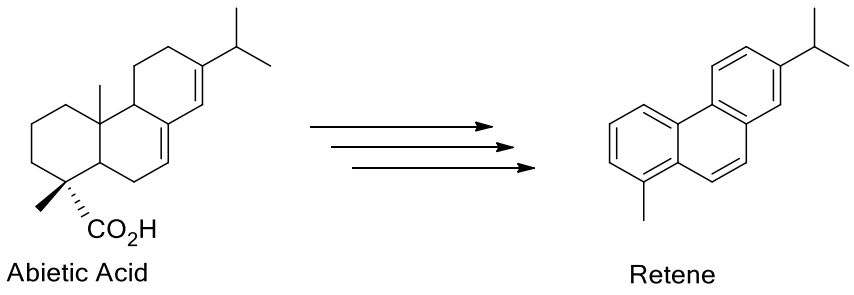

***Figure 6**: Terpenoid biomarkers produced by heating treatment of natural resin* [27].

The discovery of highly preserved organic residues indicates that they were either protected or that their molecular structures are resistant in some way to degradation. Organic residues can be protected from microbiological degradation in porous environment such as mineral matrices. Moreover, more polar substances are generally more inclined to biological decay

[27]. For example, the discovery of perfectly preserved dyes onto textiles illustrates the high stability of some characteristic compounds of dyes such as indigo and indiburin, in comparison to the high decomposition of textiles which were retrieved as mineralized materials [27]. The analysis of degraded biomarkers will account for differential preservation states and environments providing additional information about the history of the residue [23, 24].

1.4 The Royal Tomb of Qatna

During the new excavations of the Royal Palace of Qatna, Syria, in 2002, archaeologists found the perfectly preserved Royal Tomb. The Royal Palace was built during the Middle Bronze Age between 1650 and 1550 BC and was destroyed in 1340 BC by the Hittites [19, 25]. The Royal Tomb was located deep below the foundation of the Palace, directly underneath the courtyard, in the north-eastern quarter. The courtyard was the main residential unit of the Royal Palace reserved for the kings. Because of its symbolic location, the grave was interpreted as a ‘palace of the dead kings’ based on the model of the ‘palace of the living kings’ [29].

The tomb was composed of four grave chambers formed into the rock and an ante-chamber. The access to the Royal Tomb was a long corridor allowing direct access to the ante-chamber from the center of the Royal Palace. The 40 m long stairs that constituted the corridor led to three different doors which could have been carefully locked before climbing down to the ante-chamber. The access to the ante-chamber was only possible by means of wooden ladders which could have been removed when necessary. All these installations proved that the Royal Tomb was hidden and thus highly protected. The entrance to the main chamber of the tomb was surrounded by two ancestor statues and three smaller side chambers encircled this latter in a trefoil layout [29].

***Figure 7**: The Royal Tomb of Qatna* [29].

The destruction of the Palace in 1340 BC left out a perfectly sealed Royal Tomb leaving numerous undamaged artifacts found at the presumed location of their last use. The inventory and analysis of more than 2000 objects within the grave was interpreted as an assemblage of different burial activities and cults of the dead. It is assumed that the burial of a member of the Royal Family was accompanied by long, multi-stage rituals. Living and dead members of the sovereignty were reunited for sharing a last meal [23].

1.5 Chromatography in archaeology

Numerous analytical techniques have been used in archaeological chemistry to identify the nature and origin of organic residues. Among them, the chromatographic techniques have provided insight into detailed chemical characterization. Organic residues are typically made of complex mixtures requiring the separation into individual chemical components for identification. This is the main reason why, during the past decades, GC has been the most widely used, in particular coupled to MS. GC-MS is a powerful technique for the analysis of the numerous biomolecular components of such residues. This technique permits the separation and structural identification of each component, even for minimal amount of residues analyzed [30]. These biomolecular components, called archaeological biomarkers, are used to relate the structure or the chemical fingerprint to the original materials and mixtures known to have been utilized in the past. For example, resins can be distinguished from beeswax based on their chemical composition [27].

To date, the use of GC-MS in archaeology is mainly for the analysis of high molecular weight compounds such as fatty acids. Therefore, derivatization was done to increase the volatility of such polar compounds. Methylation, silylation and hydrogenation are three different methods of derivatization in which polar functional group are replaced by less polar analogous. However, extraction prior to derivatization is very critical for archaeological samples. In numerous cases, the artifact on which organic residues are recovered should be preserved for further analysis but derivatization would alter the samples. An alternative is to look only at the volatile organic compounds; hence avoiding the extraction step. Headspace solid-phase microextraction (HS-SPME) is an efficient sampling method which can be used in a solvent-free manner permitting the analysis of the volatile components without sample alteration. Although HS-SPME, without any derivatization, has not been widely utilized for archaeological analysis, this technique has drawn some attention since the last decades [20, 26, 27].

So far, GC-MS analyses of organic residues reported focused on qualitative studies. Archaeologists are mostly interested in the identification of the residues recovered and their origin. Nevertheless, quantitative analyses are possible using internal standards directly added within the sample. Information on the amount of the different compounds used within the residues could thus be obtained [27].

Although GC-MS methods are widely used in archaeology, chromatograms provided by such analysis are often highly complex. Components can overlap and therefore, their distinction can be challenging. Recent studies have demonstrated that the use of GC×GC-TOFMS provided a higher separation, resolution and sensitivity regarding organic residues analysis recovered from archaeological artifacts [22].

Chapter 2: Materials and methods

This chapter presents the different instruments and methods used in this work. Experimental parameters, sample preparation and data treatments will also be explained.

2.1 One dimensional gas chromatography

Pioneered by J.P. Martin and R.L.M Synge, GC is a powerful separation technique for volatile and semi-volatile compounds [32]. Since the introduction of capillary columns by Golay, the potential of GC was proved in many studies for qualitative and quantitative analysis. A GC system is composed of an injector, a column mounted in an oven and a detector (Figure 8). Samples are injected onto the column by means of an inert gas stream [33].

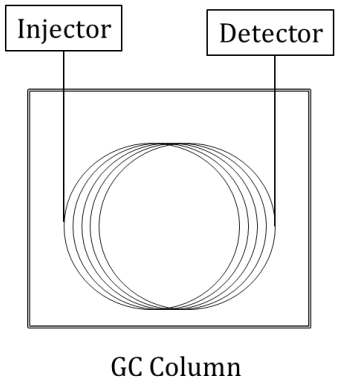

***Figure 8**: Schematic GC set-up* [33].

The separation of the components is based on the differences in volatility between analytes. According to their differences of vapor pressure, analytes will elute at different times during the GC analysis. However, in more complex sample mixtures the vapor pressure of the analytes may not differ sufficiently to be chromatographically separated. The resolution of such co-eluting components would require the application of an additional separation criterion [33]. To increase the chromatographic separation resolution of complex samples such as petrochemicals, pesticides, dioxins, food, polychlorinated biphenyls (PCBs) or perfume, multidimensional GC methods were developed [34].

2.2 Comprehensive two-dimensional gas chromatography

Comprehensive two-dimensional gas chromatography technique, commonly named GC×GC, was developed by Professor John B. Phillips and his student Z. Liu in the early 1990s. The

improved resolving power of multidimensional technique is based on its several dimensions of separation [35].

In 1990, Giddings established two principles to have a comprehensive separation in a GC×GC system [33]:

1) The entire sample components must be subjected to, at least, two different and independent dimensions of separation.
2) The resolution achieved in the first dimension has to be maintained in the second dimension and has to be preserve during the entire separation process.

The first Gidding's principle introduces the concept of orthogonality. This concept refers to the association of two GC columns composed of different stationary phases and thus presenting different selectivities. Traditionally, a non-polar column is used in the first dimension and a more polar column is used in the second dimension [33].

Accordingly, a GC×GC system (Figure 9) is similar to a 1D GC system but is composed of two different capillary GC columns connected by a modulator.

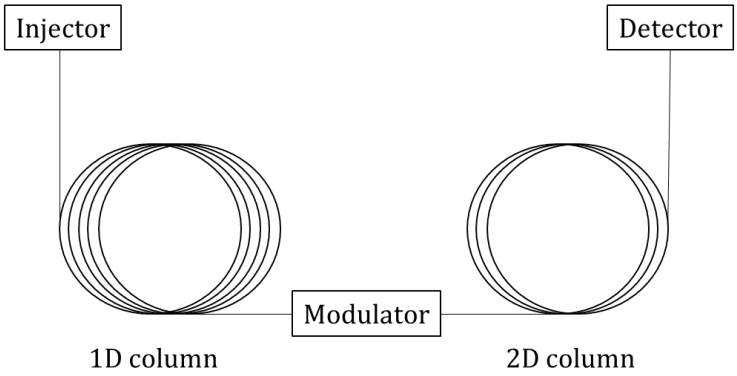

***Figure 9**: Schematic GC×GC set-up* [33].

The two capillary GC columns made of different stationary phases enable the separation of analytes that would co-elute in 1D GC and thus increases the number of theoretical resolvable peaks. Therefore, two solutes co-eluting in the first separation could be separated in the second dimension depending on the interactions encountered with the second stationary phase. Consequently, GC×GC will offer a significant enhancement of peak capacity over traditional 1D GC [31, 34].

The modulator plays a central role in this separation process. Small fractions of the effluent from the first dimension column are trapped and focused for a fixed period of time, called the modulation period (P_M), before being injected onto the second dimension column [22]. In order to preserve the sharp peaks produced by the modulator and achieve complete separation, the second dimension analysis is performed in fast GC conditions. Hence, the use of shorter column, typically 0.5-2 m long, in the second dimension satisfies the high-speed requirement [36].

GC×GC requires detectors with fast acquisition times. The high-speed condition of the second dimension column produces very narrow peaks (50 to 600 ms). Since the proper reconstruction of a chromatographic peak necessitates at least 6 acquisition points, an ideal detector should offer response times in the range of 20 - 100 Hz [33].

The resulting chromatogram can be visualized in a two dimensional (2D) or in a three dimensional (3D) plot. In both cases, the first and the second-dimension retention time (^1t_R, ^2t_R) are displayed in the x and y-axis, respectively. In 3D chromatograms, the intensity of the peaks is represented in the z-axis, while in 2D chromatograms the intensity is represented by colors which can be seen in the contour plot in Figure 10 [36].

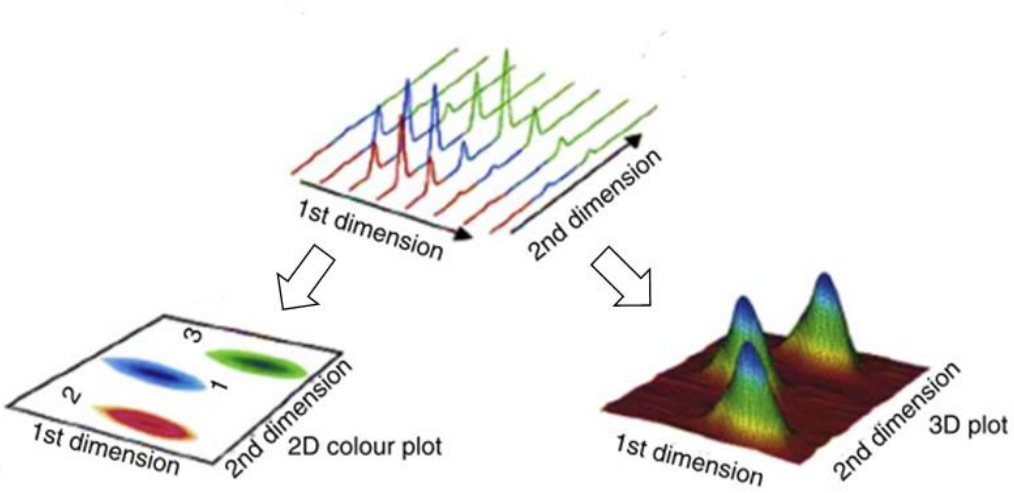

***Figure 10**: Different ways of GC×GC chromatogram visualization* [33].

2.3 Sampling method

In this work, the sampling method used was HS-SPME. Practically, a coated fiber is exposed to the headspace of the sample for a fixed period of time [33, 34]. The headspace is the gas phase which is in equilibrium with the sample. Then, the analytes are extracted and concentrated to the fiber coating and transferred to the GC injector [38]. The desorption time in the injector depends on the type of the sample. In addition of saving preparation time, this method improves minimum detection limits and preserves resolution [38].

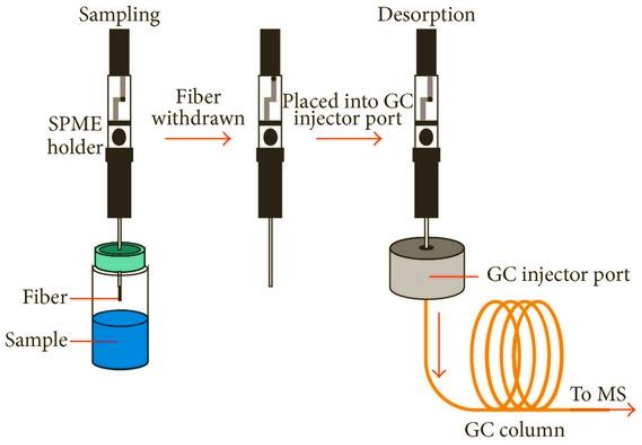

***Figure 11**: Headspace Solid-Phase Microextraction GC-MS* [39].

2.4 Injectors

As for 1D GC, there are several types of injectors and inlets available and several ways of injection. For the analysis of volatile organic compounds (VOCs) by HS-SPME sampling, a split/splitless injector is the most suitable. As there was no solvent injected onto the column, the injection was done in splitless mode. Moreover, the analysis is very sensitive because splitless injection is adapted for trace level analysis.

2.5 Column

To fulfil Giddings's orthogonality rule, a GC×GC system use two different and independent separation mechanisms. Usually, the first column has a non-polar stationary phase while the second column has a polar stationary phase [33]. This arrangement corresponds to a normal orthogonality. However, the opposite configuration can also be used, referring to a reverse orthogonality. In the first case, analytes are separated according to their vapor pressures thanks to a constant increase in temperature in the first dimension. Then, the separation in the

second dimension is based on the interaction between the analytes and the stationary phase of the second column. Consequently, this separation is carried out in isothermal conditions to satisfy the orthogonality principle [33].

The secondary column can be placed in a separate oven integrated into the main GC oven allowing independent temperature programs for the second dimension separation. The temperature of the secondary oven is usually a few degrees above the temperature of the main GC oven, preventing the influence of the vapor pressure.

2.6 Modulator

The fundamental function of modulators for an efficient GC×GC separation has increased their development in the last years. Several types of modulators, which can be classified in two main categories, are commercially available: valve-based and thermal modulators. Although their method of proceeding is different, valve-based and thermal modulators role is the same and is to trap, focus and inject small amount of eluent from the 1D column to the 2D column [29, 32].

The most commonly used are the thermal modulators. Since their introduction in 1991, they have been improved to finally reach the dual-stage quad-jet modulator. Their essential focusing effect improves the sensitivity and produces sharp peaks, which increases significantly the peak capacity [38, 39].

In this work, the LECO Pegasus 4D HRT used is equipped by a cryogenic dual-stage quad-jet modulator. Practically, those modulators consist of two cold jets and two hot jets creating two distinct trapping zones (Figure 11). The cold jet uses dry nitrogen (N_2) chilled with liquid nitrogen for cryo-focusing, while the hot jet is operated with air heated to high temperatures. Firstly, analytes are trapped at the end of the first dimension by a N_2 cold jet. Then a hot jet is activated to release these analytes to the second activated cold jet. This is the refocusing stage. Finally, the analytes are released into the secondary column by means of a second hot jet while another effluent of analytes is trapped at the end of the first column. This cycle is repeated during the whole chromatographic analysis.

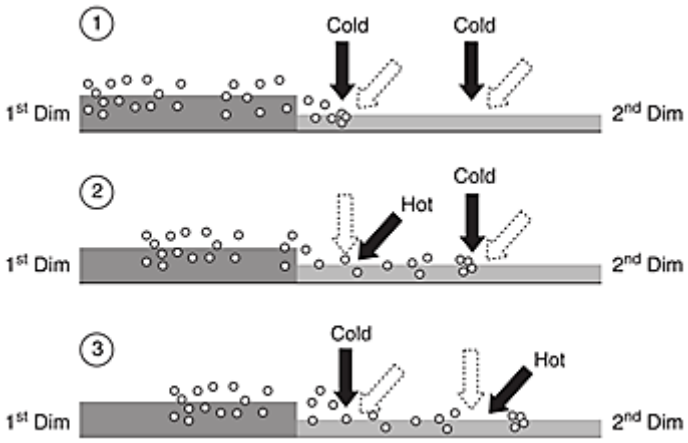

***Figure 12**: Theoretical principle of a dual-stage quad-jet modulator* [33].

Peaks eluting from the modulator should not be wider than the quarter of the 1D peak width. Therefore, an optimized P_M should be in the range of 2-8 s [33].

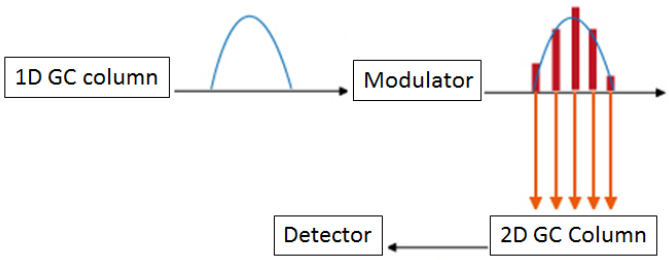

***Figure 13**: Principle of peak modulation of GC×GC* [40].

2.7 Detectors

The detector is another important component of a GC×GC system. The type of detector will depend on the analysis required but has to offer in each case a high sampling rate.

As explained in the section 2.2, peaks eluted from the second dimension column are very narrow (50 to 600 ms). Therefore, the detectors should have fast acquisition rates to guarantee the exhaustive reconstruction of the second dimension chromatogram.

In this work, a time-of-flight mass spectrometer (TOF-MS) was used as detector because it provides a high acquisition rate up to 500 spectra per second enabling the complete reconstruction of chromatograms [33]. MS adds a new dimension of separation, based on the mass spectra, therefore increasing the identification power of GC×GC. Structural information can be obtained while comparing mass spectra of produced fragments to NIST and Wiley libraries.

Moreover, this detector collects a full mass spectrum during each acquisition in contrast with quadrupole detectors that scan the different mass-to-charge ratios during signal acquisition. Thanks to the simultaneous detection of all ions, TOF-MS analyzers enable the analysis of a broad mass range and provide an enhanced sensitivity [33].

2.8 Sample treatments

Two different kinds of samples were analyzed. First, archaeological samples from the Royal Tomb of Qatna, in Syria, were provided (Table 1). Bones, textiles, soft tissues, wood samples and organic sediments were collected from different chambers within the Royal Tomb, placed into SPME vials and analyzed by HS-SPME-GC×GC-TOFMS.

Table 1*: Types, location and codes given to the samples recovered from the Royal Tomb in Qatna.*

Sample type	Location	Samples codes
Bones	Chamber 1	Q24, Q25, Q26, Q27
	Chamber 3	Q19, Q23
	Chamber 4	Q01, Q12
	Chamber 4 in sarcophagus	Q14, Q15, Q16, Q18
Textiles	Chamber 1	Q29, Q30
	Chamber 4	Q05, Q07, Q08, Q09, Q13, Q31, Q32
	Chamber 4 in sarcophagus	Q17
Soft tissues	Chamber 4	Q11, Q20, Q21
Wood	Chamber 4	Q02, Q03, Q04, Q06, Q10
Organic sediments	Chamber 3	Q22
	Chamber 4	Q28

Another set of samples was provided by Traceolab, University of Liège, and studied by the same technique (Table 2). Several types of natural resins were collected and analyzed as non-degraded raw materials. Moreover, resins were placed onto flint and burned for different times at different temperatures. Each kind of resins was placed onto both faces of the flint. Then, the flint was place horizontally in the ground 10 cm under the fire. The samples were heated at 144 °C for 5 h, at 340 °C for 8 h and finally at 144 °C and at 340 °C for 13 h. After the combustion, resins which were directed towards the ground were analyzed. Resins were extracted from the flint with ethanol and placed into sealed vials with magnetic caps.

***Table 2**: Types, codes and heating treatments of the samples received from Traceolab laboratory.*

	Sample name	**Sample code**	**Heating treatment**		
			144 °C for 5h	340 °C for 8h	340 °C for 13h
References of resins	Pinus Sylvestris	5.01	/	/	/
	Picea	5.02	/	/	/
	Fabaceae 'Birbira'	5.03	/	/	/
	Juniper berry tree	5.04	/	/	/
	Euphorbia	5.05	/	/	/
Reference of protein glue	Tendons glue	5.06	/	/	/
	Deer glue	5.08	/	/	/
	Sheep glue	5.09	/	/	/
Combusted samples	Resin + wax		5.10	5.11	5.12
	Resin + wax + ochre		5.13	5.14	5.15
	Birch bark tar		5.16	5.17	5.18
	Tendon glue		5.19	5.20	5.21
	Pork		5.22	5.23	5.24
Archaeological samples	ADHES158	5.25	/	/	/
	Star car 91234	5.26	/	/	/
	Star car 89634	5.27	/	/	/
	Bushman	5.28	/	/	/

2.9 Experimental section

2.9.1 SPME extraction

The samples were placed into sealed vials with magnetic caps. Each vial was incubated at 40 °C for 10 min. Then, the sample vials were extracted for 15 min using a 50/30 µm Stableflex 24 Ga SPME fiber coated with a mixture of divinylbenzene, carboxen and polydimethylsiloxane (DVB/CAR/PDMS) (Supelco®, Bellefonte, PA, USA). The fiber was exposed to the headspace of each sample with a penetration depth of 21.00 mm. Finally, desorption of the fiber was performed in splitless mode for 180 s at 250 °C with a penetration depth of 54.00 mm.

2.9.2 GC×GC-HRTOFMS analysis

All samples were analyzed using a Pegasus 4D HRT (LECO® Corporation, St. Joseph, MI, USA), a gas chromatography system coupled with a high resolution time-of-flight mass spectrometer, equipped with a secondary oven and a quad-jet dual-stage thermal modulator. A P_M of 4 s was used with a 0.60 s hot pulse time.

The temperature of the main oven was increased from 35 °C to 240 °C at a rate of 5 °C/min. The secondary oven temperature offset was +5 °C in relation to the ^{1}D GC oven temperature and the modulator temperature offset was +15 °C relative to the ^{2}D oven. The set of column used was a mid-polar Rxi-624Sil MS (Restek® Corporation) and a polar Stabilwax (Restek® Corporation) for the first and the second dimension columns, respectively (Table 3). The ^{1}D column is composed of 6% cyanopropylphenyl and 94% dimethyl polysiloxane, while the ^{2}D column is composed of polyethylene glycol. High purity helium (Air Liquide®, Liège, Belgium) was used as carrier gas with a constant flow rate of 1.00 mL/min (throughout the run).

***Table 3**: Physical characteristics of utilized stationary phases.*

Stationary phase	Dimension	Length [m]	Internal diameter [mm]	Film thickness [µm]
Rxi-624Sil MS	1	30.00	0.25	1.40
Stabilwax	2	2.00	0.25	0.50

Acquisition was controlled using LECO ChromaTOF-HRT software version 4.2.3.1. An acquisition delay of 300 s was used. The mass acquisition range was 29–450 *m/z* with an acquisition rate of 200 Hz and a 2 kHz extraction frequency. The ion source temperature was 250 °C. The ionization energy was 70 eV and an emission current of 1.0 mA was used.

2.10 Data treatment

Data were collected through ChromaTOF version 4.2.3.1 software and were analyzed using GC image (Zoex Corporation) software. The ChromaTOF software was used for acquisition, reporting and exporting data. The exported data were then treated in GC image. This software was used for the analysis of the obtained chromatograms by providing baseline correction, alignment and blob detection. This pixel-based approach enables point-by-point analysis which can be used for further chromatographic comparisons and statistical analyses such as

principal components analysis (PCA) and hierarchical cluster analysis (HCA) [42]. This software product possesses efficient tools, presented in section 2.11, to extract more easily useful information from acquired GC×GC data.

2.11 Statistical tools

Due to the complex output provided by the GC×GC-TOFMS analyses advanced data treatment is required to subtract relevant information.

The first approach consists on the creation of a template realized in GC image software. To generate the template, samples were grouped in different categories. Qatna samples were classified according to their different natures (bones, soft tissues, wood, textiles and organic residues from vessels), while the heated resin samples from Traceolab were classified according to their different heating treatment. Templates were generated by the superimposition of the different chromatograms and were used for further application to their respective sample set, followed by statistical analysis. The group formation did allow a comparison of the components found in each category in order to define chemical features that distinguish sample classes.

Once the template was applied, the Fisher ratio (F-ratio) was calculated to reduce data and facilitate further data treatment. The F-ratio is a statistical tool that reduces complex datasets by comparing the variance of a compound within its established class and its variance in other classes [36, 37]. Therefore, a compound presenting a high F-ratio should be specific to a certain sample class.

$$F = \frac{Between - group\ variance}{Within - group\ variance} = \frac{S^2inter}{S^2intra}$$

***Equation 1**: Fisher Ratio*[43].

The importance of the F-ratio can be easily understood since a single GC×GC analysis can provide up to a thousand analytes within one sample. Therefore, the handling of big datasets can be extremely challenging due to the massive amount of data. Moreover, relevant information can be hidden within less important information. Therefore, F-ratios were calculated for each compound in the peak table. These values were then compared to the Fisher ratio cut-off (F_{crit}). F_{crit} is defined by the number of classes within a dataset, the degrees of freedom and a significance level α and hence can vary between different studies [45]. Only compounds presenting a F-ratio above F_{crit} are selected for further analysis since they

represent compounds of interest to detect patterns in the different samples. Such technique is efficient for handling GC×GC datasets because it enables data reduction while keeping pertinent information [39, 40].

The obtain peak table still require some refinement. Peaks known as column bleed were removed from the peak table. Column bleed is due to the degradation of GC column during analysis and is characterized by silicium compounds such as hexamethylcyclotrisiloxane.

Finally, these peak tables generated for each sample will be used in The Unscrambler® X program to perform PCA. PCA is a multivariate statistical tool whose purpose is to identify patterns in data and represent the data in such a way as to highlight their similarities and differences. Datasets can be reduced to principal components and visualized in two or three dimensions by using principal component axes [46].

Chapter 3: Results and discussion

3.1 Traceolab samples

The investigation of the different state of preservation of the organic residues recovered from the Royal Tomb of Qatna requires knowledge on the different degradation pathways. In addition, characterization and differentiation between the adhesives used in ancient times will be undertaken to correlate the type of adhesive with a characteristic degradation pathway. For these reasons, the different adhesive mixtures provided by Traceolab were analyzed to get insight into these thermally induced degradation pathways.

3.1.1 Non-treated adhesive mixtures

Pinus sylvestris, *Picea*, *Fabaceae Birbira*, *Juniper Berrytree*, *Euphorbia* and three protein glue samples were analyzed as raw adhesive materials. Figure 14 shows diverse chromatograms of the non-treated adhesives. Although, the resin and glue samples gave complex volatile signatures, characteristic patterns were obtained according to the type of adhesives. The establishment of a VOCs pattern of the different adhesives is fundamental to characterize properly organic residues recovered on archaeological artifacts. In addition, the degradation pathway of adhesives could be related to their nature. Therefore, it is valuable to determine VOCs profile of raw samples prior to the study of heated adhesives.

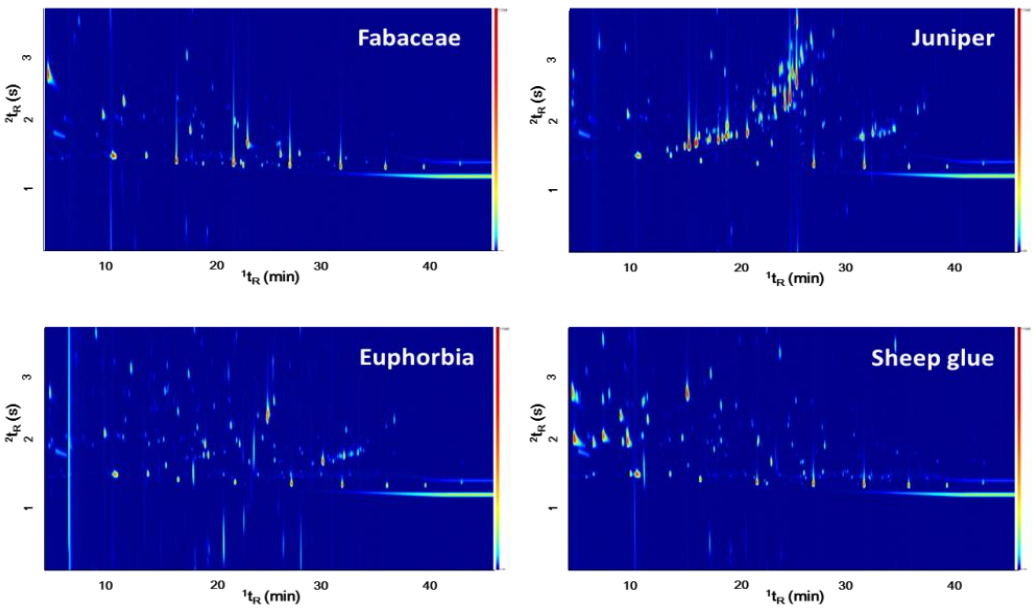

***Figure 14**: Total ions chromatograms (TIC) of four non-treated adhesives.*

To differentiate the adhesives according to their composition, the different samples were subjected to PCA. As can be seen in the scores plot in Figure 15, the protein glue samples (tendon, deer and sheep glues) are forming a close cluster. This clustering is mainly due to the extremely low concentration of terpenoid components within the glue samples and an overall higher abundance of acids, esters, alcohols, benzene derivatives and ketones. This can be expected as terpenoid derivatives are characteristic of plant resins. Regarding *Pinus Sylvestris* (5.01) and *Fabaceae Birbira* (5.03), their composition is close to the glues containing a really low amount of terpenoid compounds. Both samples were analyzed several times for method optimization. Therefore, the numerous extractions of the VOCs onto the fiber led to the overall relatively low amount of VOCs detected. Regarding *Picea*, *Juniper Berrytree* and *Euphorbia*, their VOCs profiles enable their distinction. The comparison of these resins reveals that each resin contained a high amount of characteristic terpenoid compounds ($C_{10}H_{16}$) regarding *Picea* and *Juniper Berrytree* and sesquiternoid compounds ($C_{15}H_{24}$) regarding *Euphorbia*. *Picea* was characterized by the presence of pinene, limonene, carene, fenchene and pinocarveol. Cyclohexene, 4methylene-1-(methylethyl)- and 1,5,8-menthatriene were also identified in high amount. *Juniper Berrytree* resin contained terpineol, sabinene, bornanone and nonanal. Both *Picea* and *Juniper Berrytree* were characterized by a similar quantity in carene and fenchene. The *Euphorbia* resin contains specific sesquiterpenes (panasinsene and isocaryophyllene), terpineol (eucalyptol and thujanol) and a high amount of 2-hydroxypropriophenone.

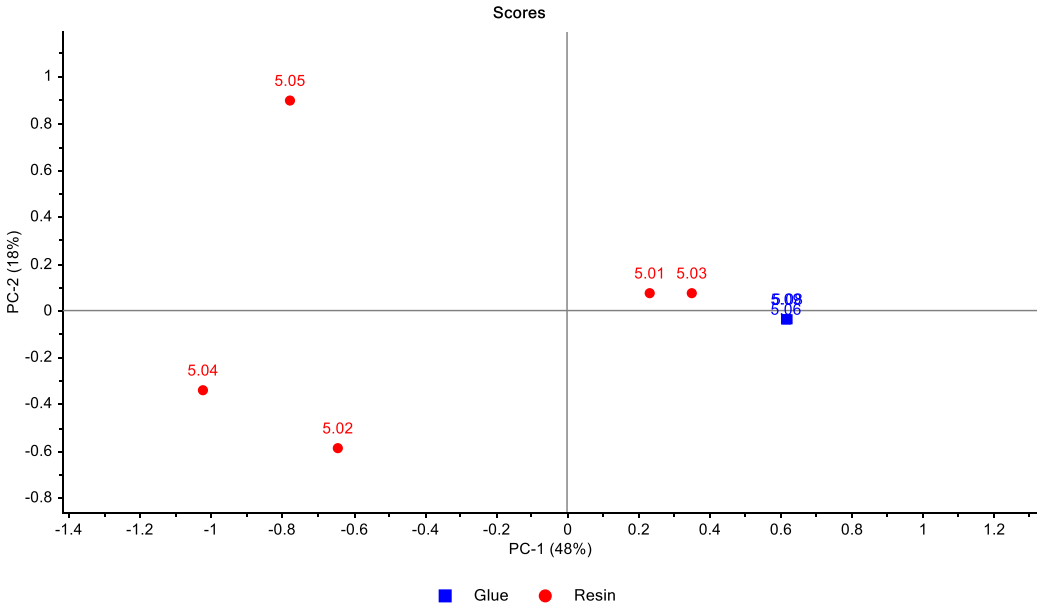

***Figure 15**: PCA scores plot of the different adhesives: resins (red) and glues (blue).*

Although characteristic terpenoid and sesquiterpenoid analytes were detected in each kind of resins, their presence can be extremely difficult to interpret. Indeed, as previously stated, terpenes and sesquiterpenes represent one of the broadest families of naturally occurring chemical compounds in a large variety of plant species.

The chemical composition of the different glues was also investigated. The heat map in Figure 16 displays the results. This heat map is composed of hierarchical diagram on the left side which represents the correlation between the sample clusters and the chemical composition of these samples. Each horizontal line of the heat map represents a sample while each column accounts for a specific compound responsible for the clustering. The different analytes are ordered by decreasing F-ratio; hence compounds on the left side have stronger influence on the hierarchical diagram structure than the ones on the right. A color key, in the upper left side, goes along with the heat map indicating the relative abundance of the analytes in each sample.

As can be seen, the three glue samples were characterized by specific analytes and can be distinguished from each other. The analytes can be classified in 5 categories, namely terpenes, acids/esters, aldehydes, aromatics and hydrocarbons. Numerous terpenoids compounds were identified in tendon (5.06) and sheep (5.09) glue, even though their concentration was much lower than in the resins samples. The main terpenoid derivatives identified within the tendon glue sample are limonene and 4-carene, while α-pinene, sabinene and terpineol were present in higher amount in sheep glue. In addition, tendon glue was high in 1,5,8-menthatriene, benzyl alcohol and butanoic acid, metyl ester. In contrast, deer glue (5.08) was distinguished from tendon and deer glue samples by the identification of aldehydes (nonanal and heptanal) and hydrocarbons (heptane, 3-ethyl). The deer glue sample contained also sabinene, carene and 1,5,8-menthatriene but the amount of these analytes is significantly lower than in the two other samples.

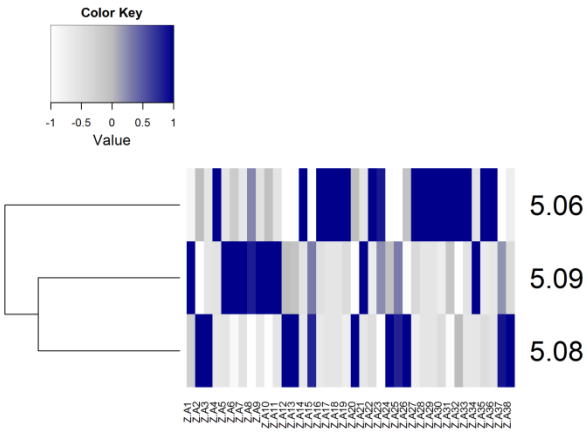

***Figure 16**: Heat map representation of hierarchical cluster analysis (HCA) using the F_{crit} value for the compounds detected within the glue samples.*

These analyses of non-heated adhesives show the possibility to differentiate resins from glues samples and also differentiate the different samples by their characteristic VOCs composition. However, it would have been beneficial to have replicates of resin and glue samples in order to strengthen the results obtained by the statistical analysis. The replicates would also have been valuable to confirm the presence of specific terpenes and sesquiterpenes within a resin sample type and the specific analytes of glue samples.

3.1.1.1 Extraction temperatures

Different extraction temperatures were investigated using the non-heated sheep glue sample, in order to compare the VOCs profiles. By increasing the extraction temperature, less volatile and more polar organic compounds can be trapped on the fiber and thus identified. Figure 17 displays the divergent profiles obtained for an extraction temperature of 40 °C (left) and 80 °C (right). For the extraction at 40 °C, the intensity is much higher at low retention times in the first dimension, while the opposite is observed for extraction at 80 °C and much more chemical compounds were detected for high retention times. The extraction at 80 °C led to the detection of linear aldehydes from C_4 to C_{14} and long chain ketones from C_{11} to C_{16}, which were not characterized by the extraction at 40 °C. In addition, numerous long chain linear and branched hydrocarbons were identified from 30 min in the first dimension retention time.

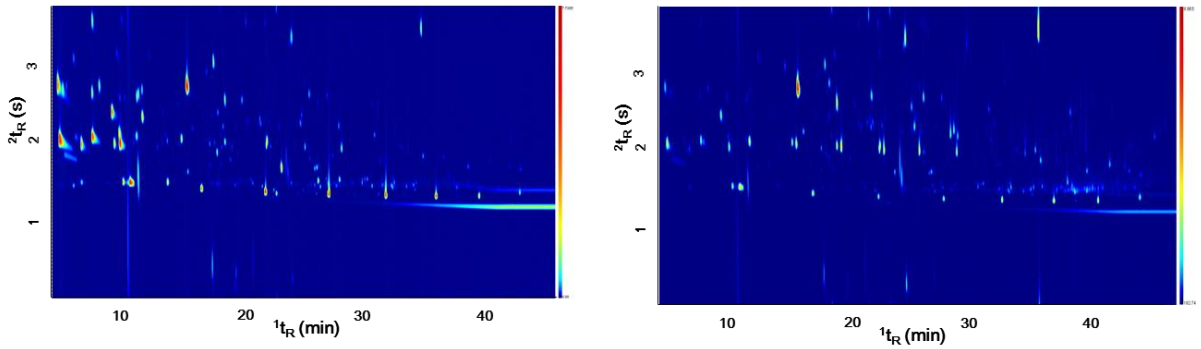

***Figure 17**: TICs of sheep glue by using an extraction temperature of A) 40 °C and B) 80 °C.*

The higher extraction temperature enabled to obtain additional information on the terpenoid derivatives present within the sample. Table 4 lists the different terpenoid derivatives identified, with a match factor generally above 600, for extraction temperatures of 40 °C and 80 °C. In both cases, d-limonene and α-terpineol were identified. Nevertheless, by using a higher extraction temperature, it was possible to characterize sesquiterpenes ($C_{15}H_{24}$) and diterpenes ($C_{20}H_{32}$). In addition, different monoterpenes, sequiterpenes and their derivatives e.g. myrcenol, borneol, aromandendrene, α-farnesene and ε-muurolene and terpineol were identified due to their higher boiling point. Indeed, by using high extraction temperatures, less volatile analytes are released from the headspace and then trapped onto the fiber [48]. However, information on the more volatile analytes is lost when using increasing temperatures. In addition, in some cases, degradation of the sample due to the use of high temperatures could lead to the formation of artifacts [49].

In order to optimize the extraction, temperatures of 50 °C and 60 °C were investigated. These temperatures enabled the detection of other terpenoids. At 50 °C, 3-carene as well as several sesquiterpenes (aromadendrene, isocaryophyllene, α-amorphene, d-germacrene and α-farnesene) were identified in addition to α-pinene, d-limonene and β-thujene. Regarding the extraction temperature of 60 °C, similar monoterpenes that the ones recovered at 50 °C were identified, while significantly less sesquiterpenes were identified (aromandendrene and ε-muurolene). However, terpineols such as myrcenol and ocimenol were additionally characterized.

A temperature of 40 °C was suitable for the analysis of highly volatile terpene derivatives, while higher temperatures enabled the characterization of less volatile sesquiterpenes and in extreme cases diterpenes. By using extreme extraction temperatures such as 80 °C, information about the presence of highly volatile monoterpenes was completely lost which

should be avoided by keeping the extraction temperature below 60 °C. In addition, archaeological artifacts are often brittle and cannot be heated above 50 °C to preserve samples for further analysis. Therefore an extraction temperature of 40 °C was applied.

***Table 4**: Different chemical compounds recovered in the sheep glue sample using extraction temperatures of 40 °C and 80 °C. D-limonene and α-terpineol were characterized for both extraction temperatures.*

Extraction Temperature	Compound name	Chemical formula	Boiling point	$^{1}t_R$ [min]	$^{2}t_R$ [s]	Match
40 °C	α-Pinene	$C_{10}H_{16}$	155 °C	16.000	1.73	734
	β-Thujene	$C_{10}H_{16}$	150 °C	17.730	1.80	761
	Sabinene	$C_{10}H_{16}$	163 °C	17.800	1.82	637
	Sabinene hydrate	$C_{10}H_{18}O$	200 °C	25.267	2.51	603
80 °C	Myrcenol	$C_{10}H_{18}O$	224 °C	14.467	2.08	590
	Borneol	$C_{10}H_{18}O$	213 °C	25.533	2.93	849
	Aromandendrene	$C_{15}H_{24}$	258 °C	31.667	1.88	625
	α-Farnesene	$C_{15}H_{24}$	260 °C	31.933	1.89	551
	ε-Muurolene	$C_{15}H_{24}$	268 °C	34.067	1.98	617
	Cembrene	$C_{20}H_{32}$	/	44.200	2.25	713

3.1.2 Thermally degraded adhesive mixtures

Controlled heating of experimental samples may help in the characterization of archaeological organic remains. The comparison between archaeological remains and reference samples may provide additional information on the state of preservation. Figure 18 already demonstrates the significant chemical changes after the heat treatment to 340 °C for 8 h. Visual inspection revealed that after being heated, only blackish organic residues remained.

***Figure 18**: Mixture of Picea resin and wax A) before heat treatment, B) after a heat treatment a 340 °C for 8 h.*

In addition, Figure 19 displays the ability to distinguish the volatile signature of the resin samples based on their heating treatment differentiated by temperature and length. The intensity and the number of detected compounds in the *Picea* resin significantly decreased after a heating treatment at a temperature of 144 °C for 5 h (Figure 19-B). Only 31 % of the compounds were detected. As can be expected, the temperature of the heating treatment had also a significant impact on the number of VOCs detected. The heating treatment at 340 °C for 8 h of the *Picea* resin sample (Figure 19-C) resulted in a decrease in intensity and only 16 peaks, corresponding to 13 % of the non-treated resin, were detected. TIC of the heated epicea at 340 °C for 13 h contained few additional peaks (Figure 19-D).

For all the analyses, characteristic peaks that are coming from column bleed were identified. It has to be emphasized that the intensity of peaks coming from column bleed can vary from one analysis to another. In addition, the fiber used can trap some highly sticky chemicals coming from other analysis or from the laboratory itself and be contaminated with these chemicals for the following analyses.

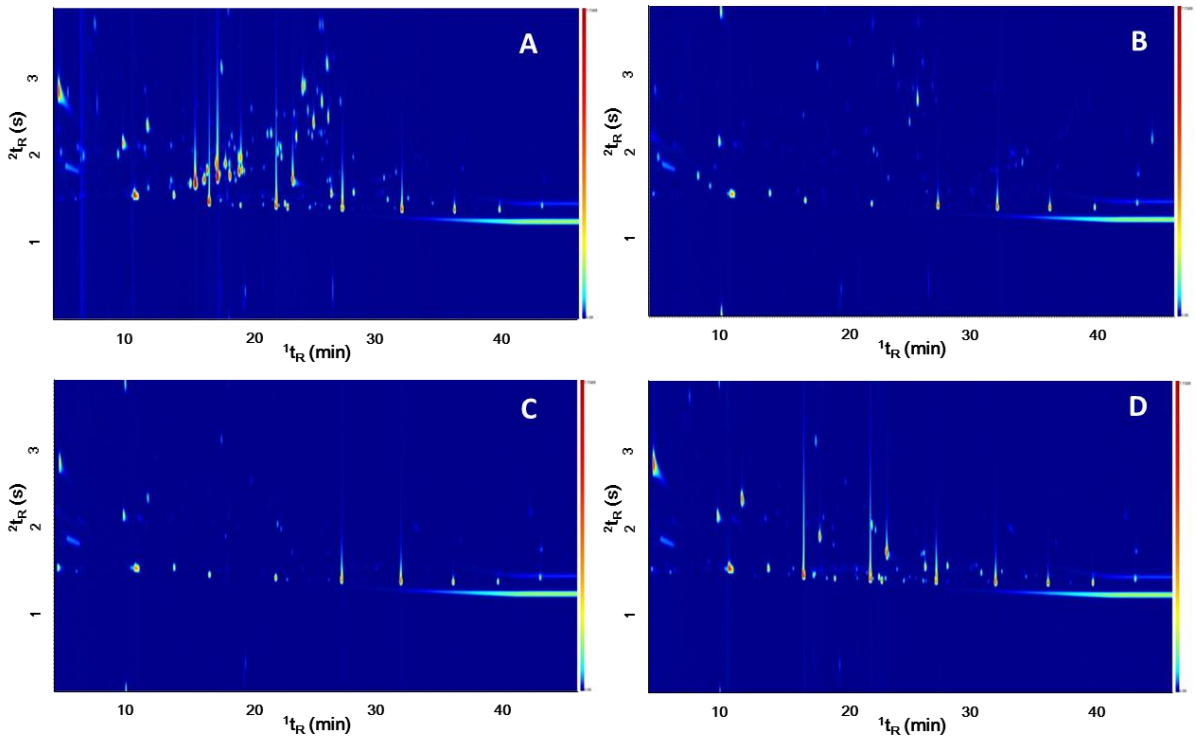

***Figure 19**: Total ions chromatograms (TIC) of A) raw epicea resin, B) epicea resin heated at 144 °C for 5 h, C) epicea resin heated at 340 °C for 8 h and D) epicea resin heated at 340 °C for 13 h.*

The chromatogram in Figure 20 represents a fiber blank obtained with the same fiber as the one used for the analysis. The majority of the peaks detected (Table 5) are coming from the column bleed. Column bleed is characterized by silane and siloxane chemical compounds and

this is mainly due to the ageing of the column. Additional peaks are coming from diverse contaminations of the fiber. Therefore, these peaks were not taken into account for the analysis of the samples.

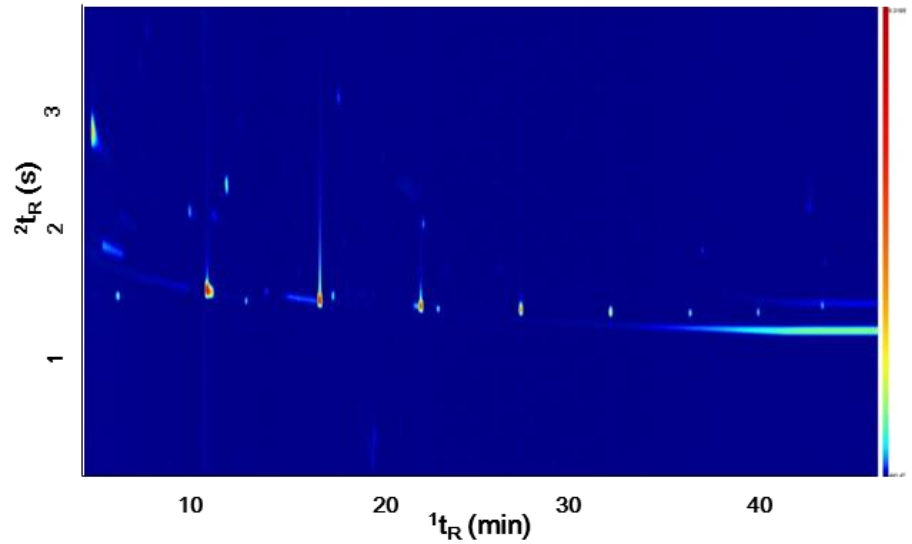

***Figure 20**: TIC of fiber blank.*

***Table 5**: Compounds listed as column bleed (siloxanes) and as fiber contaminants in the two chromatograms of fiber blanks.*

Compound name	^1t_R [min]	^2t_R [s]	Match
Trimethylsilanol	5.400	2.91	866
Toluene	10.400	2.27	859
Hexamethylcyclotrisiloxane	11.333	1.60	925
1,3-diisopropoxy-1,3-dimethyl-1,3-disilacyclobutane	12.333	2.48	646
Octamethylcyclotrisiloxane	17.133	1.50	905
Nonamethylcyclotrisiloxane	18.400	2.00	840
Decamethylcyclopentasiloxane	22.333	1.47	893
Octamethylcyclotetrasiloxane	23.600	1.80	853
p-Trimethylsilyloxyphenyl bis(trimethylsilyloxy)ethane	26.600	1.61	729
Dodecamethylcyclohexasiloxane	27.467	1.43	855
Tetradecamethylcycloheptasiloxane	32.133	1.41	767
Hexadecamethylcyclooctasiloxane	36.200	1.40	801
Tetradecamethylheptasiloxane	39.733	1.41	709

The comparison of the amount of chemical compounds detected after the different combustion temperatures and times is displayed in Figure 21. The number of detected compounds varied greatly according to the sample types. As expected, the number of peaks detected in the non-heated resin was much higher than after combustion. However, the increase of temperature and length of the heat treatment was not always correlated to the decrease in chemical compounds detected. On the contrary, for three different adhesives the longer and the higher the heating treatment, the more chemical compounds were identified.

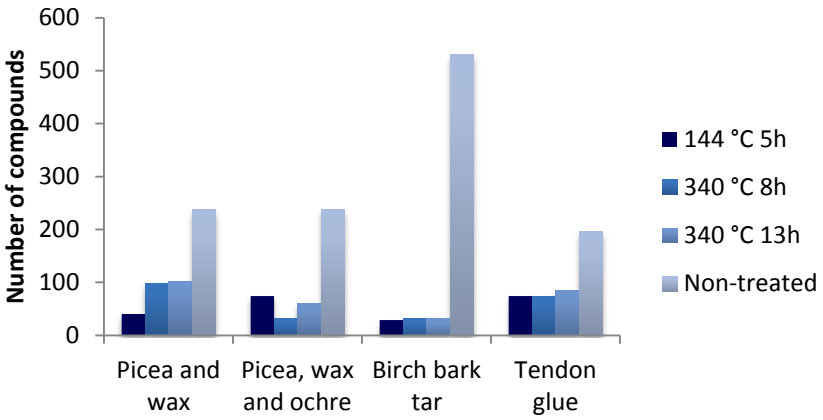

***Figure 21**: Number of chemical compounds detected by GCxGC in the resinous materials for the different heating treatment. Siloxanes, silanes and fluoro compounds were removed.*

In addition, the presence of additives such as wax and ochre had an impact on the VOCs profile (Figure 22). Indeed, the heat treatment of *Picea* mixed with wax and *Picea* mixed with wax and ochre led to the identification of different analytes. The heating to 144 °C of *Picea* mixed with wax led to a high amount of aromatics and linear hydrocarbons, while when mixed with ochre, the quantity of alcohols, naphthenics, ethers, acids, esters and ketones was greater. On the contrary, when the mixture of resin and wax was heated to 340 °C, the amount of alcohols, aldehydes, acids, esters, aromatics and linear hydrocarbons was significantly larger than for the mixture with the ochre. Some studies have shown that the mixture of resin with ochre leads to less sticky adhesives which dried more rapidly and were less brittle [21]. It would have been beneficial to have a sample of the two different non-heated mixtures to see if their VOCs profiles display significant differences. And therefore, to see if the presence of ochre in the *Picea* resin has an impact on the resin only when this latter is exposed to a heat source or not.

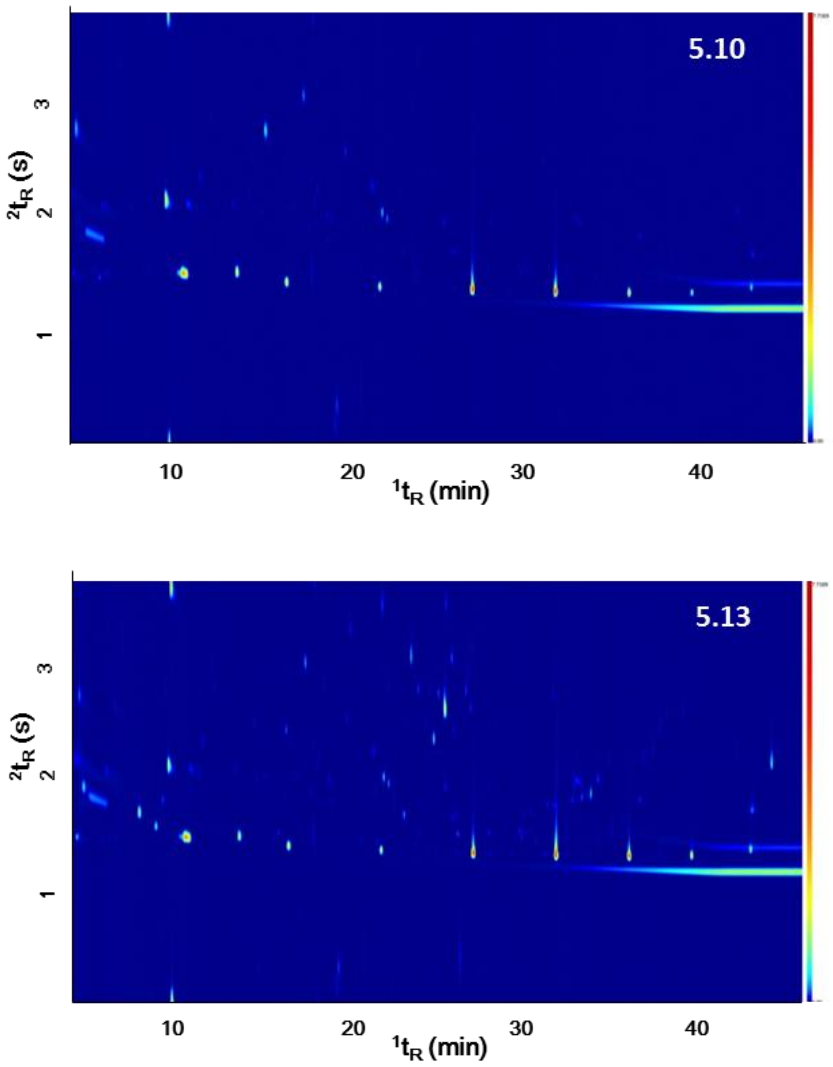

***Figure 22**: TIC of Picea resin heated to 144 °C for 5 h mixed with wax (5.10) and mixed with wax and ochre (5.13).*

To investigate the impact of the heating temperature and length on the adhesives, PCA analysis was performed (Figure 23). Two groups were defined and the reference sample consisting of the non-heated *Picea* resin was rejected from the two clusters. Samples heated at 144 °C for 5 h, except sample 5.16, were grouped on the right side of the scores plot, while the majority of the samples heated up to 340 °C were grouped on the left side. The corresponding correlation loadings indicate, in general, that the less heated samples were characterized by a higher amount of compounds.

The group on the right side is composed by the majority of the *Picea* resin samples and is characterized by a high amount in terpenes, aromatics and hydrocarbons. Among terpene derivatives detected, some analytes were also identified in the non-heated *Picea* resin, namely pinene, carene, pinocarveol and fenchone. Fenchone corresponds to the oxidized form of

fenchene that was characterized in *Picea* resin. In addition, 1,5,8-menthatriene and cyclohexene, 4-methylene-1-(methylethyl) were also characterized as major chemical compounds. Sample 5.11 representing *Picea* and wax heated to 340 °C for 8 h and sample 5.15 being the mixture of *Picea*, wax and ochre heated to 340 °C for 13 h were composed of a significantly lower amount of terpenes derivatives.

Due to the grouping of the *Picea* samples with the two glue samples heated to 144 °C for 5 h, it seemed that the heat treatment has a higher impact on the chemical composition of the resins. Indeed, raw glue samples were characterized by a much lower amount of terpenes derivatives than raw resins samples. However, after the same heat treatment at 144 °C, resin and glue samples present a similar quantity in terpene derivatives.

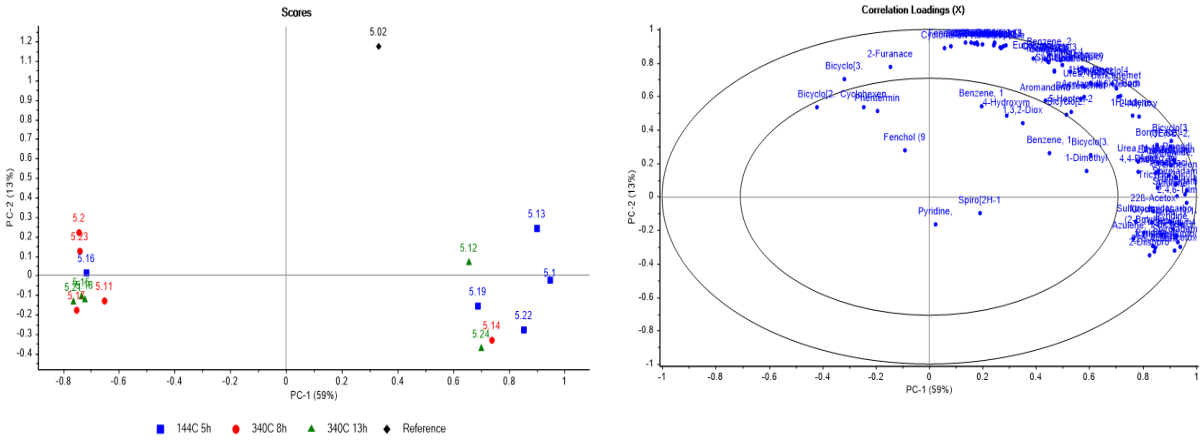

***Figure 23**: PCA scores plot and the corresponding correlation loadings of the non-treated epicea resins (black) and of the different heated resins samples at 144 °C for 5 h (blue), at 340 °C for 8 h (red) and at 340 °C for 13 h (green).*

As the different type of non-heated resins can be differentiated based on their chemical composition and that the addition of wax or ochre can have an impact on the VOC profile of resin, it may be possible to distinguish the heated resins samples based on their nature. Therefore, the comparison of the different samples according to their type was performed by PCA (Figure 24). The non-heated *Picea* resins (yellow), the mixture of *Picea* with wax (blue) and the mixture of *Picea*, wax and ochre (red) were all located on the right side of the PCA. However, they are not forming a close cluster which is probably due to the heat treatment differing from one sample to the other and/or the presence of ochre. As already observed in Figure 23, pork glue sample heated to 144 °C for 5 h was close to the *Picea* resin. In addition, pork glue heated to 340 °C for 13 h was also forming a cluster with the *Picea* resin. *Birch*

bark tar and tendon glue samples were grouped together on the left side of the PCA comprising the pork glue heated to 340 °C for 8 h.

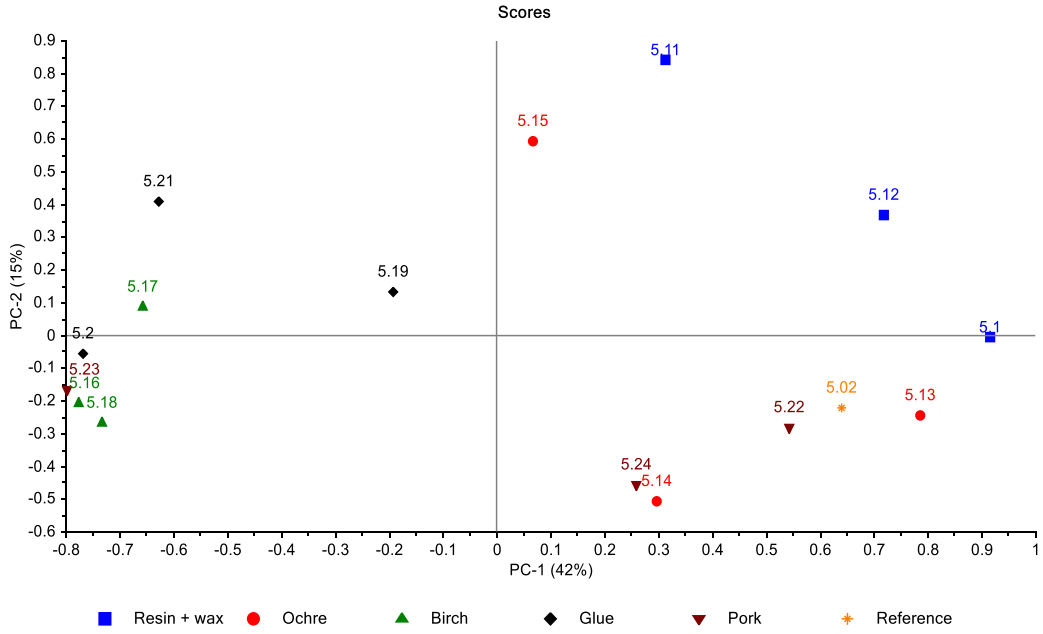

***Figure 24**: PCA scores plot of the different heated adhesives: Picea resin and wax (blue), Picea resin, wax and ochre (red), birch bark tar (green), tendon glue (black), pork glue (brown) and Picea resin (yellow).*

The main chemical compounds identified in both *Birch bark tar* and tendon glue samples were α-pinene, thujenol, 2-bornanone and p-cymene-8-ol. Nevertheless, these compounds were also present in high amount in the Picea resin samples. The presence of 2-allyloxyanisole, fenchol, adamantanecarboxylic acid and 5,10-dioxatricyclodecane in *Birch bark tar* and in tendon glue enabled their separation from *Picea* resin samples. In addition, *Birch bark tar* and tendon glue can be distinguished from each other. On one side, *Birch bark tar* contained a high amount of α-methyl-α-vinyl-2-furanacetaldehyde, santolina triene and tricyclene. And on the other side, tendon glue was characterized by high quantity of β-pinene, 4-caranone, sabinene and butyrolactone.

3.1.3 Archaeological samples

In addition to the intentionally heated resins, four archaeological samples were provided by Traceolab (Table 6). These samples consist of stone tools onto which organic remain where microscopically identified. The analysis of these archaeological artifacts led to different chromatographic profiles, even though the intensity was relatively low.

***Table 6**: List of archaeological samples and their corresponding sample codes.*

Sample names	Sample codes
Lommel ADHES158	5.25
Star Car 91234	5.26
Star Car 89634	5.27
Bushman	5.28

The HCA, performed to evaluate the differences between the four samples, is displayed in Figure 25. The variance can be explained with only fifteen analytes which were present in high concentration in these samples. Although sample 5.25 was characterized by a generally lower amount in analytes compared to the two star cars (5.26 and 5.27) and the bushman (5.28), it was especially high in α-D-xylofuranoside, methyl 2,5-di-O-methyl-, a sugar derivative. The bushman sample was high in the majority of these 15 chemical compounds. Among them, α-terpineol, caranol, isocaryophyllene and 2-pentanone, 3-methyl were the most abundant. Regarding the two star cars, they presented a high amount in butyrolactone.

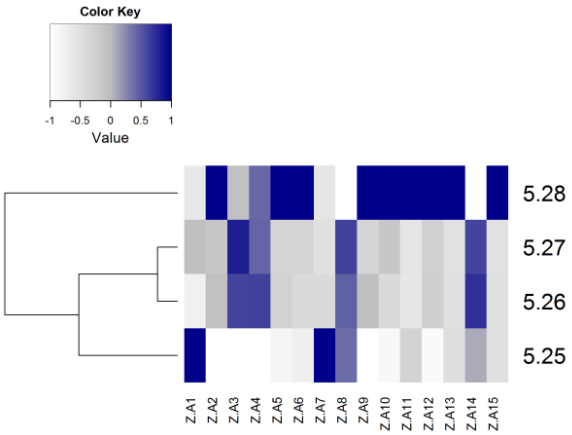

***Figure 25**: Heat map representation of hierarchical cluster analysis (HCA) using the F_{crit} value for the compounds detected within the archaeological samples.*

These significant differences between the archaeological samples can be due to (i) the nature of adhesive used or (ii) the state of preservation of each sample. First, the analytes identified within the archaeological samples were compared to those characterized in non-heated resin and glue samples. Even though their amount was extremely low, few terpenes were detected (Table 7). As already exposed by HCA, the two star cars (5.26 and 5.27) were composed of similar terpenes derivatives. However, their particularly low amount may be comparable to the few terpenes recovered within the glue samples. Regarding sample 5.25, it was characterized by numerous monoterpenes and terpineols. The great diversity and the really

low amount of identified terpene derivatives make their relation to a specific type of resin challenging. The high content of terpineols in sample 5.28 was comparable to the *Euphorbia* resin. Nevertheless, caution has to be taken because of the lack of information about distinctive volatile terpenes between the types of adhesives.

***Table 7**: Terpene derivatives identified within the four archaeological samples.*

	5.25	**5.26**	**5.27**	**5.28**
Monoterpenes				
3-Carene	X			
α-Pinene	X			X
d-Limonene	X	X	X	X
Verbenone	X		X	X
Carvone	X			
Myrtenol	X			
α-Campholenal	X			
Terpineols				
α-Terpineol	X			X
Fenchol	X			
Sabinene hydrate		X	X	X
Eucalyptol				X
Caranol				X

Second, the archaeological samples were compared to the different non-heated and heated resins to investigate their state of preservation. Statistical analysis reveals significant differences between the archaeological samples and the non-treated resin. This could be explained by a notable higher amount of terpenoid derivatives within the non-treated resins and glues. Therefore, the heated adhesives were used to perform another statistical analysis (Figure 26).

The two star cars were still forming a close cluster, which reinforced the hypothesis of the use of a same nature of adhesive. Furthermore, they are grouped with the majority of the resins heated to 144 °C for 5 h and are specifically close to the pork glue sample 5.22. Their chemical similarity to the pork glue may explain their relatively low amount of terpenes. However, further analysis on more replicates should be performed to confirm this possibility. The Bushman sample was also on the left side of the PCA with the less heated adhesives, while sample 5.25 was on the opposite side grouped with the samples heated to 340 °C.

Therefore, it may be assumed that samples 5.26, 5.27 and 5.28 were better preserved than sample 5.25.

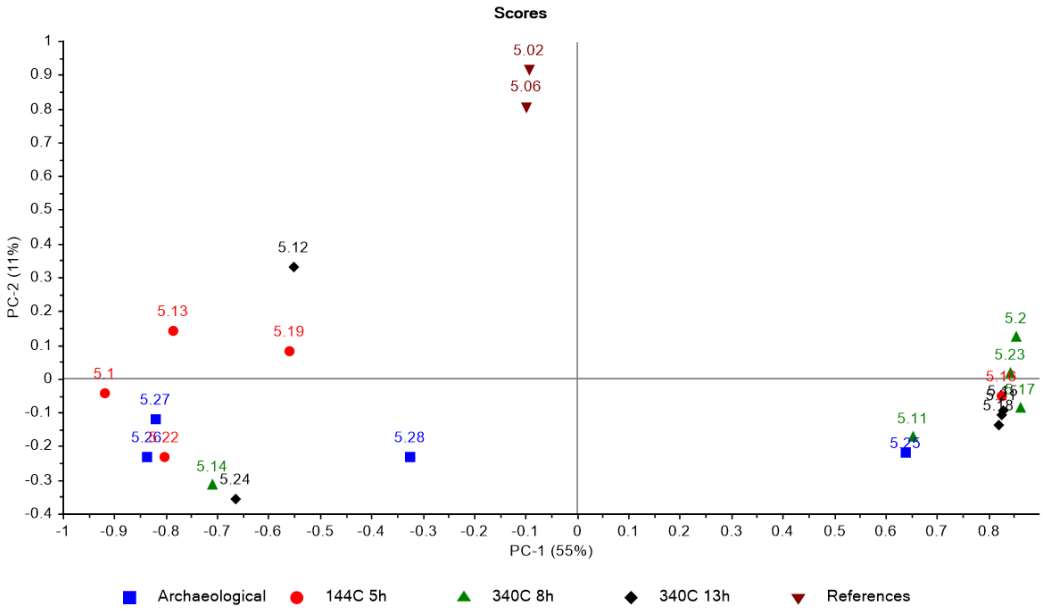

***Figure 26**: PCA scores plot of the archaeological samples (blue), the heated resin to 144 °C for 5 h (red), the heated resin to 340 °C for 8 h (green), the heated resins to 340 °C for 13 h (black) and two non-heated resins (brown).*

3.2 Archaeological samples from Qatna

The investigation of the different nature of resins and their degradation would help in determining the state of preservation of the archaeological samples. Nevertheless, it has to be emphasized that the two sets of samples are greatly different and thus the comparisons may be challenging. Indeed, the archaeological bone, wood, textile, soft tissue and vessel content samples were not all specifically treated with adhesives. In addition, because the samples date back to more than three thousand years, lots of VOCs and volatile terpenes may have evaporated.

The analysis of the different organic compounds recovered from the Royal Tomb in Qatna provided complex chromatograms presenting up to 600 peaks. According to the nature of the sample, the chromatographic patterns were highly different. Therefore, to compare all the samples and establish correlations between samples of a same class, PCA was performed. Only the compounds presenting a F-ratio higher than 2.73, corresponding to the F_{crit}, were used for the statistical analysis. This approach reduced the number of components to 16 % of the original data set, which enables to target the major chemical compounds. As can be seen

in Figure 27, the principal components (PCs) accounted for 55 % (PC-1) and 11 % (PC-2) of the variability and the separation between bones and textiles samples can be visualized. Soft tissues were grouped within the textiles samples, while wood were spread in the PCA.

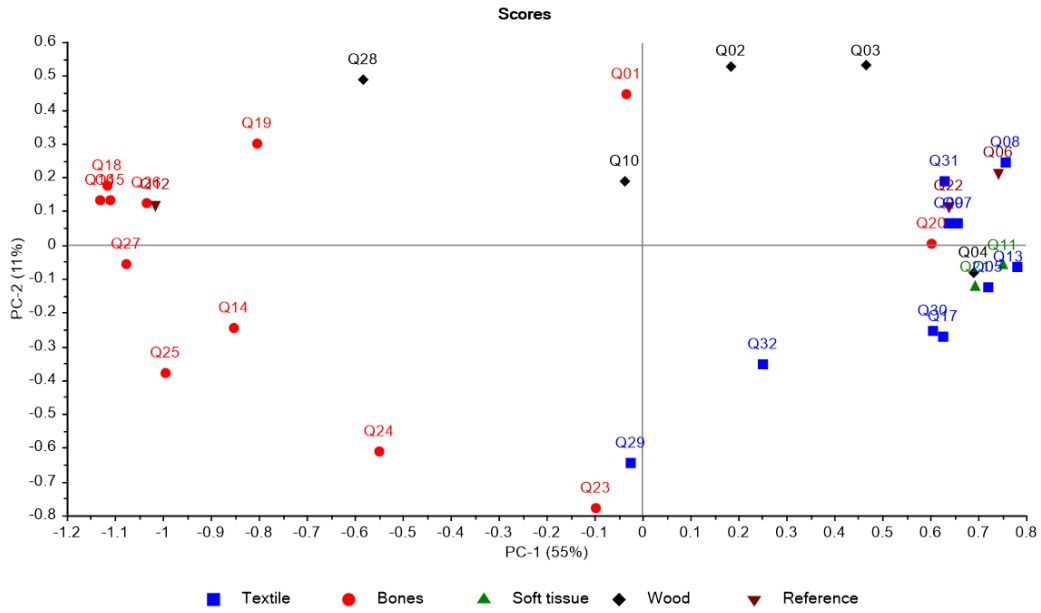

***Figure 27**: PCA scores plot applying α=0.05 for all the samples recovered in the Royal Tomb of Qatna. Principal components accounted for 55 % PC-1 and 11 % PC-2.*

The variation in the chromatographic patterns, confirmed by the PCA analysis (Figure 27), within a same class of samples was also notable. As an example, two chromatograms of different bone samples are displayed in Figure 28.

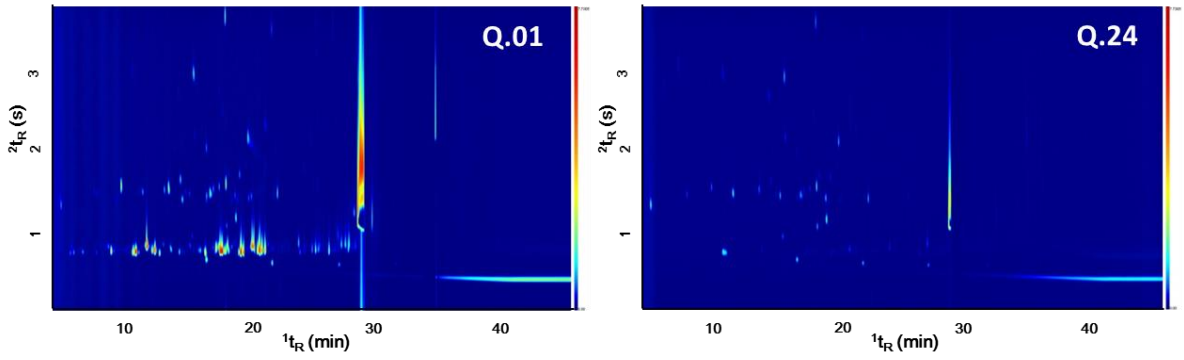

***Figure 28**: TICs of two bone samples recovered from chamber 4 (Q.01) and from chamber 1 (Q.24).*

The chromatograms in Figure 28 were chosen because the amount of sample analyzed was similar. However, the samples were recovered in two different locations within the Royal Tomb. Sample Q01 was recovered in chamber 4 while the sample Q24 was recovered in chamber 1. Therefore, the differences between the chromatograms could be explained by two

hypotheses. On one hand, the location of the sample could have an impact on the chemical composition of a sample. On the other hand, after excavation the different samples were subjected to different kinds of treatments influencing their state of preservation.

The investigation of the impact of the location on the samples was performed by PCA (Figure 29). According to this analysis, it can be concluded that the location did not influence the chemical composition of the samples in a significant way. As an illustration, the numerous samples recovered from chamber 4 did not show any particular grouping and hence no specific chemical correlations.

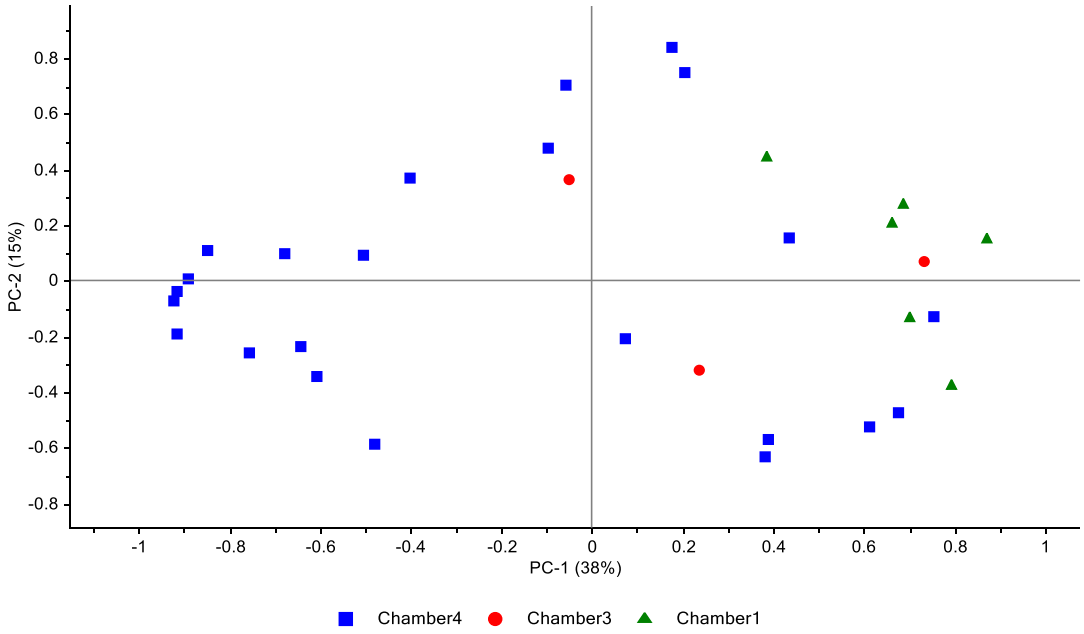

***Figure 29**: PCA scores plot applying α=0.05 for all the samples recovered in the Royal Tomb of Qatna. Principal components accounted for 38 % PC-1 and 15 % PC-2.*

Because the location of the sample did not have any impact on the sample characteristics, it was assumed that their state of preservation vary within the samples. To extract valuable information about this state of preservation, the set of samples analyzed was reduced to bone and textile samples. Indeed, due to the complexity of the dataset and the number of samples, it was necessary to focus on particular samples. Bones and textiles were chosen for several reasons. Firstly, the number of samples enables reliable statistical analysis. Secondly, the chromatographic patterns of the different bone samples are highly different from one sample to another, which could be an indicator of their nature and/or state of preservation. In addition, previous studies have pointed out the use of resinous compounds to preserve clothes which were also directly in contact with some bone samples [23]. Therefore, it will enable a direct comparison between the chemical compounds recovered on textile and/or on bone

samples with the different heated resins provided by Traceolab. Finally, the group of M. James, N. Reifarth and R. Evershed has discovered dyes components within textiles samples [23]. Therefore, an expectation was to find dyes chemical compounds within the textiles to show the efficiency of the method compared to classical analyses.

3.2.1 Bone samples

The purpose was to establish different groups according to the chemical features of the different bone samples. These relations between the samples could help to determine the level of degradation and/or the different natures of the bone samples. After microscopic examination, it was assumed that the sample Q.20 was a bone, probably coming from an animal due to its shape, while Q.11 and Q.21 were considered as soft tissues; therefore, they were included in the analysis (Figure 30). However, this was not clearly established, thus, relation between these samples and the bone samples was also investigated.

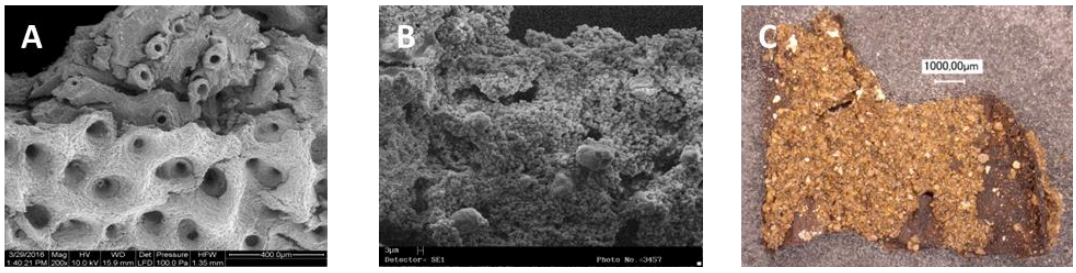

***Figure 30**: Microscopic analysis of sample A) Q.11, B) Q.20 and C) Q.21 recovered from the Royal Tomb.*

HCA was performed on the 22 analytes obtained after using the F-ratio method and applying F_{crit} on the initial dataset. Figure 31 displays the heat map obtained. Among the three groups that can be discerned, samples Q.11, Q.20 and Q.21 formed a separate cluster. Regarding the compounds responsible for the clustering, different chemical classes were identified: alcohols, ketones, linear hydrocarbons, naphthenics and aromatics. These relevant compounds from the heat map (Figure 31) were not recovered within these three samples, which displayed their different chemical composition. Among the components that differentiate Q.11, Q.20 and Q.21 from bones, 46 % were characteristic of bones [50]. This confirms the fact that Q.11, Q.20 and Q.21 are not any kinds of bones and therefore, that they all may be part of soft tissues. Regarding the bone samples, two groups can be distinguished. The analysis of samples Q.15, Q.16, Q.18, Q.26 and Q.27 displayed a high concentration in characteristic components of bones. Therefore, it can be assumed that these bones were better preserved

than the other group of bones constituted of samples Q.01, Q.14, Q.19, Q.23, Q.24 and Q.25. This hypothesis, however, requires more investigation.

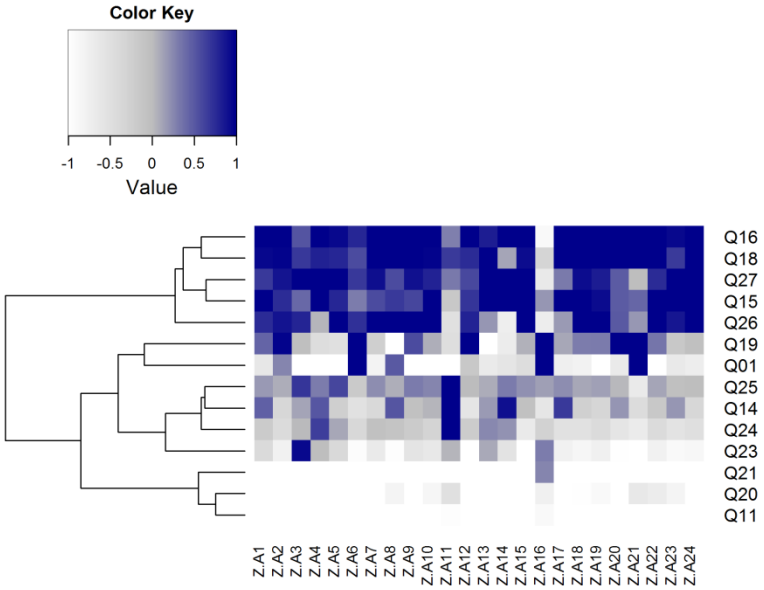

***Figure 31**: Heat map representation of hierarchical cluster analysis (HCA) using the F_{crit} value for the compounds detected within the bones samples.*

The analysis of bones samples based only on their chromatographic pattern was challenging because the amounts analyzed were varying from 0.071 g to 1.545 g. Therefore, the intensity of peaks in chromatograms obtained for each sample cannot be directly linked to the state of preservation of bones. In addition, the extraction of organic remains was performed using petroleum ether. The total ion chromatogram of petroleum ether is presented in Figure 32. As it can be seen, the pattern provided is complex and petroleum ether is a major source of contamination for all the samples. In addition, the type of petroleum ether used could have been changed between the samples, which make the analysis even more complicated. However, focusing on resinous components is a great alternative since these analytes cannot be recovered in petroleum solvent.

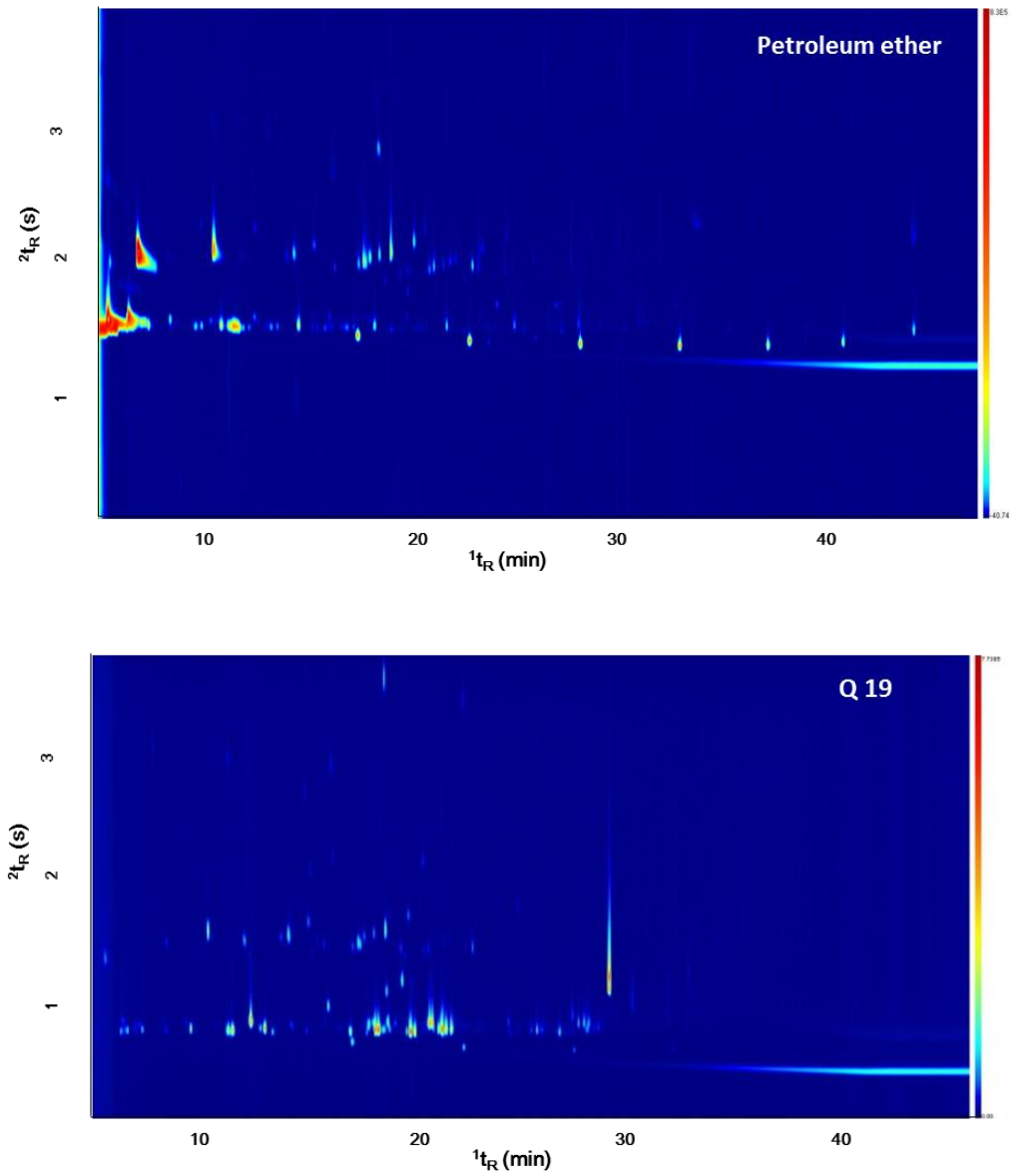

***Figure 32**: TICs of petroleum ether (top) and bone sample Q19 (bottom) illustrating the contamination due to the extraction using petroleum ether as solvent.*

The detection of different resinous compounds will also help for the analysis of the state of degradation by comparison with the thermal degradation pathway of resins provided by Traceolab. The monoterpene, α-pinene, was recovered in all organic residues recovered in the Royal Tomb and therefore was not considered as specific component in this analysis. Other terpenes were detected in only four different samples and listed in Table 8. The four samples, in which monoterpenes were detected, were recovered in the sarcophagus in chamber 4. Interestingly, chemical composition of sample recovered in chamber 4 in the sarcophagus was significantly different than the chemical composition of samples coming from the burial table.

***Table 8**: Monoterpenes compounds detected within the bone samples.*

Compound name	Q.14	Q.15	Q.16	Q.18
Camphor				X
Carene		X		X
Eucalyptol	X	X	X	X
Levomenthol	X	X	X	X

It has to be stated that the burial rituals in the Royal Tomb of Qatna can be divided in four main phases. For this work, the two first stages are of particular interest and could explain the differences of chemical composition between the burial table and the sarcophagus. During the first stage, called the primary burial, the deceased is placed in a large wooden box, above the burial table, where the decomposition occurs. The dead person is dressed, covered with oils and earth pigment and then the body is heated to 200 °C [23]. The secondary burial consists of the re-deposition of the bones, removed from the skeleton, in the sarcophagus. Skeletons recovered from the sarcophagus in chamber 4 were incomplete and in disorder. This phase is often accompanied by symbolic rituals whose goals are to venerate, care and nourish the deceased [23]. Up to 16 individuals were recovered in the two sarcophagi in chamber 4 and 1. Therefore, the previously heated remains were all mixed in the same place where other rituals probably involving food offering and/or ointments took place. From the high number of bone remains which were all treated with resinous materials, it can be expected to detected more terpenoid compounds than onto the burial table.

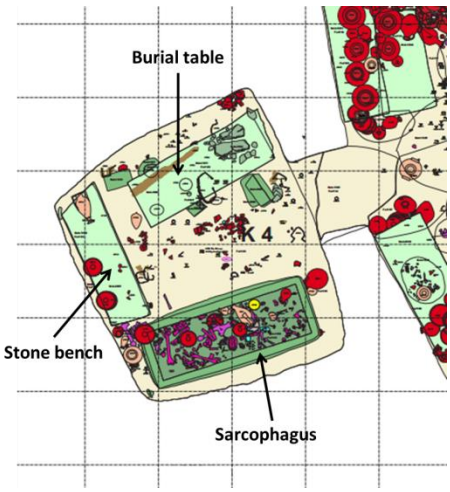

***Figure 33**: Location of the burial table and the sarcophagus in the chamber 4 of the Royal Tomb.*

3.2.1.1 Comparison with the heated adhesives from Traceolab

The different bone samples were compared to the heated resins and glues by PCA (Figure 34). The divergent natures of the bones samples and of the voluntary heated adhesives led to two separate clusters. In order to establish the different state of preservation of the archaeological samples, it would be valuable to characterize heated bone samples onto which different resins were applied. Indeed, the matrix used can have an impact on the VOCs identified. Since it has not been proven that all the samples were thermally treated, different ageing tests could also be performed. Such analysis may provide additional information about the state of preservation of the archaeological samples.

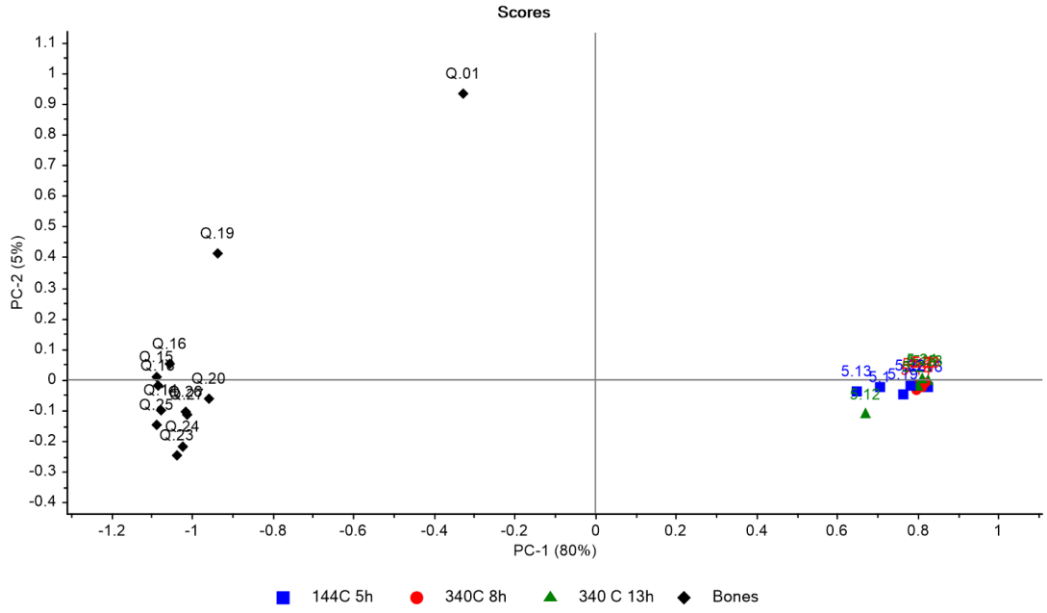

***Figure 34**: PCA scores plot of the different heated adhesives provided by Traceolab (blue, red and green) and the bones samples recovered in the Royal Tomb of Qatna (black).*

3.2.2 Textile samples

The majority of the textile samples were recovered from the burial table in chamber 4. During the excavation of the tomb, only one complete skeleton has been discovered still in anatomically correct order. Below these remains several layers of textile, in a mineralized form, were found [51, 52]. In addition, sedimentary textile remains were discovered on a wooden bench in chamber 1. Analysis by scanning electron microscopy and X-ray diffraction confirmed the gypsum mineralized form of the different textiles [23, 51]. On the burial table in chamber 4, up to 40 different types of fabrics were identified on top of each other, measuring not more than half a millimeter [23, 51]. The textile remains, directly connected to

the human bones, had a blackish resin-like appearance instead of being mineralized in a gypsum form. Chemical analysis revealed the treatment of these textiles with a mixture of oils, Royal Purple dye pigments and heating processes at roughly 200 °C in some cases [51]. The analysis of these textile fragments along with their comparison to the heated resin provided by Traceolab, could confirm the different states of preservation of the textile residues, their treatment with resinous materials during burial and eventually the presence of dye pigments.

Into the complex sediment of textiles recovered in the burial table were incorporated human remains, plant remains and also wood residues coming from the coffin placed on top of the burial table [23, 51]. Therefore, the gypsum mineralized textile samples were compared to bone and to the presumed soft tissues, labelled as unknown, to see if human remains were intimately mixed to the textile samples or on the contrary, if the chemical composition of textile samples enables their distinction from bones.

The PCA scores plot in Figure 35 displays the significant difference between bones and textiles samples. Indeed, bones samples are located in the left side, while textiles, grouped with soft tissues, are located in the right side of the PCA. However, sample Q.23 was closer to the textiles than to the bone groups. This could mean that its degradation was so important that almost no volatile organic compounds characteristics of bones remained within the sample.

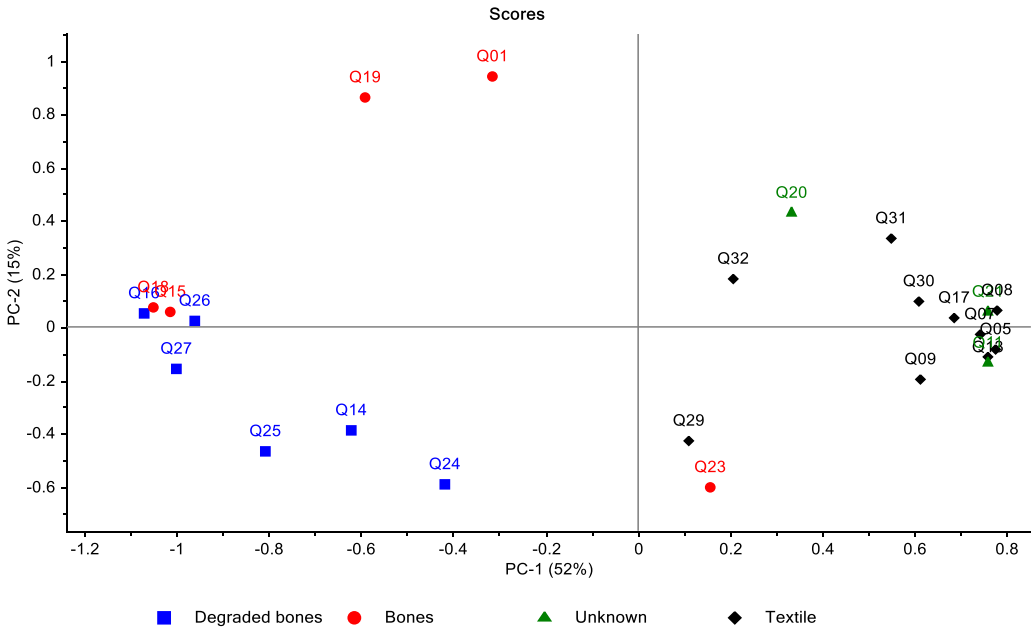

***Figure 35**: PCA scores plot of bones (red and blue), textiles (black) and soft tissues (green). The principal components account for 67 % of the total variance.*

In the same way as bones, mineralized textiles were characterized by different chromatographic pattern which may also be linked to their state of degradation. Textiles covering the dead body were treated with resinous materials and earth pigment before being heated to 200 °C [23, 51, 52]. Assuming that only textile remains from the burial table in chamber 4 (Q.05, Q.07, Q.08, Q.09, Q.13, Q.17, Q.31, Q.32) were thermally treated, their distinction with textiles from chamber 1 (Q.29, Q30) should be possible. In addition, several resinous components, from the treatment of the body during the first burial, should be detected.

The PCA scores plot in Figure 36 supports this hypothesis. Indeed, samples from chamber 4 and chamber 1 are forming two separate clusters. The samples of the burial table are grouped in a distinct area, with the exception of sample Q.32, of the PCA scores plot which accounts for 62 % of the variance. In addition, the textile sample Q.17 recovered in the sarcophagus seems to possess a chemical composition similar to the sample Q.31 from the burial table. The distinction between samples from chamber 1 and 4 resided in a low concentration in alkane, aromatic and ketone derivatives. In addition, although α-pinene was detected in all samples, it was present in a higher concentration in the samples grouped in the right bottom side of the PCA i.e. Q.05, Q.07, Q.08, Q.09 and Q.13. Others resinous compounds were not identified as specific components of textiles coming from the burial table.

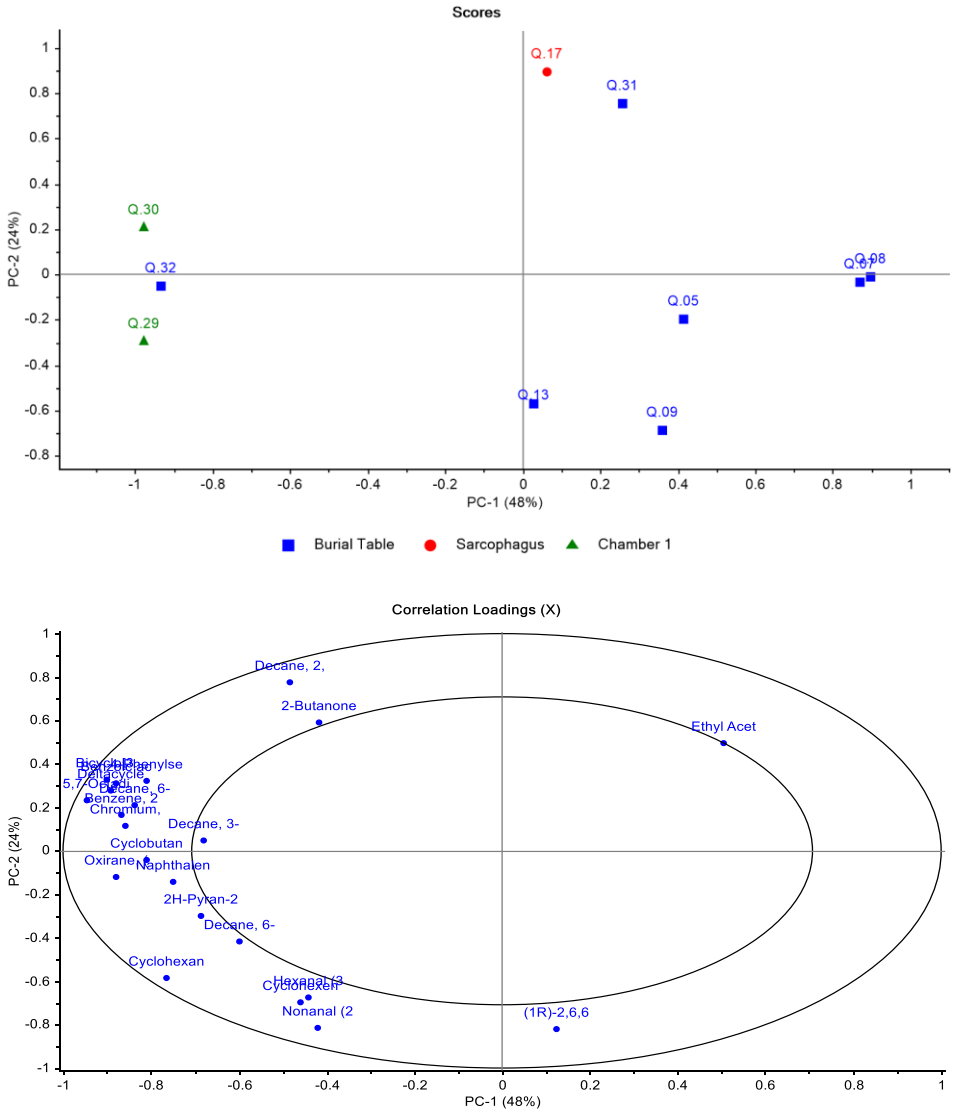

***Figure 36**: PCA scores plot (top) and the corresponding correlation loadings (bottom) representing the different location, burial table (blue), sarcophagus (red) and chamber 1 (green) of the textiles within the Royal Tomb.*

However, in addition to α-pinene, other terpenes were identified in two of the mineralized textiles, samples Q.09 and Q.29. The supplementary terpenes detected in Q.09 were verbenone, camphor, eucalyptol and thujene. It has to be noticed that eucalyptol was identified in all bone samples from the sarcophagus. Moreover, the combination of camphor and eucalyptol was found in the bone sample Q.18 recovered in the sarcophagus. Thus, the sample Q.09 could correspond to the layer of textile directly in contact with the deceased and hence being subjected to the same ritual treatment. Previous analysis by infrared spectroscopy also led to the conclusion that this sample was treated with a mixture of resinous substances and earth pigments [23, 51]. The chemical analysis from the inner side of the earth pigment shows a distinctive loss of hydroxides which could be a trace for heating processes at around 200 °C [23, 51, 52]. Regarding the sample Q.29, verbenone and limonene were characterized.

However, these terpenes were not identified in any of the bone samples. This textile sample, recovered in chamber 1 could have had a different use than recovering the dead body.

Although textile samples from the burial table were all located in the right side of the PCA in Figure 36, they were not forming a close cluster. Therefore, it was assumed that the states of preservation between the samples are differing. The heat map in Figure 37 shows the differences in chemical composition between the samples.

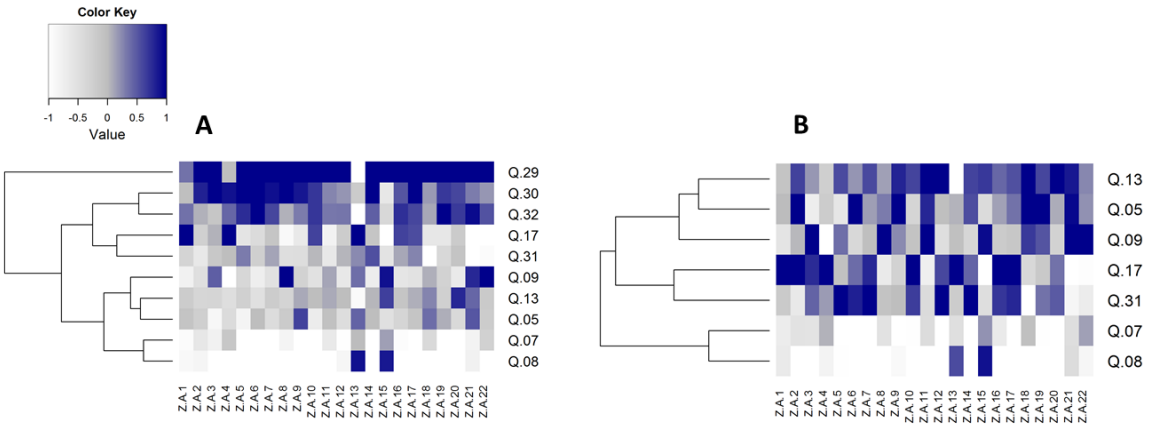

***Figure 37**: Heat map representation of hierarchical cluster analysis (HCA) using the F_{crit} value for the compounds detected within the textile samples.*

This HCA confirms the PCA analysis showing 5 groups within the textile samples (Figure 37-A) and only the compounds of interest, with a F-ratio higher than the F_{crit} were used. As samples Q.29 and Q.30 were coming from chamber 1 and sample Q.30 presented a similar chemical composition, they were excluded from the HCA to further investigate the correlation between samples coming from chamber 4 (Figure 37-B). The samples were divided in three groups composed of Q.05, Q.09 and Q.13 for group 1, Q.17 and Q.31 for group 2 and Q.07 and Q.08 for group 3. Group 1 was composed of a particularly high concentration in aldehyde. The group 2 was separated from group 1 mainly because of its lower content in α-pinene. Regarding the group 3, samples contained less alcohol, alkane, aromatic and ketone derivatives than the two other groups (Figure 38).

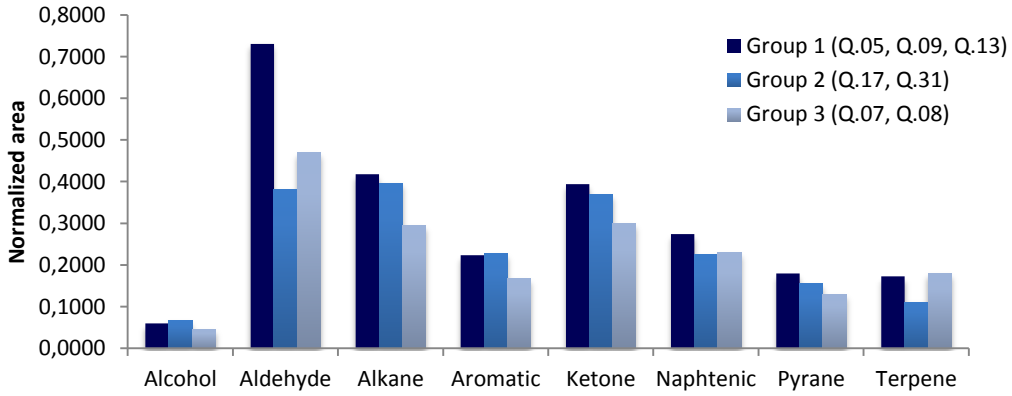

***Figure 38**: Chemical composition of the textiles samples, excluding Q.29, Q.30 and Q.32, using only relevant chemical compounds responsible of the separation between groups.*

Finally, the identification of dye pigments was investigated. Previous analyses have proved the presence of Royal Purple dyestuff within the textile fabrics [51, 52]. In addition, it has been shown that this dyestuff was characterized by two major components, namely indigo and indiburin [23]. However, these two molecules are not volatiles which makes their direct analysis by HS-SPME GC×GC-MS challenging. It has been highlighted that the presence of indole and 2-aminobenzoic acid were linked to the presence of indigo [53]. The analysis of textile samples Q.05, Q.07, Q.08, Q.13, Q.17 and Q.32 reveals the presence of indole derivatives (C_8H_7N) and anthranilic acid derivatives (2-aminobenzoic acid, $C_7H_7NO_2$). Therefore, the presence of these compounds supports the assumption of the use of Royal purple dye in funeral context.

Chapter 4: Conclusion

Headspace SPME-GC×GC-TOFMS proved to be a promising method for the characterization of organic residues recovered on archaeological artifacts. Due to its non-destructive nature, this method can be performed in the sequence of other analyses, which is of particular interest regarding archaeological samples. It allowed the characterization of the volatile profile of organic residues recovered in minimal amount without sample preparation. In addition, specific volatile signatures were successfully distinguished between the sample types.

Different types of raw adhesives (*Pinus sylvestris*, *Picea*, *Fabaceae Birbira*, *Juniper Berrytree*, *Euphorbia* and the glues) were analyzed. These samples were successfully differentiated based on their VOCs profiles. Generally, the glue samples were characterized by a lower amount of terpenes than resin samples. In addition, each kind of samples was differentiated by specific terpenoid and sesquiterpenoid compounds.

By increasing the extraction temperature, the number of extracted terpenoids was increased. However, an excessive extraction temperature can lead to the formation of artifacts and irreversibly damage samples. Therefore, for samples where alteration must be avoided, the extraction temperatures should be maintained below 60 °C.

In addition, the voluntary heated adhesives were analyzed. The heat treatment greatly impacted their composition and led to various volatile signatures. The intensity and the number of detected compounds varied according to the temperature and the length of the treatment. Furthermore, the type of analytes detected after being heated relied heavily on the nature of the resin. Nevertheless, the majority of samples heated to 144 °C were distinguished to those heated to 340 °C by their higher amount in chemical compounds and in particular terpenes, aromatics and hydrocarbons.

Based on the analysis of the samples VOCs composition, it was possible to distinguish organic remains recovered on archaeological stone tools and relate the presence of specific terpenes to specific adhesives. Their comparison with the heated adhesives has also provided insight into their state of preservation.

Regarding the archaeological artifacts recovered in the Royal Tomb of Qatna, the VOCs profile of the heated adhesives was used to investigate their state of degradation. The great

heterogeneity within the sample types made their comparison challenging. Although, significant chemical variations were identified between the types of samples, these differences cannot be directly linked to the state of degradation of the samples. Indeed, numerous factors may be responsible for these differentiations, notably the various amount of organic remains analyzed, the different natures of samples and the complexity due to specific treatment of some samples with petroleum ether.

Finally, it has to be emphasized that all storage, laboratory transfer, analyses and samples treatment prior to HS-SPME-GC×GC-TOFMS analysis induced a great risk of contamination. Therefore, keeping detailed records about sample history after excavation is particularly important.

Chapter 5: Perspectives

This pilot study induced a lot of new open questions and there are numerous future possible approaches for further investigations. First, it would be valuable to investigate the analysis of replicates to strengthen the statistical analyses and to confirm the presence of specific terpenoids within a sample type. In addition, the creation of a database including the VOCs composition of diverse reference resins and protein glues would be useful to identify characteristic terpenoids of a type of adhesives and also to evaluate the adhesive used on archaeological artifacts.

In order to study the degradation process over time, additional ageing studies should be performed. Indeed, the samples can have been exposed to extremely diverse environments leading to significant variations in chemical composition. Therefore, to understand the degradation of VOCs in archaeological context, several laboratory experiments accelerated by diverse factors (temperature, humidity, low oxygen content) should be performed. In a similar perspective, it would be worth establishing a collection of reference samples from specific sites to compare them to real archaeological artifacts. Furthermore, it would be beneficial to perform these ageing studies on diverse natures of samples in order to investigate the influence of the matrix on the organic residues degradation.

Finally, analyses of archaeological samples by HS-SPME-GC×GC-TOFMS should be conducted at the beginning of the archaeological workflow to prevent the risk of contamination as much as possible.

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