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GC-MS and PCA analyses of diterpenoids degradation state in 21 human mummies of Ancient Egypt dating from New Kingdom to Graeco-Roman Period

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Abstract

GC-MS analyses were performed on 25 samples of balms from 21 human mummies, kept in the “musée des Confluences” of Lyon. This study was focused on the diterpenic molecules detected in the organic residues constituting the balms. This chemical family was studied because diterpenoids of Pinaceae resins could be altered due to a number of different chemical reactions, and so they could provide precious information about ageing and/or anthropogenic factors. To obtain greater detail from the results and to illustrate the significance of them, statistical processing and analysis on chromatographic data was applied and has permitted the clear definition of different steps and/or processes of degradation of the abietane type diterpenoid compounds. The statistical results showed that there was a clustering of the samples that correlate with 7-oxo-dehydroabietic and hydroxy-dehydroabietic acids, and another group in correlation with abietic acid. The PC1/PC3 plan gave more information on the state of the samples. Through this plan, 7-oxo-dehydroabietic acid molecules and hydroxy-dehydroabietic acids were positively linked, according to their formation path, contrary to the PC1/PC2 plan. Finally, using

PCA, 5 samples were not degraded and they were well conserved in contrast to the 20 other balms. Among these latter balms, 15 of them have been naturally altered by aging and 5 of them have undergone thermal anthropogenic degradation.

Key words: Ancient Egypt, Human mummification balms, Gas Chromatography-Mass Spectrometry, Principal Component Analysis, diterpenoids, degradation processes.

1. Introduction

Within the framework of a multidisciplinary research project called *Human Egyptian Lyon Confluences Mummies (HEL YCOM)-Mourir pour renaitre*, mummification balms of 21 human mummies were analyzed.

Mummification has been studied since the XIXth century to understand the ritual practices in Ancient Egypt [1–5]. Nowadays it is known that in order to preserve the bodies of the deceased, complex mixtures of organic substances, called balms, were applied on the body of the deceased after it has undergone different stages such as desiccation, evisceration, excerebration etc... A wide variety of natural substances can be used in the production of balms, including plant oil or animal fat, resins, bitumen, beeswax [6–12]. These substances have certain pharmacological properties such as being hydrophobic, antibacterial, antifungal and odoriferous but also they have a sacred, magical and symbolic character during funerary ritual use, in connection with the religious beliefs in rebirth and the Osirian myth [1,12,13]. Because of the molecular complexity of these materials, their identification in an archaeological sample represent a significant challenge. Indeed, these materials could undergo oxidation, degradation and alteration processes, naturally over time (aging) or due to anthropogenic factors. Degraded molecules could provide an important role in the characterization and identification of the balm components [14,15]. Thus, in order to understand the methods of preparation and the common use of each ingredient but also related to the natural and unintended degradation of the material over time, it was necessary to evaluate the involved reactions [16–18]. Currently the most common technique used is the Gas Chromatography coupled to Mass Spectrometry (GC-MS) [15,19–22] because this analytical tool is able to separate and identify a very large number of organic compounds, even in the form of

traces, from complex matrices. In particular, GC-MS could detected many molecules belonging to Pinaceae resins and more particularly the abietane compounds which could be degraded in many different ways [23–26]. The characterization of these compounds could provide precious information about the preparation of balms as well as the types of degradation they could undergo. In addition to the GC-MS, it was possible to apply statistical data processing. The use of statistical analysis has permitted the graphic presentation of the information allowing the clear illustration of the significance of the data, not easily apparent from the data presented in the table. The most common statistic is Principal Component Analysis (PCA) [27–29]. Graphically the PCA provided a visualization of variables and data or both simultaneously on a biplot representation [30].

2. Research aim

The aim of this study was, by applying PCA analysis on the data obtained by GC-MS, to define the degradation state of resins contained in 25 balms samples of 21 mummies. The objective was to obtain a graphical representation to clearly define the state of degradation (natural and/or anthropogenic) in the studied archaeological samples. In the end, this will allow us to learn more about the aspect of funeral practices and the preparation of resins, for example by using a heat treatment.

3. Material and method

3.1. Archaeological material

13 mummies' heads and 8 complete mummies were chosen randomly for balms analysis. The mummies' heads were brought back from Egypt by the anthropologist Ernest Chantre [31], who had constituted the museum collection of Osteology with 250 Egyptian heads and skulls. Several mummies have been previously beheaded, and only their heads have been brought in France, to enrich the museum anthropological collection. As a result, much information about the mummification processes of these individuals, their places of burial, their possible identity, were forever lost [10].

1 The mummies came from Upper Egypt where Charles-Louis Lortet, the first dean of
2 the faculty of Medicine of Lyon, then director of the “musée d’Histoire naturelle” in
3 Lyon during forty years, had made excavations [32].

4 In this study, 21 individuals, divided into 13 mummy heads, 8 whole bodies and
5 composed of 13 men, 6 women, 1 girl and 1 head with indeterminate sex, of different
6 ages were analyzed (Table 1). Originating from the archaeological sites of Upper
7 Egypt (Thebes, Esneh and Kom Ombo), the mummies cover a broad temporal
8 spectrum, ranging from the New Kingdom to the Graeco-Roman Period. 25 balms
9 samples were taken either directly on the mummified skin, on the textile, or inside the
10 balm mass, while respecting the mummy’s integrity. Some samples were taken on
11 the mummy (external samples), some others, inside the skull or the abdominal cavity
12 (internal samples). The operator was wearing gloves and took the sample after
13 selection of the mummy’s part with a scalpel.

14 Two samples were obtained, per individual, for 4 of them. Samples on the inner part
15 of the neck (N° 17) and on the outer part of the skull (N° 18) were taken from the
16 heads reference 30000148. Samples on the inner part of the neck (N° 10) and the
17 mandible (N° 11) were taken from the heads reference 30000286. Samples of the
18 mummies 90001257 were performed on the inner part of the abdominal pad (N° 43)
19 and on the outer part of the abdominal cavity (N° 69). And samples of the mummies
20 90001402 were performed on the top of the skull (N° 8) as well as on the pubis (N° 9)
21 for the second one.

23 3.2. Standard molecules and reference materials

24 Commercial standard molecules such as dehydroabietic, 7-oxo-dehydroabietic acids
25 and retene came from Sigma-Aldrich. Two diterpenic resins were used as
26 references. The first one a fresh colophony resin (Kremer Pigmente GmbH & Co KG)
27 and the second one an aged resin (Kolophonium hellst, Kremer Pigmente GmbH &
28 Co KG) but kept away from light and oxygen.

30 3.3. GC-MS apparatus

31 The analyses were carried out with a ThermoFisher apparatus equipped with a 8-slot
32 AI3000 automatic injector and a Focus GC chromatograph coupled to an ITQ 700 ion

trap type mass spectrometer. The capillary column used was a GOLD TG-5MS Thermo trace (5% diphenyl and 95% dimethyl polysiloxane) with an internal diameter of 0.25 mm, a length of 30 m and a film thickness of 0.25 μm . The carrier gas used was helium with a constant flow rate of 1 $\text{mL}\cdot\text{min}^{-1}$. The injector, the transfer line and the ion trap were respectively at 250, 300 and 200 $^{\circ}\text{C}$. The electronic impact source had an ionization energy of 70 eV, an ionization time of 25,000 μs and a scan in the mass range of 40-650 m/z . 1 μL of solution was injected in splitless mode. The software used was Xcalibur and the identification of the different compounds was performed by comparing the retention times and mass spectra with commercial standard molecules and from the NIST 08 V.2 database.

3.4. Analytical procedure

10 mg of sample were extracted with 1 mL of dichloromethane (DCM) (Merck) under ultrasound for 10 min and then centrifuged at 6000 rpm for 10 min. The supernatant was set aside to extract the solid pellet again. This step was performed 2 more times. The three fractions thus collected were then combined and evaporated to dryness under a stream of nitrogen. After having undergone a trimethylsilylation reaction with 200 μL of BSTFA/TMCS (99/1, v/v) (Sigma Aldrich) and heated at 70 $^{\circ}\text{C}$ for 30 min, the mixture was again evaporated to dryness and then dissolved in 0.2 to 1 mL of a hexane/DCM mixture (1/1, v/v) and filtered on a polytetrafluoroethylene cartridge (0.45 μm , VWR). 1 μL of the solution was injected into GC-MS.

The oven temperature program started at an initial temperature of 50 $^{\circ}\text{C}$, constant for 2 min, then increased from 10 $^{\circ}\text{C}/\text{min}$ to 200 $^{\circ}\text{C}$, followed by a second increase of 2.5 $^{\circ}\text{C}/\text{min}$ to 310 $^{\circ}\text{C}$ and finally a final rise of 8 $^{\circ}\text{C}/\text{min}$ to 330 $^{\circ}\text{C}$, after which temperature was maintained for 3 min. The total analysis time was 67 min.

3.5. Statistical processing of data

The Principal Component Analyses was performed with XLStat software using Pearson correlation that automatically standardizes the data prior to computations to avoid inflating the impact of variables with high variances on the result.

1

2 4. Results and discussion

3 All of the samples and both reference materials, a fresh diterpenic resin (Rf) and a
4 diterpenic resin slightly aged (Ra) were analyzed by GC-MS. This study has been
5 **focus** specifically on the diterpenic compounds and **more particularly** abietanes
6 molecules have been considered because pimaranes molecules are less sensitives
7 to the degradation due to their relative stability [33]. Abietane structures have
8 conjugated double bonds, which could react in particular conditions. Understanding
9 the reaction mechanisms of abietanes degradation, known in the literature, has
10 permitted the interpretation of statistical data. Through a non-exhaustive summary of
11 possible reactions (Fig. 1) it was possible to **realize a scheme** representing the
12 alteration pathway of each type of markers detected in the 25 samples of
13 mummification balms.

14 The chromatographic results of a fresh diterpenic resin, a slightly aged colophony
15 resin and a mummy balm containing a high proportion of diterpene resin were
16 compared (Fig. 2). On the chromatogram of the fresh resin (Fig. 2A), it was possible
17 to identify dehydroabietic acid (DHA), abietic acid in very large quantities and traces
18 amounts of DHA derivatives such as 3-hydroxy-DHA, 7-hydroxy-DHA and 7-oxo-
19 DHA. Abietic acid can be considered as a marker of a fresh resin because it is a
20 biomarker initially present in Pinaceae resin and in large quantities.

21 The chromatographic analysis of the slightly aged resin stored in the laboratory
22 **revealed** the presence of abietic acid **in small quantities and a large quantity of** DHA
23 (Fig. 2B). Abietic acid tends to decrease or even disappear over time leading to
24 **formation** of DHA (A1) by **dehydrogenation/aromatization** [16] (Fig. 1). From the DHA
25 several pathways are possible depending on the type of formulation and the type of
26 degradation. The action of oxygen in the air could lead to oxidation reactions of A1. It
27 is a natural process that will preferentially lead to the formation of 7-hydroxy-DHA
28 (A3), 15-hydroxy-DHA (A2), 3-hydroxy-DHA (A5) and 7-oxo-DHA (A4) by
29 incorporation of hydroxyl groups or ketones [34] (Fig. 1). **These molecules were**
30 **detected in small quantities in the resin aged**. Thus for the analyses DHA will be used
31 as a marker of slight oxidation.

32 The chromatogram of the sample No. 53 contained a small quantity of DHA, a very
33 large quantity of DHA derivatives with notably 7-oxo-DHA and 15-hydroxy-7-oxo-

DHA (Fig. 2C). Indeed, isomerization and oxidation of A2, A3 and A4 are possible to give 7,15-dihydroxy-DHA (A6) and lead to 15-hydroxy-7-oxo-DHA (A7) [35] (Fig. 1). 7-oxo-DHA (A4) and 15-hydroxy-7-oxo-DHA (A7) can be used as a marker of a resin having undergone a strong oxidation i.e. a strong natural ageing with time.

All of the other archaeological samples were analyzed. The molecules detected were DHA, 3, 7 and/or 15-hydroxy-dehydroabietic acid (hydroxy-DHAs), 7-oxo-DHA, 15-hydroxy-7-oxo-DHA and retene. The formation of this last compound is shown in Figure 1. When the material was heated at temperatures exceeding 300°C, DHA (A1) it underwent a decarboxylation reaction and gave 18 and 19-norabietantriene (A8). An aromatization of A8 was realized to achieve tetrahydroretene (A9). A theoretical study permitted consideration of another possible pathway starting from A1. These molecules undergo dehydrogenation to give the dehydro-dehydroabietic acid (A11) which could undergo an aromatisation to give an intermediate A12 and thus lead to A9 [16]. Finally, the molecule intrinsic to resin, dehydroabietane (A13, although dehydroabietane is not a significant component in fresh Pinaceae resins) [36], could undergo aromatisation and give simonellite (A14) [37]. Simonellite, by decarboxylation, leads to A9 [38]. Thus tetrahydroretene (A9) could be formed in three different pathways, as presented above. This molecule undergoes a complete aromatization leading to retene (A10) [39], the ultimate stage. This molecule is thermodynamically the most stable and is a marker of thermal degradation by intense heating or pyrolysis. All of the relative proportions of the peak area of diterpenic molecules were in Table 2.

Most of the interpretation presented in this paper could have been stated without the statistical analysis/processing but because of the great number of the archaeological samples and to obtain more useable data, statistical processing was performed using data in table 2. Concerning PCA it was chosen to take into account 4 variables, the abietic acid as a marker of fresh resin, DHA as a marker of **a resin with a good preservation**, retene as a marker of anthropogenic degradation by intense heating and 7-oxo DHA as well as hydroxy-DHAs as markers of aging natural degradation. The principal component analysis indicated that 2 main components (PC) explained 76.14% of the variability, with 49.25% for PC1 and 26.89% for PC2. The variance explained by the main components is directly related to the correlation between the original variables. In the present research, there was a significant correlation

1 between the different abietanes molecules. On the biplot representing axes PC1 and
2 PC2 (Fig. 3), there were 4 variables: abietic acid, DHA, hydroxy-DHAs/7-oxo-DHA
3 and retene. They were considered in order to obtain information about the natural
4 and/or anthropogenic degradation of the analyzed mummies. Two variables were
5 correlated between them corresponding to DHA and abietic acid. This correlation
6 could be explained because these two molecules provided similar information. As a
7 reminder, abietic acid is the marker of a fresh resin and DHA is the marker of a resin
8 with a **good preservation**. Samples 18, 40, 63, 64 and 65 contained a large
9 proportion of DHA (A1), which implied good preservation of these **balms**. It was also
10 possible to observe that the two reference resinous materials, fresh resin (Rf) and
11 aged resin (Ra), were correlated respectively with the two variables abietic acid and
12 DHA as expected. Samples 1, 2, 9, 12, 14, 16, 41, 43, 46, 47, 48, 53, 58, 66 and 69
13 were correlated with the variable hydroxy-DHAs/7-oxo-DHA, which implied a
14 degradation by oxidation of these samples. Then five samples: 4, 8, 10, 11 and 17
15 were correlated with the variable retene, so these samples have undergone
16 anthropogenic degradation, which means an intense heating process of the resin.

17 Concerning methyl dehydroabietate ester (A15) [40], [35] it was obtained when the
18 resinous wood was heated, methanol was stirred and reacted with carboxylic
19 functions of diterpenoids leading to methyl ester formation. It should not rule out the
20 possibility of a strongly heated resin also producing methyl dehydroabietate in balms.
21 This molecule was detected in the samples 2, 11, 12, 17, 18 and 64. Methyl
22 dehydroabietate ester was not considered as a parameter for the statistical study
23 because this compound was associated with wood tar, so it was a marker of
24 preparation mode and not a marker of degradation.

25 In parallel, it could be noted that for a same mummy with two samplings in different
26 places, the obtained results were not necessarily identical. Indeed, concerning the
27 mummy balm 30000148, two samples were taken: the first one from the inner part of
28 the neck (N° 17) and the second one from outer part of the skull (N° 18). Both of
29 these samples did not undergo the same type of degradation. For N° 17, the balm
30 was definitely heated to over 300°C during its formulation, while sample referred to
31 as sample 18 was slightly degraded. Similarly, for the samples N° 8 and 9 from the
32 mummy balm 90001402, the balm from the mummy's skull (N° 8) was heated, while
33 the other one from the pubis (N° 9) has undergone natural aging. However, this

observation should be moderated, although lower in retene than sample 17, sample 18 also suggests it has been heated, but perhaps less so. And, again, with sample 9 the chemical evidence does suggest some degree of heating, albeit appreciably less so than sample 8 from the same mummy. Samples 18 and 9 have undergone greater oxidative degradation compared to 17 and 8, respectively. The explanation of this difference could be attributed to the fact that internal balms required a greater heating to obtain a better liquefaction in order to fill all the cavities, after evisceration. So, the application of balms was made using a two-stage operation.

5. Conclusion

This present study has permitted, by using statistical analysis, the assessment and determination of the different states of degradation concerning the diterpenoids. Statistics were efficient because of a consequent number of samples studied and so the results obtained could be considered meaningful. This study could serve as a useful model, by simply adding a sample, therefore the nature of its degradation could be defined in this way.

Among the 25 balm samples studied by GC-MS, 5 of them were relatively well preserved because of the dominant presence of dehydroabietic acid. Fifteen (15) of them have undergone natural aging, specifically oxidation, being characterized by 7-oxo-dehydroabietic acid and hydroxy-DHAs. Finally, 5 samples were subjected to very intense heating with a minimum of 300°C in view of the retene detected as a large proportion of the abietane diterpenoids. In addition, the results of samples collected from a same mummy from different places has revealed that the employed Pinaceae resin contained different chemical markers, reflecting different states of degradation and evidence for differential processing. It was probably due to the presence of different balms applied during the process of mummification. In this case, the characterization of the different kinds of abietane markers was a better method of assessing balm recipes.

The sample analyses provided an absence or a slight oxidation indicating a very good conservation of the balms, certainly due to the storage conditions of the mummies. The resins having undergone anthropic degradation such as an intense

1 heating, could have two different origins depending on the balm formulation: (i) the
2 resin alone was heated before mixing it with the other ingredients of the balm or (ii) a
3 global heating was applied to all of the substances contained in the balm during its
4 production.

5 The interest was to study specific biomolecules found in balms in order to obtain
6 information on the degree of degradation and/or evidence of any anthropogenic
7 treatment to learn more about funeral practices in Ancient Egypt. The research
8 presented here only allows one balm component to be assessed in this way and so it
9 may also be useful to apply statistical analysis/processing to other balm components
10 also susceptible to oxidative changes and thermal degradation (e.g. fatty acids,
11 triterpenoids).

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Figure captions:

Table 1: Data of archaeological samples

Table 2: Relative percentage of peak areas from diterpenic molecules.

Figure 1: Proposition of path degradation and alteration of abietanes (*Pinaceae* resins).

Figure 2: TIC chromatograms of fresh diterpenic resin (A), slightly aged diterpenic resin (B) and sample N° 53 (C).

Figure 3: Biplot diagram of original variables ordering and scores in the first two principal components.

1 Table 1: Data of archaeological samples.

No sample	Inventory nr	Specificity	Sexe	Age	Localisation	Period
1	30000114	Head	Female	Old	Thèbes	Ptolemaic to Roman Period
2	30000152	Head	Female	Teenager	Thèbes	Graeco-Roman Period
4	30000134	Head	Male	Adult	Gournah	Third Intermediate Period to Late Period
aa8	90001402	Body	Male	Adult	Unk.	Third Intermediate Period to Late Period
9	90001402	Body	Male	Adult	Unk.	Third Intermediate Period to Late Period
10	30000286	Head	Male	Mature adult	Esneh	Third Intermediate Period to Late Period
11	30000286	Head	Male	Mature adult	Esneh	Third Intermediate Period to Late Period
12	30000143	Head	Male	Adult	Thèbes	Third Intermediate Period to Late Period
14	30000110	Head	Male	Adult	Thèbes	Roman Period
16	30000139	Head	Female	Young adult	Gournah	Ptolemaic to Roman Period
17	30000148	Head	Male	Old	Thèbes	Unk.
18	30000148	Head	Male	Old	Thèbes	Unk.
40	90001258	Body	Male	Adult	Gournah	Graeco -Roman Period
41	90001169	Body	Male	Young adult	Thèbes	Late Period to Ptolemaic Period
43	90001257	Body	Male	Adult	Gournah	Unk.
46	90001948 A	Head	Male	Adult	Gournah	New Kingdom to Third Intermediate Period
47	90001259	Body	Female	Adult	Kôm Ombo	Ptolemaic to Roman Period
48	90001265	Body	Male	Mature adult	Unk.	Late Period to Ptolemaic Period
53	90001598	Body	Female	Adult	Unk.	New Kingdom
58	90001953 A	Head	Male	Adult	Gournah	New Kingdom to Third Intermediate Period
63	30000142	Head	Male	Adult	Gournah	Late Period to Ptolemaic Period
64	90001955 A	Head	Female	Adult	Gournah	Unk.
65	90001954 A	Head	Unk.	Adult	Gournah	Ptolemaic to Roman Period
66	90001597	Body	Female	Adult	Unk.	Late Period to Ptolemaic Period
69	90001257	Body	Male	Adult	Gournah	Unk.

1

2 Table 2: Relative percentage of peak areas from diterpenic molecules.

No sample	Retene	DHA	7-oxo/hydroxy DHAs	Abietic acid
1	0	16.01	83.99	0
2	0	0	100	0
4	18.3	34.85	46.84	0
8	36.4	5.9	57.7	0
9	10	0	90	0
10	28.41	0	71.59	0
11	21.5	30.3	48.2	0
12	0	21.43	78.57	0
14	4.44	21.14	74.43	0
16	0	16.17	83.83	0
17	28.5	39.5	32	0
18	7.54	43.28	49.18	0
40	0	62.18	30.47	7.35
41	0	34.88	65.12	0
43	0	10.54	89.46	0
46	15.74	0	84.26	0
47	1.47	7.7	90.83	0
48	0	26.93	73.07	0
53	0.05	8.13	90.51	0
58	0	30.36	69.64	0
63	0	78.93	21.05	0
64	0	58.9	33.33	0
65	0	29.82	67.05	0
66	0	0	100	0
69	1.25	4.62	94.14	0
Rf	0	17.36	2.71	79.91
Ra	0	51.2	23.46	24.7

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4 *Rf: fresh diterpenic resin; Ra: slightly aged diterpenic resin.*

Figure 1: Proposition of path degradation and alteration of abietanes (*Pinaceae* resins).

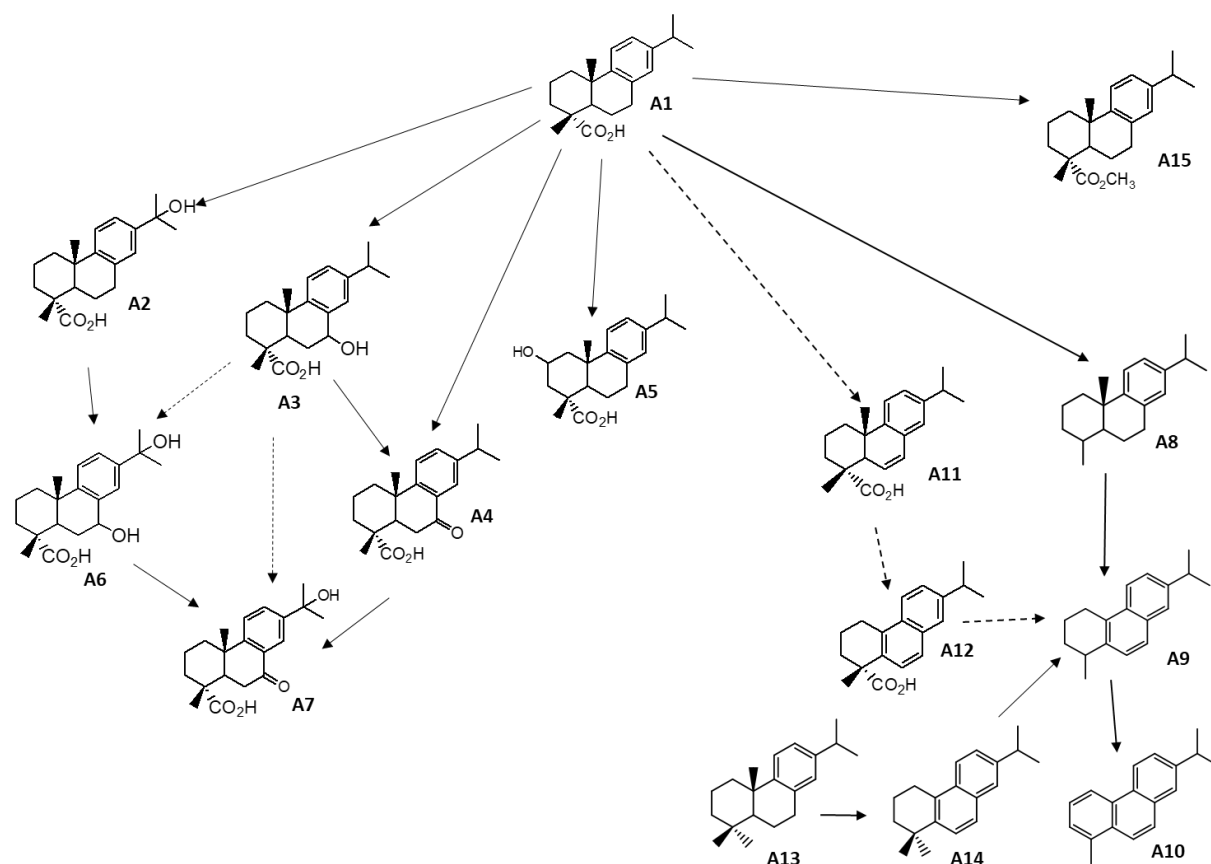


Figure 2: TIC Chromatograms of fresh diterpenic resin (A), slightly aged diterpenic resin without light and oxygen (B) and sample N° 53 (C).

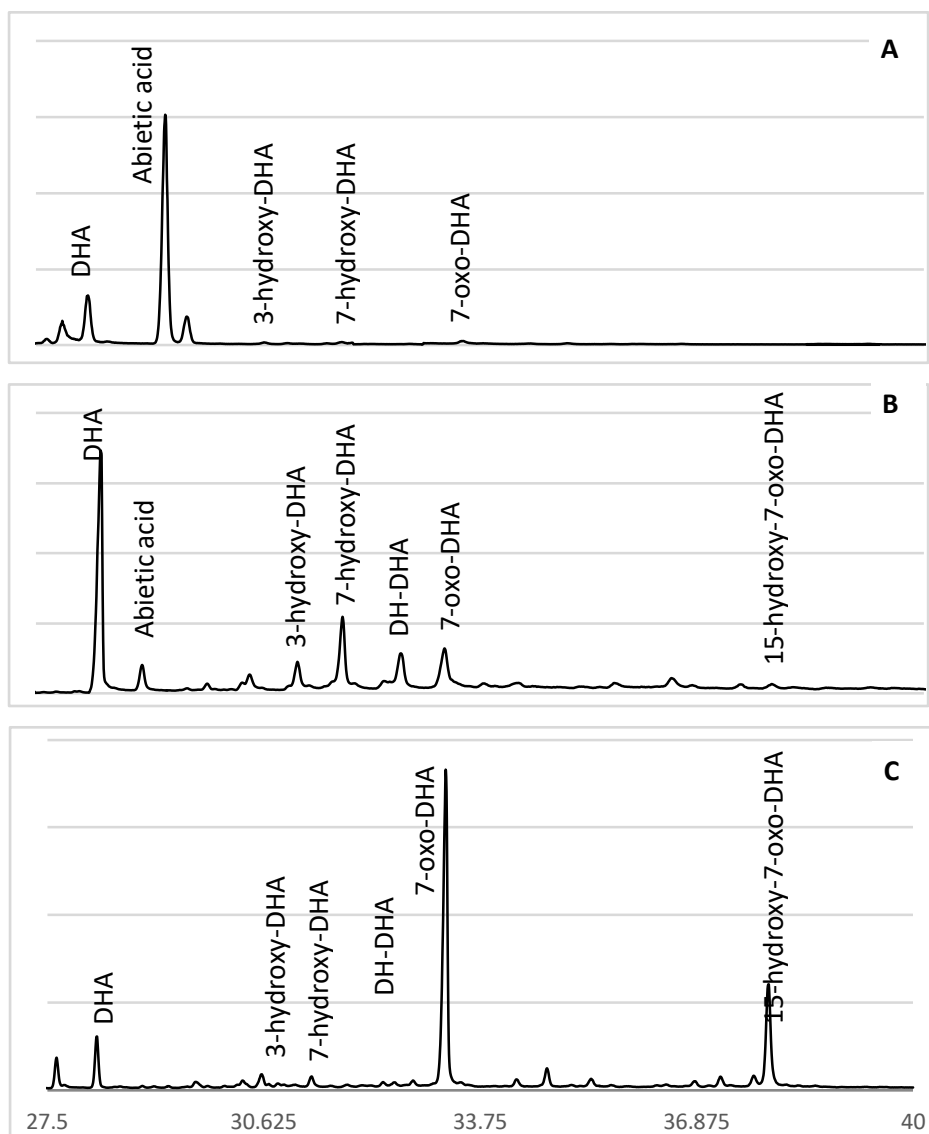


Figure 3: Biplot diagram of original variables ordering and scores in the first two principal components.

