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Characterization of thermally assisted hydrolysis and methylation products of polyphenols from modern and archaeological vine derivatives using gas chromatography–mass spectrometry

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Abstract

If some ceramics, such as vinary amphorae attest the consumption and trade of wine in the Roman world, the first wine productions in Occident often stay undocumented. Chemical analysis of organic materials preserved in archaeological vessels is the only way to bring new lights about the elaboration and the consumption of this fermented beverage. To determine the preservation state of wine and other grape derivatives residues, we proceeded to in situ tetramethylammonium hydroxide (TMAH) treatment followed by thermally assisted hydrolysis and methylation—gas chromatography—mass spectrometry (THM–GC–MS). The objectives of the study were (i) the understanding of the pyrolytic mechanisms of proanthocyanidins and (ii) the assessment of the usefulness of thermochemolysis for the identification of phenolic markers from polymeric solid deposit in modern wine bottle and Roman amphorae. THM–GC–MS was revealed to be an efficient method for the characterization of fruit derivatives even if mixed with another organic material, such as pitch used to ensure the watertightness of the ceramic container. The preservation of tannins during millennia in archaeological context is here enlightened for the first time by using analytical pyrolysis. The proposed identification of vinary residues is now based on the detection of the association of more than 30 pyrolytic markers derived from di- and trimethoxylated benzenoid compounds. THM–GC–MS represents a new method for the rapid detection of wine traces in ancient ceramics, adapted to tiny samples (<0.1 mg), allowing to precise the role and function of pottery during antiquity.

Keywords: Thermochemolysis; GC–MS; Tannins; Wine; Grape seeds; Roman and Gallo-Roman periods; Archaeology

1. Introduction

Chemical analyses of organic residues preserved in ancient ceramic vessels play a major role for reconstructing the evolution of culinary customs and diet through time. Until now, most of the studies have focused on the characterization of ancient lipids from animal origin, such as dairy products [1,2], animal fats [3] or beeswax [4,5], or from plant remains containing either fatty acids [6] or di- and triterpenoids [7,8]. However, among the molecular constituents possibly preserved in ancient pottery, phenolic compounds have often been underestimated although their degradation products, phenolic acids, have been detected in some

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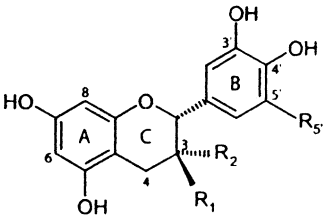
Table 1
General scheme of grape monomeric flavan-3-ols



R_1	R_2	$R_{5'}$: H (procyanidol)	$R_{5'}$: OH (prodelphinidol)
OH	H	(+)-Catechin, C	
H	OH	(−)-Epicatechin,	(−)-Epigallocatechin, EgC
H	*O*-galloyl	(−)-Epicatechin-3-*O*-gallate, ECG	(−)-Epigallocatechin-3-*O*-gallate[a], EgCG

(A, B, C) are ring labels, (4,6,8) carbon positions for C4–C6 or C4–C8 interflavanic linkages involved in the formation of proanthocyanidins.
[a] EgCG is not present as main constituent in grape but is studied in this work as a polyphenolic standard.

Roman amphorae [9,10]. Such organic compounds are well distributed in the plant kingdom [11] and may be used as biomarkers of wine. This fermented beverage has played a very important economic, social and sanitary role in ancient societies as shown by Latin and Greek texts and Graeco-Roman mythology. According to the Greeks, vine and wine were gifts from the god Dionysus; but the first wine productions, notably in Etruria,[1] are not documented. The detection and identification of the phenolic compounds still preserved in archaeological ceramic vessels would be a great opportunity to bring new lights on the quality and the wine-making process of this fermented beverage during Antiquity.

The oldest chemical evidences for the consumption of vine derivatives were shown by McGovern and coworkers: the identification of calcium salt of tartaric acid by infrared spectrometry and liquid chromatography on the inner side of a Neolithic pottery in the site of Hajji Firuz Tepe (Iran, 5400–5000 B.C.) and in various other ceramic vessels allowed the authors to determine the use of ancient pottery for the storage of a vine derivative or the wine-making process [12–16]. Pioneer analyses of archaeological potsherds from the Roman shipwreck *Madrague de Giens* (70–60 B.C.), performed by Formenti and coworkers, revealed the preservation of both tartaric acid and phenolic derivatives as traces: alkaline fusion by KOH at 300 °C released tartaric and gallic acids, grape biomarkers [9,10] in different kinds of amphorae (Lamboglia 2, Haltern 70 and especially Dressel 1B).

Although various polyphenolic constituents, such as alizarin, purpurin or other anthraquinones and gallic acid were already identified in dyes from ancient textiles using HPLC [17,18], very few analytical studies were dedicated to the research of polyphenols in archaeological wine residues. These studies were mostly based on the detection of tartaric acid, although few phenolic compounds were shown to be preserved at a trace level.

However, phenolic acids, derived from benzoic and cinnammic acids, and from flavonoids including monomeric (flavanols, flavones, flavanonols, anthocyanidins, etc.) and polymeric compounds (proanthocyanidins also named condensed tannins) are characteristic of vine derivatives as well as of other fruits. In grape seeds, oligomeric and polymeric tannins were shown to be procyanidins characterized by a double hydroxylation (3′,4′) of the B ring, consisting of three flavan-3-ol units: (+)-catechin (C), (−)-epicatechin (EC) and epicatechin-3-*O*-gallate (ECG). Prodelphinidins, consisting of flavanol units with 3′,4′,5′ trihydroxylated B-ring, especially epigallocatechin (EgC), occur mainly in grape skin tannins (31–41% of the constitutive units) [19] and, with lower abundance, in stem tannins (2.2–10.1%) [20] (Table 1). Tannin constitutive units are linked to each other through C4–C8 or C4–C6 B-type interflavanoid bonds and some of

[1] Etruria is the Italian coastal area between the Tibre river (Roma) and the Arno river (Pisa, Firenze), occupied by the Etruscans since the XIIth century B.C., a civilization who controlled the West Mediterranean area before the emergence of the Roman Empire.

them are acylated by a gallic moiety on the 3-*O* position. Their degree of polymerization (DP_n) ranges from 2 to 15 [21], whereas in grape skin, DP_n is from 3 to 80 with <10% as oligomers up to decamers [19,20].

In order to assess the preservation degree of polyphenols from various archaeological samples and to study the possibility of using polyphenols as biomarkers of ancient fermented beverages, we implemented THM in an analytical procedure adapted to the specificity of such samples which are usually highly altered and preserved in low amount. To better interpret the results obtained from archaeological samples containing wine from Roman amphorae, a series of commercial standards and of contemporary natural products (wine and grape seeds), as well as archaeological grape seeds preserved in wet and dry contexts, were also analysed.

Although different soft chemical depolymerization procedures (Bate-Smith test [22,23] and acidic depolymerization in the presence of α-toluenethiol, i.e. thiolysis [24,25], or phloroglucinol [26]), currently used for the study of contemporary wine, were investigated on archaeological samples in this study, they were shown to be inefficient for the analysis of such residues, probably due to their high degree of degradation and polymerization. However, an improved extension of the pyrolysis method involving a thermally assisted hydrolysis–methylation (THM, also named thermochemolysis [27]) of the sample with tetramethylammonium hydroxyde (TMAH) was used for the structural elucidation of these degraded materials. Coupled with GC–MS technique, thermochemolysis was revealed to be well adapted to the study of polymerized phenolic compounds from archaeological samples, leading to the identification of several phenolic molecular markers arising from procyanidin and prodelphinidin precursors.

2. Experimental

2.1. Samples

2.1.1. Standards, solvents and reagents

The following polyphenols were analysed: (+)-catechin (C), (−)-epicatechin (EC), epigallocatechin (EgC) and epigallocatechin-3-*O*-gallate (EgCG), from Sigma Aldrich, MI (Table 1). These compounds are of interest as model monomeric polyphenols because of the structural composition of natural fruit polyphenols. In grape, the main flavan-3-ols units of the proanthocyanidins are catechin, epicatechin, epicatechin-3-*O*-gallate (ECG) and epigallocatechin (EgC) [20]. Due to the lack of commercial availability, ECG was replaced by its 3′,4′,5′-trihydroxylated derivative, EgCG. Solvents (HPLC grade) were purchased from Prolabo, TMAH (25% in MeOH) and trifluoroacetic acid (TFA) from Sigma Aldrich (ACS reagent grade).

2.1.2. Modern and archaeological vine derivatives

Various grape seeds and skins were harvested at commercial maturity and analyzed to provide reference analytical data for the study of archaeological materials by the THM method. Details of the samples are presented in Table 2. Red and white wines were selected as modern reference for this work, with different ages (young or aged wines, from 5 to 10 years old) and diverse preservation degrees (wine and wine

Table 2
Modern grape seeds, grape skins and wines analyzed

Code	Name and origin	Nature
Chard	*V. vinifera* var. Chardonnay	Grape seed
Pinot	*V. vinifera* var. Pinot noir	Grape seed
Chas	*V. vinifera* var. Chasselas blanc	Grape skin
Musc	*V. vinifera* var. Muscat of Alexandria	Grape skin
Bjls	Beaujolais, 1999	Red wine
Pom	Lalande de Pomerol, Château Haut Caillou, 1998	Red wine
Med[a]	Médoc, Château Cardonna-Lahourcade 1996	Red wine
Bdx[a]	Bordeaux rouge collection privée Cordier, 1998	Red wine
Smr	Saumur, 1999, oxidized in vinegar	Oxidized red wine
Smr-d	Solid deposit from Saumur, 1999	Brown deposit
Mscdt	Muscadet, 1999	White wine
Tur	*Turriculae* 1999, white sweet wine made according to the Pline the Elder recipe [51]	White sweet wine
Tur-d	Solid deposit from *Turriculae* 1999	Brown deposit
Cal[a]	10 years old Red Port wine, Calem	White sweet wine
Cal-d[a]	Solid deposit from the red Port wine Calem	Brown deposit

[a] Wine-making process including the *élevage* in oak barrels.

Table 3
Sampling of archaeological grape seeds and wine

Code	Provenance (department)	Nature	Period
Latt	*Lattara*, Lattes (34)	Grape seeds	Iron Age (Vth century B.C.)
Msl	Jules Verne 11, US397, *Massalia*, Marseille (13)	Grape seeds	Greek (IIIth–IIth century B.C.)
Grav	La Roque, Graveson (84)	Grape seeds	Iron Age (IIth century B.C.)
Perd	Roman shipwreck Sud-Perduto II, South Corsica (2B)	Grape seeds	Iron Age (IIth century B.C.)
Giens	Wine preserved in stoppered Dressel 1-type amphora from the shipwreck *Madrague de Giens*, Giens (84)	Wine	Iron Age (70–60 B.C.)
Defr	*Defrutum* containing olives, in a Haltern 70-type amphora from the shipwreck Port-Vendres II (66)	Reduced sweet wine	Claudius reign (41–54 A.D.)

oxidized in vinegar, reflecting the potential oxidative mechanisms during wine storage in amphorae). The chosen 10-years old red Port wine is characterized by a similar wine-making process as the *Turriculae* wine, a modern sweet white wine made in South of France from Villard blanc variety [28], according to ancient recipe detailed by Latin agronomists, especially Columella [29]. The ancient process, prohibiting the ullage[2] of the storage container, leads to the formation of a *saccharomyces* yeast film, yielding an oxidized beverage similar to modern yellow wine, Andalusian *oloroso* or Port wines [28]. Columella reports that wine must be stored in amphorae or *dolia*, these latter corresponding to storage vessels which can contain from 800 to 3000 l of liquid. With the intention to provide a good waterproofing and a sufficient sanitarization, amphorae and *dolia* had to be pitched with a heated resin obtained from crude wood or bark of *Pinus sp*. [29]. So the alcoholic beverage extracts parts of the resinous compounds and becomes flavoured [28]. Among the modern wines studied, three were *élevés* in oak barrels during the wine-making process (Med, Bdx and Cal, Table 2).

Non-carbonized grape seeds were sampled during recent excavations from various contexts: dry (Graveson), wet and terrestrial (*Lattara* and *Massalia*) or submarine (Sud-Perduto II, Table 3). Grape seeds were chosen as model ancient polyphenolic material, because of their preserved morphology allowing to identify domestic *Vitis vinifera* sp. The solid structure of the seed lets us think that phenols are better preserved through time than wine polyphenols in aqueous solution because the rigid organization of the material limits the interactions with the external sedimentary medium.

Two Roman wines were preserved during two millennia in stoppered and sealed amphorae. One comes from a vinary amphora Dressel 1-type from the shipwreck *Madrague de Giens*. This cargo boat traded a wine consisting mainly in *Caecuban*, a luxurious white wine produced in the North Campania. The other was preserved in another type of vinary amphora, Haltern 70, loading of the shipwreck Port-Vendres II coming from *Baetica* (present Andalusia). The contained beverage, called *defrutum*, is a red wine boiled down and reduced to the third [29]. The obtained sweet syrupy beverage was used for the must chaptalization or for the foodstuff preservation, olives in the present case.

2.2. Method

2.2.1. Extraction and fractionation of polyphenols from modern grape seeds, skins and wine

If grape skins present a high content of solvent extractable phenolic compounds, grape seeds and wine are mainly constituted of lipids and sugars respectively, associated with polyphenols. Due to their high proportion of triacylglycerols or polysaccharides, the extraction and isolation of polyphenols from these samples require different purification procedures to obtain a sugar and lipid-free extract.

Polyphenolic extracts from modern grape seeds were obtained by a modified procedure described by Bourzeix et al. [30] and improved by Sun et al. [31]. Briefly, crushed grape seeds (two seeds, ∼50 mg) and grape skins (∼50 mg) were extracted three times by a mixture acetone/water (8:2 (v/v), 2 ml) by

[2] Operation consisting of the addition of wine in a barrel during wine-ageing, with the intention of replacing the evaporated water and to limit the oxygen amount in the container that could spoil the wine.

ultrasonication (10 min). The extract was separated by centrifugation (10 min, 4000 rpm) and the acetone evaporated under reduced pressure at 40 °C. Lipids were removed by washing the aqueous phase with cyclohexane (3 ml × 5 ml), evaporated to dryness under reduced pressure (40 °C) by adding an equal volume of butan-1-ol.

By the same way, wine (1 ml) was concentrated by rotary evaporation (40 °C). The dry polyphenolic extracts of grape seeds, skins and wine were dissolved in 1 ml of aqueous TFA 0.05%.

The polyphenolic aqueous extract of all the samples (i.e. grape seeds and skins, and wine) was fractionated by solid-phase extraction (SPE), by passing through a preconditionned tC_{18} Sep-Pak (Waters Associates, Bedford, MA) [31,32]. Elution was carried out with 3 ml × 2 ml of HCl 0.01 M to eliminate sugars and phenolic and residual fatty acids. The Sep-Pak is well dried under a nitrogen stream. For an overall analysis of the polyphenolic material, flavanol monomers, proanthocyanidins and anthocyanins are eluted with 10 ml of methanol. This fraction was taken to dryness and the residue dissolved in water (1 ml) just before analysis.

2.2.2. Preparation of archaeological samples

Archaeological grape seeds are ground just before analysis, without lipid removal or further preparation because of their low preserved lipidic content. The samples were directly analyzed by thermochemolysis. The Roman wine, preserved in a resinated amphora, was purified by successive extractions of the soluble organic matter, an aliquot of the homogenized wine (1.0 ml) was centrifuged (10 min, 400 rpm) and both phases were separated and washed separately three times with a mixture of chloroform/methanol (2:1 (v/v)) to discard diterpenoic material coming from the resinous water-proofing agent applied on the inner side of the amphora. An aliquot of the extracted residue is treated with TMAH and pyrolyzed by the same way as ancient grape seeds.

2.2.3. Analysis

An aliquot of the aqueous polyphenolic extract (3 μl) or a weighed amount of the modern wine deposit or of an archaeological sample (∼0.5 mg) was mounted in a quartz tube containing quartz wool. Then TMAH (25 wt.% in MeOH, 3 μl) was spread evenly into the sample and the mixture was first dried at 80 °C until complete evaporation of the solvent, and immediately pyrolysed at 450 °C for 5 s using a CDS 1000 pyroprobe platinum heated filament pyrolyser (Chemical Data System, Oxford, USA). GC–MS analysis is performed with a HP5890 with an injector held at 280 °C set in splitless mode, coupled to a ThermoQuest GCQ ion trap mass spectrometer. A Chrompack CP Sil-8CB capillary column (poly (5% diphenyl/95% dimethyl)siloxane) stationary phase, 30 m, 0.25 mm i.d., 0.25 μm film thickness, equipped with a 5 m-precolumn, 0.25 mm i.d.) was used with a temperature program from 50 °C (held for 10 min) to 250 °C at 5 °C/min, to 325 °C at 10 °C/min (held 10 min) with helium as carrier gas (constant pressure 16 psi). Mass spectra were recorded at 3 μscans/s under electron impact at 70 eV (source at 180 °C), mass range *m/z* 50–500.

Phenolic compounds were identified by comparing their mass spectrum with reference compounds from the Wiley and the NIST libraries, and their retention time found in literature [33,34].

3. Results and discussion

3.1. Polyphenolic standards

Four standard flavan-3-ols (catechin C, epicatechin EC, epigallocatechin EgC and epigallocatechin-3-*O*-gallate EgCG) were pyrolysed after the addition of TMAH. Evaporation of the solvent before pyrolysis allows to avoid solvent effects when performing thermochemolysis and to study the decomposition of the quaternary ammonium salts solely. THM of these four species was studied at different temperatures and durations of pyrolysis (from 250 to 750 °C with a step of 50 °C and at 900 °C for 1, 5 and 30 s) in order to assess the influence of these parameters on the pyrolytic products and on their relative ratio. The four reference flavanols analysed released only phenolic compounds, mainly di- and trimethoxylated isomers of benzene, toluene and benzoic acid (Figs. 1 and 2). No difference was observed between the pyrograms of catechin and epicatechin, which only differ by the stereochemistry of the hydroxy group located on the central ring at the 3 position (Table 1). This

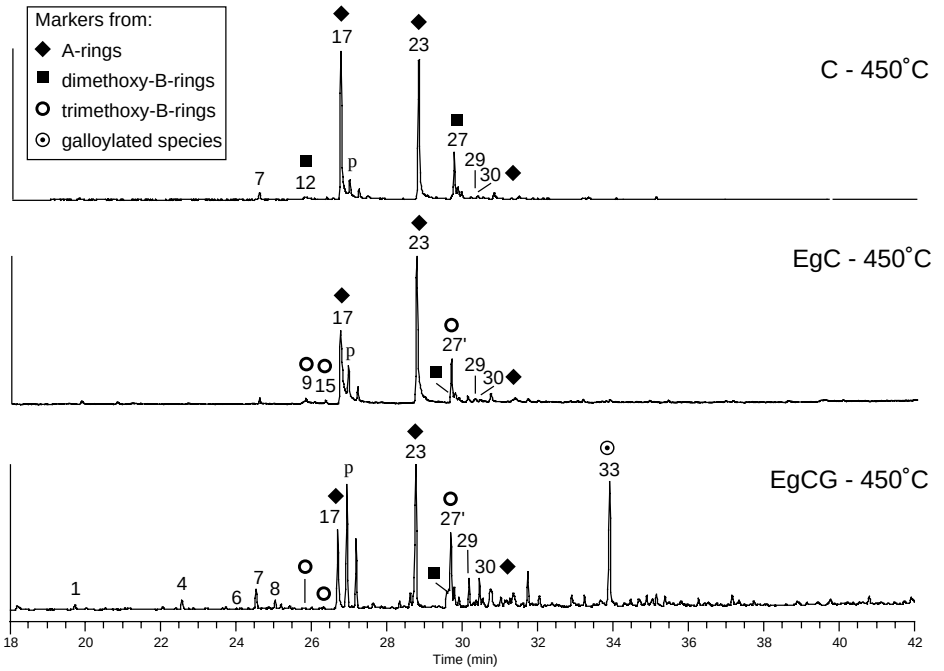


Fig. 1. Partial pyrograms of catechin (C), epigallocatechin (EgC) and epigallocatechin-3-*O*-gallate (EgCG) at 450 °C. Labels refer to compounds listed in Table 4. p: pollution marker from TMAH reagent.

shows that stereochemistry affects neither the implied mechanisms nor the proportion of pyrolytic fragments.

Whatever the temperature of pyrolysis, all the flavanol monomers studied mainly yield 2,4,6-trimethoxylated derivatives of benzene (**17**),[3] toluene (**23**) and styrene (**29**, **30**) (Figs. 2 and 3). In addition, the pyrolysis of the monomers with dihydroxylated B-ring (C and EC) leads to the formation of low amounts of 3,4-dimethoxylated derivatives of styrene (**12**) and of benzoic acid (**27**) (Fig. 1), whereas the flavanol monomers with trihydroxylated B-ring (EgC) generate low amounts of 3,4,5-trimethoxylated derivatives of benzene (**9**), toluene (**15**) and benzaldehyde (**27′**).

These observations, correlated with the absence of benzopyran derivatives, prove the C-ring cleavage which provides the formation of pyrolytic phenolic markers either issued from the A-ring (compounds **17**, **23** and **30** of Table 4 for all the commercial flavanol monomers studied) or the B-ring (compounds **12** and **27** of Table 4 for C and EC and compounds **9**, **15** and **27′** of Table 4 for EgC).

The galloylated procyanidin EgCG presents the association of both classes of trimethoxylated compounds, with an important releasing of methyl 3,4,5-trimethoxybenzoate (**33**). Permethylated gallic acid (**33**), not observed from EgC, seems to be a specific pyrolytic marker of galloylated units.

The influence of temperature on pyrolytic processes was shown to be negligible. Indeed, most of the main compounds, namely 2,4,6-trimethoxylated benzene (**17**), toluene (**23**), styrene (**30**) and methyl 3,4-dimethoxybenzoate (**27**), are formed in equal proportions, independently of the temperature (Fig. 3). This reflects the decomposition of quaternary ammonium salts formed by addition of TMAH, at temperatures above 250 °C providing sufficient thermal energy for the hydrolysis–methylation. Pyrolysis yield may

[3] Such labels refer to compound numbers listed in Table 4. In the aim to simplify the denomination of phenolic compounds, and to better distinguish the different classes of pyrolytic markers, we use the nomenclature 2,4,6- and 3,4,5-trimethoxy for all benzenoids derivatives, including benzene.

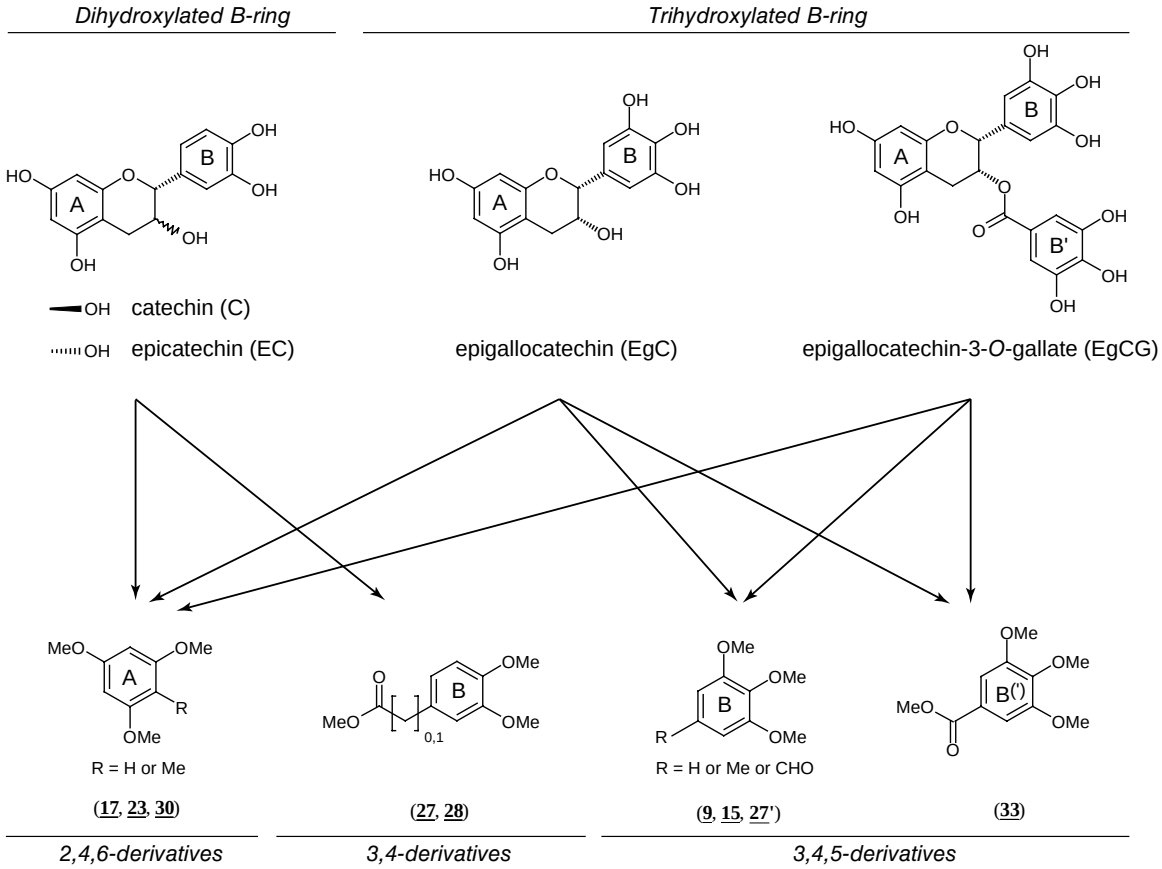


Fig. 2. Relationships between benzenoid pyrolytic products and their monomeric precursors, standard flavan-3-ols, during thermochemolysis in the presence of TMAH. Labels into brackets refer to compounds listed in Table 4.

thus be considered as independent of the heating temperature for the previous compounds, as already mentioned by Challinor for other chemical compounds, such as dicarboxylic acids from resins [27] and by Asperger et al. for lipids from waxes [35]. However, whereas the formation of permethylated gallic acid (**33**) decreases when temperature increases, partially methylated phenols with low molecular weight (methoxyphenols **1** and **3**, dimethoxybenzenes **4** and **5**, and toluenes **6**–**8**), increase as temperature increases (Fig. 3). This provokes the increase of the ratio of low molecular constituents above 600 °C probably due to the formation of free radicals in the gas phase and the extent of thermal fragmentation reactions [35,36]. Such fragments are not formed at temperatures below 450 °C.

Pyrolysis time of 1 s was shown to be insufficient to provoke the formation of all the components observed after 5 s of pyrolysis. However, increasing the time of pyrolysis above 5 s provides the same pyrograms as for 5 s.

All the analyses were performed in triplicate. In all cases, pyrograms were similar for a same sample both from qualitative and quantitative points of view, giving evidence for the good reproducibility of the method as already noticed by other authors for lipid constituents [35]. This allowed us to proceed to the relative quantification of the results obtained.

All these considerations led us to proceed to THM analyses at 450 °C for 5 s in order to limit the radical fragmentations and to favour nucleophilic additions–eliminations.

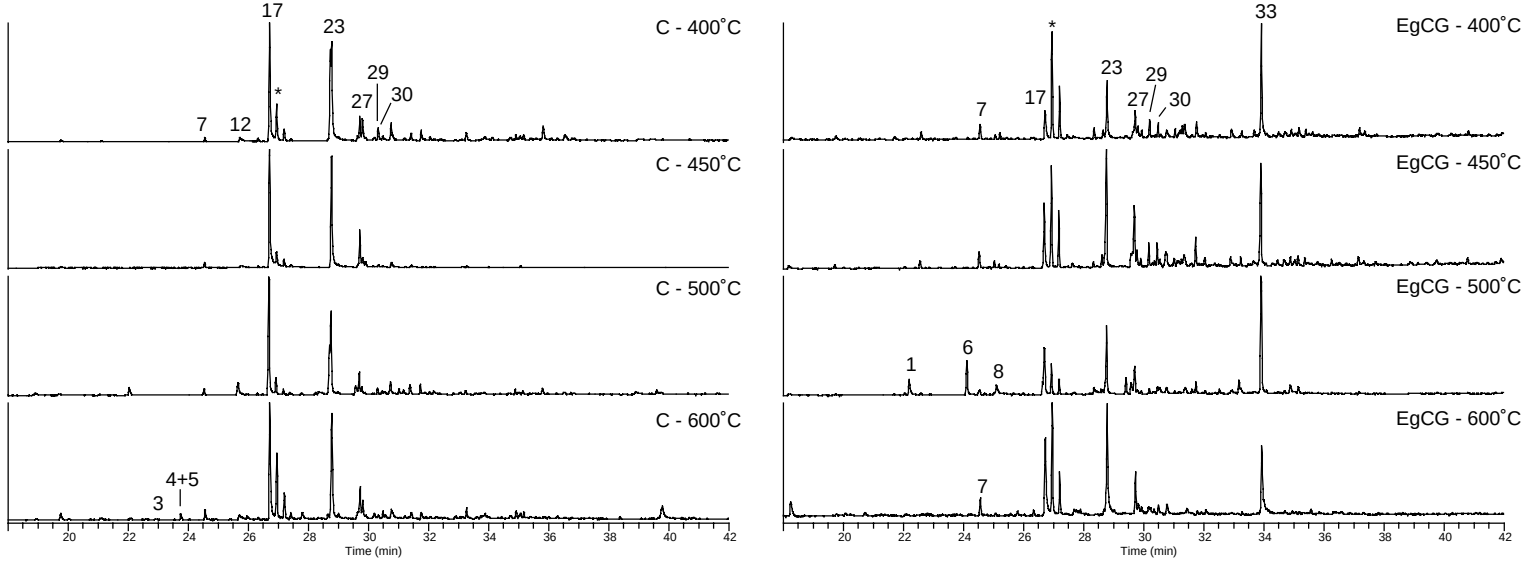


Fig. 3. Influence of the temperature of pyrolysis on the THM profile of catechin (C) and epigallocatechin (EgCG).

Table 4
Relative intensities of THM products (calculated by integrating the TIC signal) from modern grape seeds, skins and wines, and from archaeological grape seeds and wines

Peak	MW	Retention time (min)	Compound	Sources	Modern seeds		Modern skins		Modern red wines				Modern sweet wines				Ancient grape seeds				Ancient wines	
					Chard	Pinot	Chas	Musc	Pom	Med[a]	Bdx[a]	Smr	Cal[a]	Cal-d[a]	Tur	Tur-d	Grav	Latt	Msl	Perd	Giens	Defr
1	124	15.80	2-Methoxyphenol	PC, A2			1	1		5			25	22	48	34	7		2	48	9	4
2	138	16.91	1,2-Dimethoxybenzene	PC, A2			29	29	1	8	2	1	4	7	100	100	37	3	3	2		8
3	138	17.32	4-Methyl 2-methoxy phenol	PC, A2	18	18						1			39	74	1	3	3	1	1	5
4	138	17.75	1,3-Dimethoxybenzene										8	5	9	13	2					
5	138	17.86	1,4-Dimethoxybenzene							1			7	10	7	10	18					
6	152	18.45	2,3-Dimethoxytoluene												20	20	4	2	4	3	100	
7	152	18.84	2,4-Dimethoxytoluene	F1, A2, A3	4		24	24	2	4	1	2	11	13	17	30	49	3	6	9	49	12
8	152	19.28	3,4-Dimethoxytoluene	PC, A2									4	4	8	7	8					
9	168	19.60	3,4,5-Trimethoxybenzene	PD, A3	14				3	6	3	2	11	12	1		3	2				6
10	150	19.75	?	?											3	3	14			100		
11	166	20.05	4-Methoxybenzoic acid, methyl ester	Bound 4-hydroxybenzoic acid, kaempferol (?)	9		10	10	9	16	9	6		3	5	6	1	6			2	
12	164	20.42	3,4-Dimethoxystyrene		3	1	3	3	2	4	2	2	3	8			100		6	18		38
13	168	20.42	1,2,4-Trimethoxybenzene	Polysaccharide			98	97	3	44	4	3	9	6	52	95	10	9	11		2	14
14	178	20.77	1,2-Dimethoxy-4-propen-1-yl benzene	Degraded PC (?)													13				20	2
15	182	20.90	3,4,5-Trimethoxytoluene	PD, A3	2		6	6	2	24			3	6	3	6						8
16	168	20.95	Isomer of dimethoxystyrene				1	1							6	39		10	28		2	
17	168	20.98	2,4,6-Trimethoxybenzene	F1, A2, A3	18	9	2	2	14	29	19	4	100	8	6	39		10	28		2	
18	182	21.14	2,4,5-Trimethoxytoluene	Polysaccharide			100	100	3	14					9	14	1	2	2			1
19	180	21.30	2,4-Dimethoxyacetophenone	F1			10	10		14		1		2	5	6	1	2	4		18	2
20	178	21.49	Isomer of 1,2-Dimethoxy-4-propen-2-yl benzene	Degraded PC (?)													13		2		15	6
21	164	21.77	Dimethyl-p-anisaldehyde	Lignin											11	11					8	
22	166	21.82	3,4-Dimethoxybenzaldehyde	PC, A2											9	22	36	13	13	9	8	94
23	182	22.02	2,4,6-Trimethoxytoluene	F1, A2, A3	17	5	5	5	9	13	12	3	7	6		18		9	23	2	3	
24	178	22.18	1,2-Dimethoxy-4-propen-2-yl benzene	Degraded PC (?)												5	76		8			26
25	196	22.61	3,5-Dimethoxybenzoic acid, methyl ester	?	10	2	2		4	4	5	2		2	1	8		3	8			2
26	180	22.86	3,4-Dimethoxyacetophenone	PC, A2	1		9	9	2	2	2		5	6	2	9	15	2	9	2	4	28
27	196	23.24	3,4-Dimethoxybenzoic acid, methyl ester	PC, A2	96	17	17	17	78	100	90	62	24	31	9	64	21	100	100	9	6	40
27′	196	23.28	3,4,5-Trimethoxybenzaldehyde	PD, A3																		
28	210	23.56	3,4-Dimethoxybenzeneacetic acid, methyl ester	PC, A2	6		1	1	1	2	1	2	8	13	8	10					3	
29	194	23.62	Isomer of trimethoxystyrene	F1 (?)	6	4		1	8	8	4	3		4		2		13	1		11	
30	194	23.79	2,4,6-Trimethoxystyrene	F1, A2, A3	1	4	3	3	7	9	4	4		5		7	14		17		8	100
31–32	208	24.31	*cis* and *trans*-Asarone	Degraded PC (?)			2									6	12	9	10		7	88
33	226	26.95	3,4,5-Trimethoxybenzoic acid, methyl ester	Galloylated units, A3	100	100	9	95	100	75	100	100	11	100	11	79	1	26	23	1	1	44
34	208	25.19	4-Hydro-3-methoxycinnamic acid, methyl ester	Lignin													8	3	4		6	38
35	222	27.27	3,4-Dimethoxycinnamic acid, methyl ester	A2 ?, Lignin											3	10	6	7	7			23

For a better legibility, only scores >1% are shown. According to an inefficient separation, compounds **27** and **27′** are quantified together.

[a] Wines élevés in oak barrels; A2: disubstituted anthocyanins (cyanidin, peonidin); A3: trisubstituted anthocyanins (delphinidin, petudinin, malvidin). PC: procyanidins (B-ring); PC: prodelphinidins (B-ring); F1: monomeric flavanoids (A-ring).

3.2. Modern vine derivatives

First assays have been conducted on the crude wine, grape seeds and skins but in all cases, complexity of the pyrogram due to the main presence of sugars in wine and skins, and of lipids in seeds hides the polyphenols response. These samples were then analysed after different steps of purification (see experimental).

Analogous to monomeric flavanol standards, the polyphenolic extract of all the modern vine derivatives gave rise to di- and trimethoxybenzenes, toluenes, benzoates as major products and styrenes in lower amount (Table 4). The different wines obtained from various winemaking processes and with different ages yielded similar pyrograms, characterized by numerous phenolic benzenoid compounds and dominated by methyl 3,4-di- and 3,4,5-trimethoxybenzoates (respectively **27** and **9**, **15**, **33**, Fig. 4).

In order to better detect and identify isomers of trimethoxybenzenes and toluenes, we proceeded to the realization of the mass fragmentogram of ions at *m*/*z* 168 and *m*/*z* 182 (Fig. 4), corresponding to the more intense ions (here the molecular ions $M^{\bullet+}$). One must note that pyrolytic markers of sugars (**13** and **18**) are released by thermochemolysis in most of the samples, especially from liquorous wines Port (Cal and Cal-d) and *turriculae* (Tur, Table 4 and Fig. 4), despite the previous discarding of sugars by the SPE procedure. This is due to the fact that, during the purification procedure dedicated to the elimination of sugars, proanthocyanidins are coeluted with anthocyanidins, mainly present in wine as glycosylated species (anthocyanins). Consequently, the THM analysis of this fraction yields a combination of pyrolytic markers from flavonoids (from A- and B-rings of proanthocyanidins and anthocyanins, as described for standard flavan-3-ols), and from sugar moieties, especially glucose (Table 4).

Pyrolytic markers of the flavonoid A-ring are abundant. For all the 18 samples studied, including commercial flavanols and modern vine derivatives, a correlation between the intensities of 2,4,6-trimethoxylated benzene (**17**) and toluene (**23**), showing a good regression coefficient ($r^2 = 0.8$, Fig. 5), reveals that these two markers come from a common source (flavonoid A-rings) and are formed by the same mechanism. Their generalized predominance in skin, grape seeds and in wine shows them as pertinent pyrolytic markers of the A-ring of flavonoids.

3,4,5-Trimethoxylated benzene (**9**) and toluene (**15**) are present in various amounts, in grape seeds (ca. 14 and 2%, respectively) and in wines (1.0–11.9% and 0.5–6.2%, respectively), revealing the proportion of prodelphinidins. Their presence, associated with a major release of permethylated gallic acid (**33**, 100% for seeds, 74–100% for red wines, around 11% for white wines and 78–100% for their deposit), reflects the high proportion of galloylation of proanthocyanidins of seeds. In contrast, both trimethoxy-benzene and toluene are present in pyrolytic profiles of grape skins (0.6 and 5.6%, respectively) but with a relatively lower proportion than gallic acid (8.5%). This latter, formed by transesterification of galloylated units, is yielded in small amount, reflecting the low galloylation rate of proanthocyanidins from grape skins. Furthermore, the predominant formation of 3,4,5-trimethoxytoluene (**15**) compared to 3,4,5-trimethoxybenzene (**9**) may reflect the high proportion of epigallocatechin units in skins. The determination of the origin of these two markers, arising from prodelphinidins and galloylated flavanols, respectively, results from the study of trimethoxylated benzenoids in the THM pyrograms of EgC and EgCG (Fig. 1), the latter yields mainly permethylated gallic acid and only traces of 3,4,5-trimethoxybenzene (**9**) or toluene (**15**). This reflects the formation of **9** and **15** by radical scission of the C-ring from prodelphinidins, and the release of **33** by *trans* methylation of galloylated units. Other trace markers of the A- and B-rings, 2,4- and 3,4-dimethoxyacetophenone (**19**, **26**), were released from the grape skin extracts, which were not observed by THM of standard flavan-3-ols. They can be formed by homolytic scission and opening of the C-ring of flavonols or flavanonols.

Among the three categories of samples studied (purified polyphenols from grape seeds and skins, and wine), the pyrograms from grape skins are more simple because of the simplest molecular structure of their biological tissue, but they present many saccharidic pyrolytic markers. Indeed, skins are mainly constituted of polysaccharides especially cellulose, main constituent of the plant pectocellulosic cell walls, and of polyphenols (mostly proanthocyanidins and, in red grapes, anthocyanins). Their THM yields the same pyrolytic phenolic markers as standard monomers,

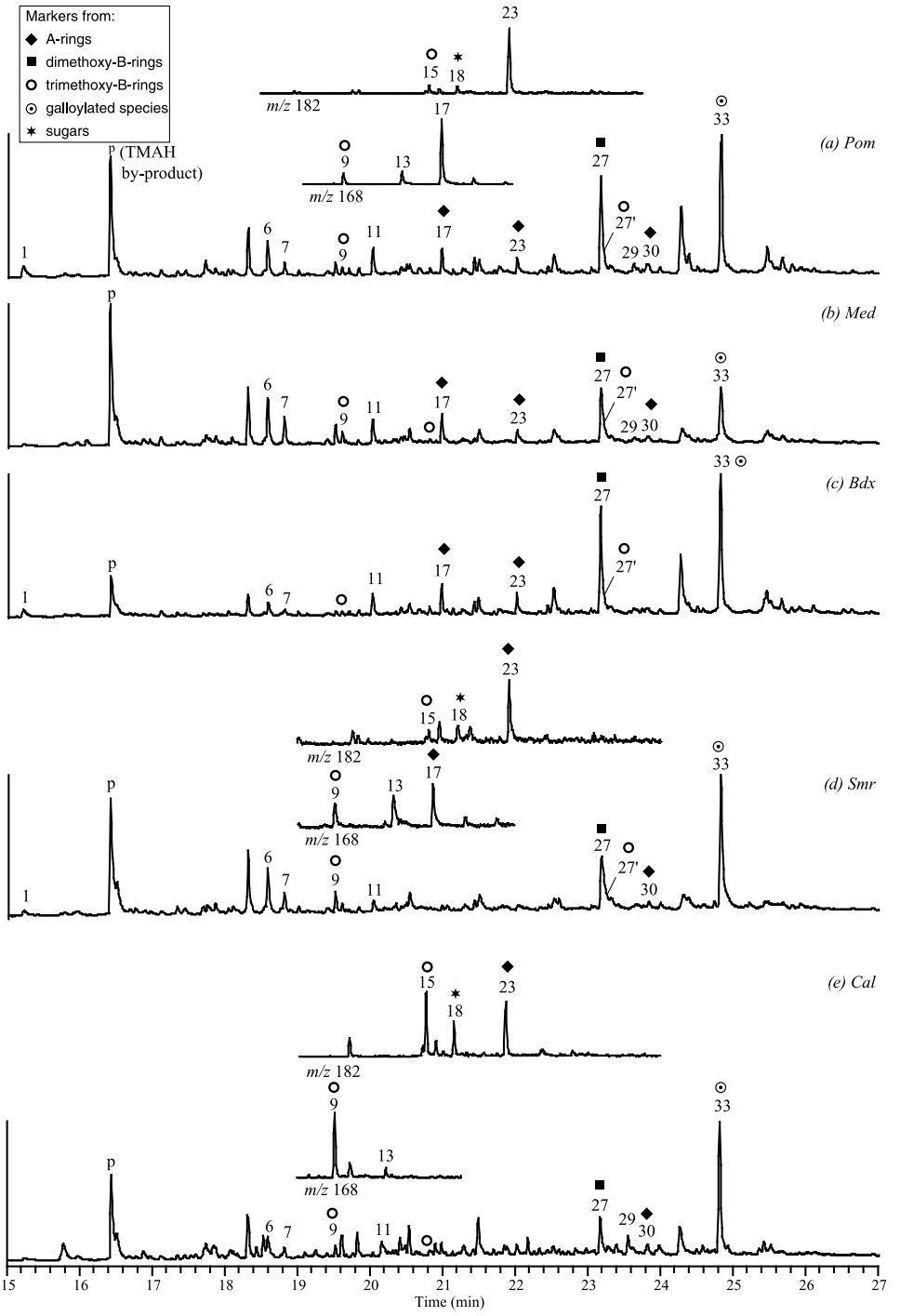


Fig. 4. Partial T hM/GC/MS pyrograms and fragmentogram traces of the ions at *m*/*z* 168 and *m*/*z* 182 of polyphenolic extracts from (a) a red Bordeaux wine Lalande Pomerol 1998, (b) a red Médoc 1996, (c) a red Bordeaux 1998, (d) an oxidized wine Saumur, and (e) a 10-years old Port wine.

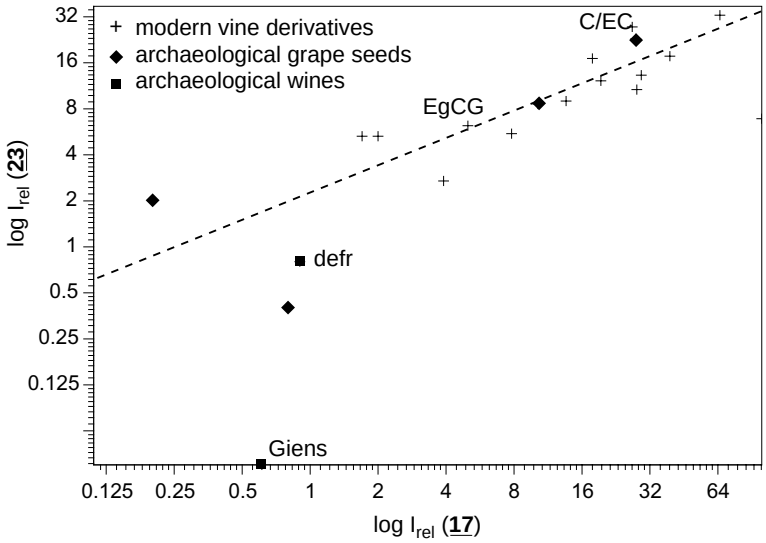


Fig. 5. Correlation between release of 2,4,6-trimethoxylated benzene (**17**) and toluene (**23**), reflecting a common mechanism for their pyrolytic formation.

along with large amounts of 1,2,4-trimethoxybenzene (**13**) and 2,4,5-trimethoxytoluene (**18**) (Table 4). These molecules are known as polysaccharidic markers, obtained from cleavage of osidic bond and aromatization of the released units [37,38]. Releasing of these pyrolitic sugar markers associated with higher-molecular weight compounds, such as tetramethyl-3-deoxyhexonic acids confirms this postulated saccharidic origin [37]. These results show that carbohydrate material could not be totally eliminated by the SPE procedure although this purification step was shown to be efficient for polyphenol concentration.

In wine samples, THM–GC–MS profiles are dominated by two main condensed tannin markers, namely permethylated gallic acid (**33**) and methyl 3,4-dimethoxybenzoate (**27**), respectively, issued from the galloylated units, and from the procyanidin B-ring or anthocyanins (peonidin). In the anthocyanin series, abundantly present in red wines and skins, B-ring markers allow discrimination of two classes according to their hydroxylation/methoxylation degree: dimethoxylated markers are related to cyanidin and peonidin, whereas, trimethoxylated benzenoids correspond to delphinidin, petudinin and mainly malvidin. One must note the particular high content of methyl 3,4-dimethoxybenzoate (**27**) in the wines *élevés* in oak barrels compared to other wines. This compound is also known to be formed by thermochemolysis of lignin [39–41]. Consequently, in the case of wines *élevés* in oak barrels, this compound may have a dual origin: the thermochemolysis of lignin from oak which was in contact with wine and that of wine itself. The pyrolytic markers of the A-ring (2,4,6-trimethoxybenzenoid compounds **23** and **30**) which are the major compounds observed with non-galloylated flavanol monomers are present in rather small amounts in pyrograms of wine and grape extracts. The low yield of A-ring markers from wine and grape extracts is attributed to the involvement of flavonoid moieties in polymers with linkages on the 6- or 8-position of the A-ring. Only A-rings of the upper units, non substituted, may yield the series of saturated 2,4,6-trimethoxybenzenoid compounds, although A-rings from the extension units of the proanthocyanidins may conduce to 2,4,6-trimethoxystyrene by a postulated concerted mechanism (Fig. 6). This hypothesis, by that the C-heteroring is preferably opened by THM rather than the interflavanic bond, is confirmed by the observation of the formation of vinylflavanols in wine during wine-making process: adducts from catechin with acetaldehyde and

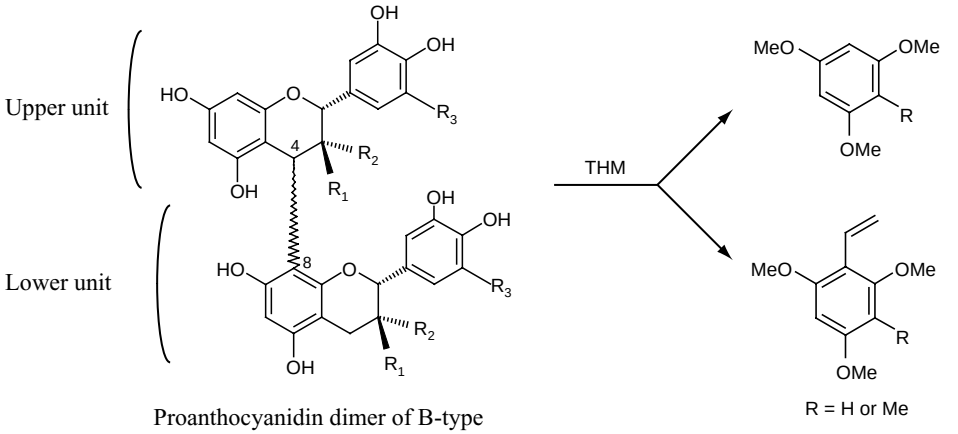


Fig. 6. Postulated A-ring pyrolytic fragments obtained from the upper unit and the extension unit of proanthocyanidins.

another flavanol, tannin or anthocyanin, can cleave by an acid-catalysed reaction to release 8-vinylflavanol (Fig. 7) [42].

Relationships between pyrolytic phenolic markers and their origin in grape derivatives is presented in the Table 4. One must note that markers of the A-rings are general markers of flavonoids although markers of the B-rings afford a discrimination between 3′,4′-dihydroxy and 3′,4′,5′-trihydroxyflavanoids, i.e. between (pro)cyanidins and (pro)delphinidins, and between both series of anthocyanins di- (cyanidin Cy, peonidin Pn, delphinidin Dp) and trisubstituted (malvidin Mv, petudinin Pt).

Port wine (Cal) and the modern *Turriculae* wine (Tur) present a slightly modified THM-profile. In comparison with other wines, they show a relative high proportion of dimethoxybenzenes (**4**, **5**) and toluenes (**7**, **8**), in association with 2-methoxyphenol (**1**) (Table 4). These markers, were only released in very small amount during THM of the standard flavanols. These compounds probably result from the hydrolytic scission of alkyl ether linkages in lignin structure: formation of dimethoxytoluenes also takes place, in association with dimethoxystyrenes [39]. In the case of Port wine (Cal), their presence can be explained by the long-time maturing period of the wine in oak wood casks (10 years for these analyzed samples). Their higher proportion in the THM profiles of the Port deposits than in the corresponding wine can also bring in light the involvement of the ellagic tannins from wood in the slow precipitation of the wine tannins.

Both sweet wines present a low proportion of permethylated gallic acid, although this marker is predominant in the THM-profiles of their deposit. It can be related to the role played by galloylated units in the precipitation of tannins and wine sedimentation [43]. Particular markers (peaks **6**, **21** and **35**), absent from other modern samples, characterize the *Turriculae* wine and its deposit. Their presence is certainly associated with the chosen wine-making process: the adjunction of some raw crush plant commodities, as fenugreek (*Trigonella foenumgraecum L.*), a ligneous plant, brings some exogen phenolic material in the wine by extraction of the ligneous material by the alcoholic beverage. Fennel (*Foeniculum vulgare*), added in the must before fermentation, is the major natural source of anisaldehyde from which **21** may be released. The presence of the 3,4-dimethoxycinnamate **35** can also reflect the wine-making process used in which the freshly pressed grape juice is directly stored in large ceramic containers for fermentation. The absence of oxygenation of the must decreases the enzymatic oxidation that caffeoyltartaric acid is very sensitive to [44–46].

3.3. Gallo-Roman grape seeds

The thermochemolysis of archaeological grape seeds has provided diverse structurally informative

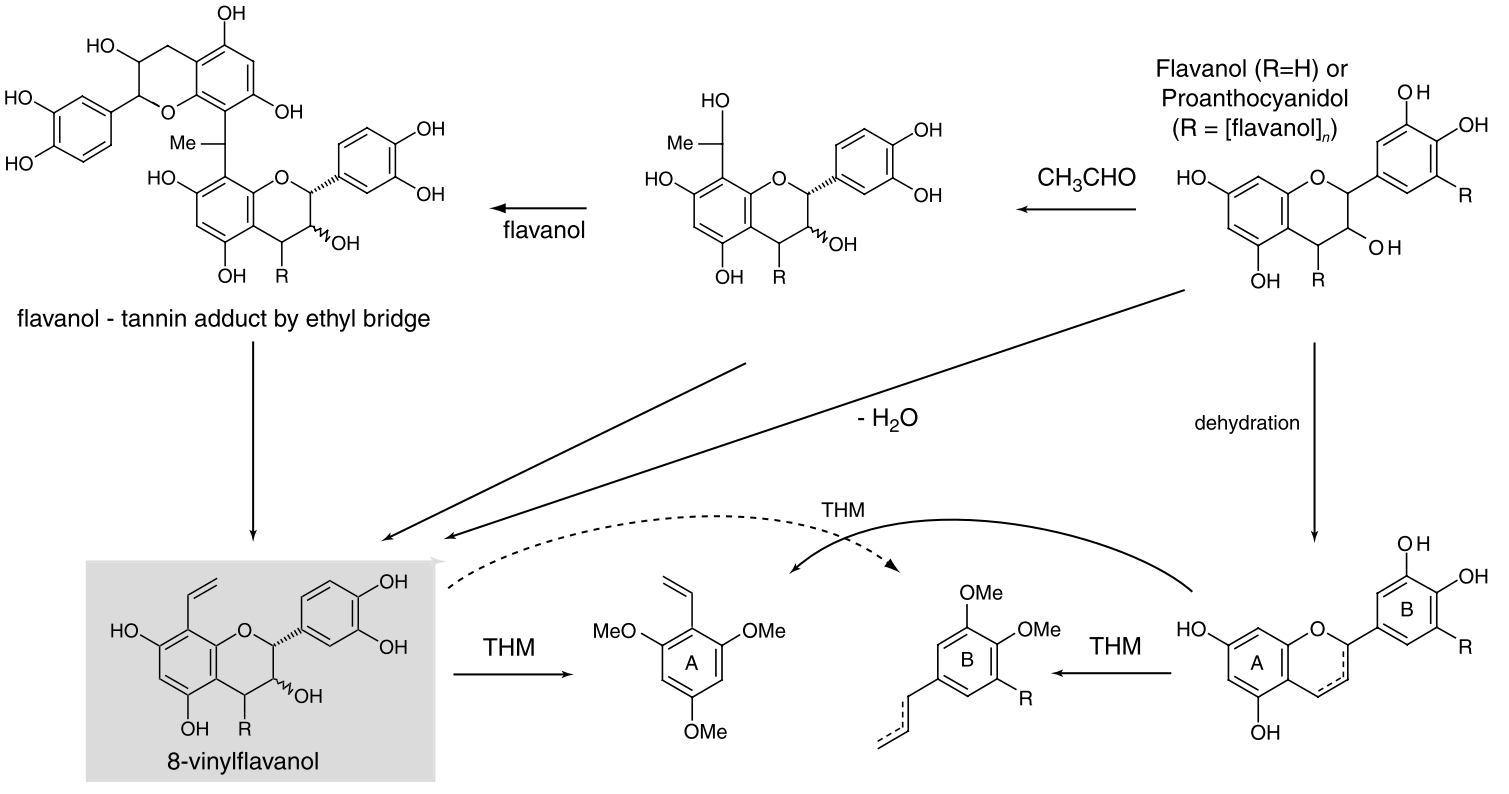


Fig. 7. Hypothetical formation of trimethoxystyrene and di- and trimethoxypropenylbenzenes from dehydrated and reduced flavan-3-ols, and from their adducts with tannins and from 8-vinylflavanol, formed during wine-making process. $R'_3 = H$ (derivatives of catechin); $R_3 = OH$ and $R'_3 = OMe$ (derivatives of EgC, only in ancient residues).

pyrolytic phenolic products (Fig. 8). Di- and trimethoxylated benzenes, toluenes and methyl benzoates are the main released constituents, along with *N*-containing heterorings and permethylated aminoacids indicative of proteinaceous source [47].

THM of these ancient vine derivatives yields different pyrograms, reflecting a valid relationship between a qualitative and semi-quantitative THM-analysis and the context where the sample was found. Most of them provide interesting pyrograms with polyphenolic pyrolytic markers. However, the grape seeds from the submarine shipwreck (Perd) were found to present a very low content of polyphenols issued from vine derivatives (Fig. 8d). This indicates that during the storage in the submarine context, almost all the polyphenolic material disappeared from these samples. The other three samples present various degrees of preservation and show different proportions of markers from cellulose and grape tannins.

Pyrolytic markers of the A- and the B-rings of the proanthocyanidins are detected and all archaeological samples show an interesting pyrogram of unsaturated phenolic compounds. If the major pyrolytic markers characteristic of modern grape seeds are released with high yield, some unsaturated degradation markers appear. Two particularly interesting compounds, namely 2,4,6-trimethoxystyrene (**30**, also formed by THM of standard monomeric flavanols) and 1,2-dimethoxy-4-propen-1(or 2)-yl benzene (**31** and **32**), are also produced by THM of ancient grape seeds.

Release of 2,4,6-trimethoxystyrene (**30**) confirms the presence of proanthocyanidins and/or of their reaction products. Due to its large source of provenance, **30** is considered as a grape seed pyrolytic marker, and more generally, as flavonoids and phenolics pyrolytic marker. Secondly, the greater amount of these unsaturated phenols (**29** and **30**) released from ancient wines rather than modern samples and their unsaturation degree suggest a degradation of the original proanthocyanidins (Fig. 7). The isomers of asarone (**31** and **32**) could be markers of the A- and the B-rings of ancient proanthocyanidins, respectively, the double bond possibly resulting from a reductive degradation of the C-ring, by dehydration of the flavan-3-ol (Fig. 7). Contrary to **30**, asarones are absent from modern material, suggesting their potentiality to represent degradation markers of tannins.

In comparison with modern grape seeds, archaeological samples release fatty acids (palmitic and oleic acids) in very low proportions. The comparative analysis by THM of modern and ancient crude materials shows that modern seeds consist mainly of triacylglycerols (especially triolein), and of polyphenols in lower amount (ca. 3–4%). Nevertheless, THM–GC–MS profiles of ancient seeds show that more than 90% of the pyrolytic extract are phenols and, at the opposite, lipids (detected as FAME) are present as trace amounts. This observation brings in light the good preservation of polyphenolic material (from fruits or wood) through time, in contrast with lipids still well-known for their stability. These results tend to show that polyphenols are rapidly submitted to oxidative process during the first steps of storage whereas they reach a complex chemical structure looking very stable towards post-depositionnal degradation mechanisms in a second time.

3.4. Archaeological wine residues

Both wine samples show a complex pyrolytic profile consisting primarily of phenolic markers and diterpenic compounds (Fig. 9). Although the free lipids of the wine residues were removed by successive extractions of hydrophobic compounds with chloroform/methanol, the THM profile reveals a typical pattern of pitch material from *Pinus sp.* wood. The *Pinus sp.* pyrolytic markers are probably issued from diterpenoid precursors partially bound with non solvent soluble substances, by ester bond that are cleaved during the THM step. If the boiled-down red wine, *defrutum*, yields phenolic components in very low amount, the *Caecuban* wine (Giens) shows the main phenolic markers, released by modern wines: markers of the A-ring (exclusively **30**), dimethoxylated B-ring (**12** and **27**), trimethoxylated B-ring (**9** and **27′**) and galloylated species (**33**). Di- (**24**) and trimethoxypropenylbenzenes (*cis*- and *trans*-asarone isomers, **31** and **32**), unsaturated phenolic markers detected as traces, seem to be characteristic of ancient vine derivatives.

From a more general point of view, some of the di- or trimethoxy pyrolytic markers identified in this study have also shown to be formed by the thermochemolysis of lignin [39–41,48,49]. Lignin being an important biopolymer of vascular plants [40], it is well distributed in sedimentary matrix, and the

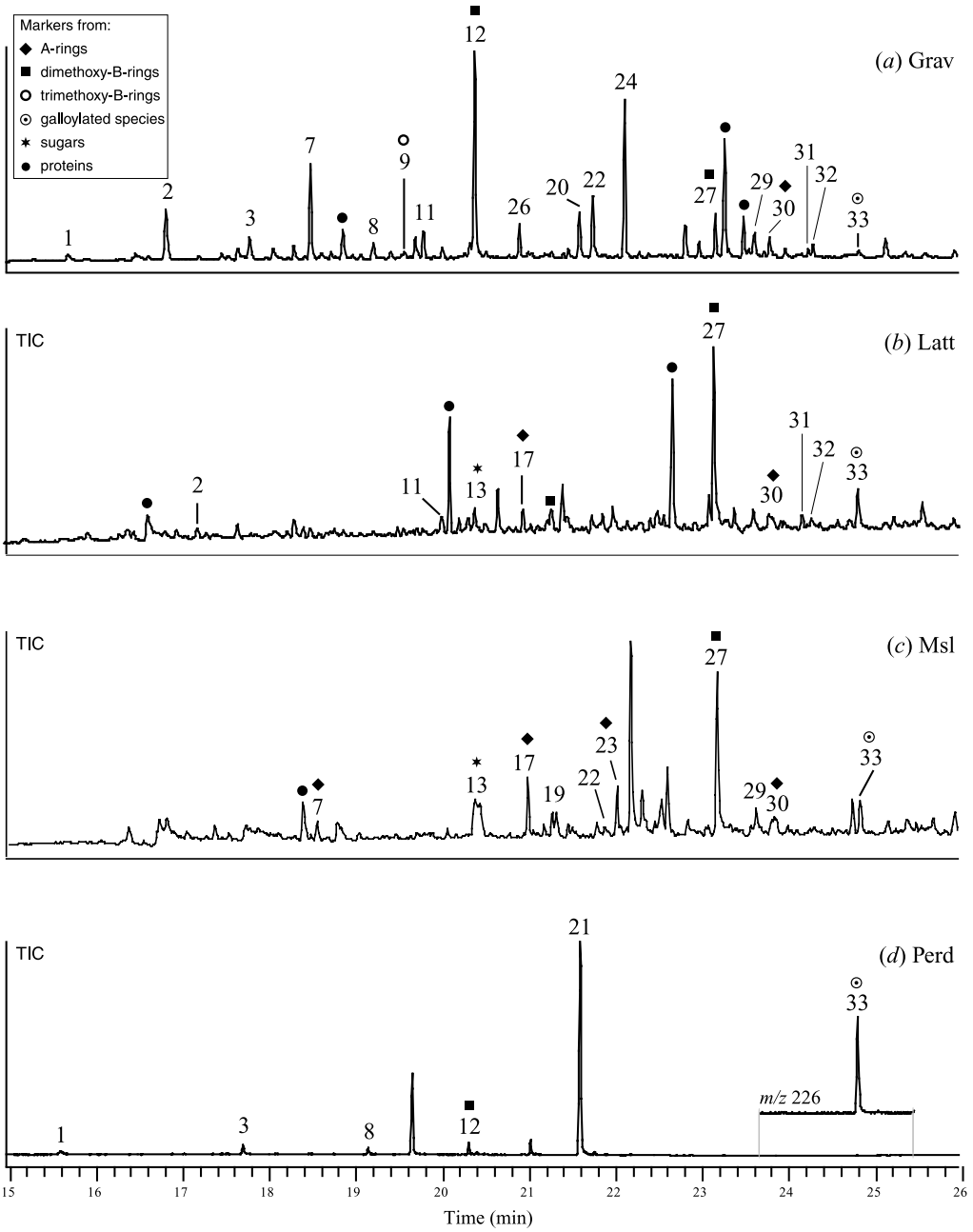


Fig. 8. Partial TIC of THM of ancient grape seeds coming from the terrestrial contexts (a) Graveson, (b) *Lattara*, (c) Marseille and from the Roman shipwreck (d) Sud-Perduto 2.

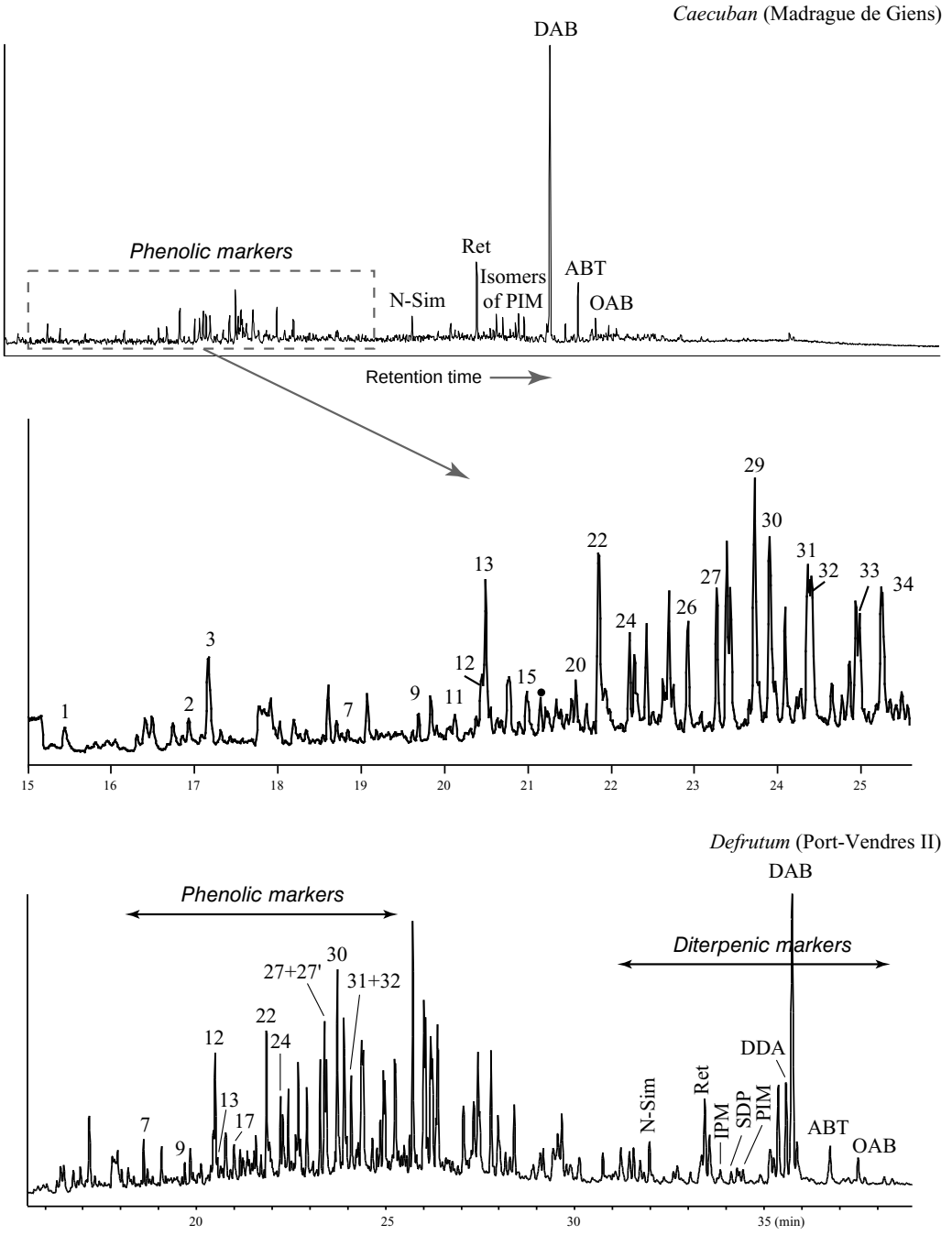


Fig. 9. Partial pyrogram (THM–GC–MS) from a Roman *Caecuban* wine and a Baetican boiled-down wine (*defrutum*).

identification of wine residues based on the pyrolytic markers released by thermochemolysis may be difficult. However, in the case of lignin, di- and trimethoxy benzenes and toluenes are always accompanied by a series of phenolic C_6–C_3 constituents [41]. Such components were very rarely encountered in our samples and we can thus consider that in most cases, the association of the phenolic markers identified were mainly issued from vine derivatives.

The ancient degraded Roman wine *Caecuban* (Giens) is characterized by the presence (*i*) of the trimethoxy styrenes (**29**, **30**) and propenylbenzenes (**31**, **32**), revealing an hypothetical reductive degradation of the C-ring as for ancient grape seeds, and

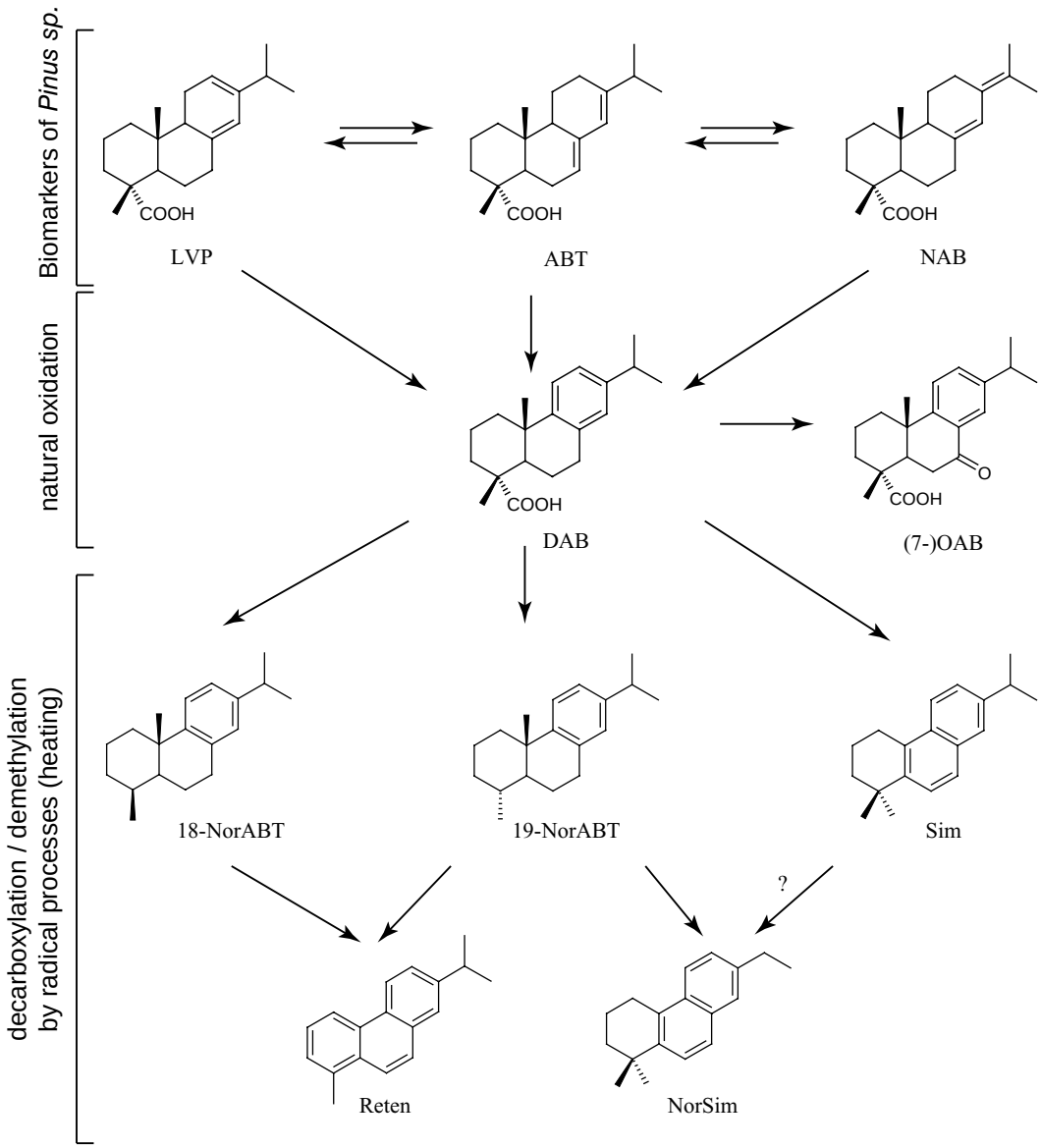


Fig. 10. Chemical structures of diterpenoid compounds identified in the Roman antic wines. LVP: levopimaric acid; ABT: abietic acid; NAB: neoabietic acid; DAB: dehydroabietic acid; OAB: 7-oxodehydroabietic acid; 18-Nor ABT: 18-norabieta-8,11,13-triene; 19-Nor ABT: 19-norabieta-8,11,13-triene; Sim: simonellite; NorSim: norsimonellite.

(*ii*) of 3,4,5-trimethoxylated compounds (**9**, **18**), characteristic markers of gallic moieties, which are also found from modern wines, especially those *élevés* in oak barrels, and from grape seeds. The releasing of such galloylated species does not reflect a potential *élevage* in oak barrel, because of the unknowledge of this technique during the Roman period, but the hypothetical diffusion of the ellagitannins from the cork stopper that closed the amphora since the end of the wine fermentation.

This new analytical protocol using THM for the structural characterization of polyphenolic materials brings more relevant proofs for the identification of ancient vine derivatives and we expand the number of detected phenolic markers, affording a more relevant characterization of wine residues in ceramics.

Beside pyrolytic markers of condensed tannins, other organic compounds have been detected in diverse amounts. The *defrutum* presents particularly important woody indicators, 3,4-dimethoxy-benzaldehyde (**22**) and styrene (**12**), issued from hydrolytic scission of the guaiacylpropyl units [39] (Fig. 9a). These lignin markers are normally present in the wine residue because of the extraction of phenolic material from the cork stopper of the amphora. The water-proofing loss of the sealant agent, a pozzolana cement, over time induces exchanges through the stopper and an increase of salinity in the amphora. The higher ionic force of the media causes the precipitation of the wood tannins [25]. As for modern sweet wines, deposits consist of highly galloylated tannins.

The *Caecuban* wine (Giens) yields other organic markers derived from diterpenic acids in larger amounts than phenolic pyrolytic markers (Fig. 9). The series of isomers of abietic acid (ABT), i.e. isopimaric acid (IPM), pimaric acid (PIM) and its main degradation markers, dehydroabietic acid (DAB) and 7-oxodehydroabietic acid (OAB), are associated with polyaromatic hydrocarbons, such as retene (Ret) and norsimonellite (N-Sim) (Fig. 10) [50]. Although the abietic series comes from resinous species specially from *Pinus sp*., the presence of retene brings in light that the resinous material detected is not a crude resin from a coniferous species, but a pitch obtained by heating and low distillation of the resin or the crude resinous wood. The association of polyphenolic markers and of resinous markers in this *Caecuban* wine (Giens) shows that ancient wines were stored for trade and before consumption in amphorae or *dolia*, the inner sides of which were always coated with a resinous pitch named *Bruttiae pix* or *crapula* by the Latin agronomists [29,51].

THM method allows the simultaneous identification of wine, and the partial determination of the wine-making process used for its better preservation. This coupled THM–GC–MS method presents some improved advantages, permitting: (i) the analysis of extractible and insoluble organic matter, resins or polyphenols by THM, and (ii) the analysis and the identification in a same procedure of components from different chemical families that are clearly separated by the GC method. In the case of archaeological wines, this method appears to be a powerful tool allowing a relevant detection of phenolic markers present in trace amounts compared to resinous markers. The pyrograms obtained allow the detection of vine derivatives, grape seeds or wines, even if mixed with other organic materials, and to assess the existence of other sources of polyphenolic pyrolytic markers, such as cellulose or lignin.

4. Conclusion

The analysis of ancient grape seeds and wine residues by THM–GC–MS represents a significant improvement from the earlier studies that used either infrared spectroscopy or GC analysis preceded by alkaline fusion. Indeed, THM–GC–MS was shown to be very efficient to release a large number of phenolic markers (more than 30), even when applied to samples of several millennia. The tiny amount of organic matter necessary for the analysis (<0.1 mg for a biological material, such as seeds or wine deposits) is very suitable for the study of archaeological samples, which are often preserved in small quantity.

This study makes possible the identification of various polymeric sources which give rise to different pyrolytic phenolic markers: polysaccharides are detected by the important release of 1,2,4-benzenoid compounds, whereas contribution of lignin may be detected by the presence of C_6–C_3 phenols with a lateral methoxylated chain. Proanthocyanidins issued from fruits provide a large variety of di- and trimethoxylated benzenoids compounds.

The preliminary analyses of standard flavanols were used to provide a useful correlation between the pyrolytic phenolic markers released by thermally assisted hydrolysis and methylation and their monomeric precursors. In particular, it was possible to distinguish the 2,4,6-trimethoxybenzenoid compounds released by the non-substituted A-ring of flavonoid monomers from the 3,4,5-trimethoxybenzenoids which are issued from the B-ring of prodelphinidins or galloylated proanthocyanidins.

The identification of such pyrolytic markers in archaeological residues is a strong indication for the preservation of the phenolic molecular structure in ancient samples, even if strongly modified by degradation processes. These results tend to show that after a rapid degradation, probably mainly due to polymerization process of polyphenolic components which occur in the months or the years just following the wine fabrication [44], the new polymeric structure seems to be quite stable, which allows its preservation for several millennia. Such results indicate that it is now possible to apply this analytical methodology to a large range of residues from various archaeological vessels to try to understand the origin of wine production in Europe and to distinguish ceramic vessels directly linked to wine production, consumption or trade, from those dedicated to other purposes.

Although all the modern and archaeological samples release the same pyrolitic markers, all the pyrograms differ by the amount of the different biomarkers. Because of the variety of these pyrogram patterns, the structural identification of each polyphenol detected is essential for detecting the presence of wine in archaeological samples. The variation of the ratio of the different polyphenolic markers may be due to the use of different grape varieties and wine-making techniques but also to their preservation in various environments.

From this study, it has also been possible to detect and identify saturated and unsaturated benzenoid pyrolytic markers, the latter reflecting the partial degradation of constituting units of the phenolic polymer. Indeed, unsaturated phenols, derivatives of styrene or propylbenzene, result from a hypothetical reductive alteration of the condensed tannins leading to the dehydroxylation without aromatization of the C-ring of flavan-3-ols and their unsaturation.

Furthermore, the use of THM–GC–MS permits an efficient separation of pyrolytic markers according to their volatility. Because of the low-molecular-weight of phenols, they can be easily separated and identified in complex mixtures, such as resinated wines where diterpenoid components are major compounds. In a larger field, such an analytical approach could be extended to the study of other materials from our Cultural Heritage, such as organic dyes. For example, ellagitannins extracted from wood (especially oak) used for textile dyeing or for leather tanning could be analysed by this method.

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