Analytical Pyrolysis and Thermally Assisted Hydrolysis–Methylation of Wine Tannin

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Condensed tannins from wines produced in Calabria, Italy, were purified by gel filtration on Sephadex LH-20. Tannin samples were subjected to pyrolysis/gas chromatography/mass spectrometry (PY/GC/MS) and to thermally assisted hydrolysis–methylation/gas chromatography/mass spectrometry (THM/GC/MS), the latter in the presence of tetramethylammonium hydroxide. The tannin fragments produced by PY/GC/MS were identified and ascribed the B ring of the monomeric flavanoids. The methyl derivative of the A ring of the monomeric flavanoids was evident in the THM/GC/MS trace, along with permethylated derivatives of phenolic acids and fatty acids. Control wines and the corresponding samples aged in oak wood barrels were compared as to PY/GC/MS quantitative data of selected pyrolysis fragments.

Keywords: *Pyrolysis/gas chromatography/mass spectrometry; wine analysis; polyphenol analysis; tannin analysis; phenolic analysis; ion trap detector*

INTRODUCTION

Condensed tannins are polyphenols based on flavanoid monomeric units. They are important in the making, color, taste, and redox properties of wines. However, condensed tannins are not a homogeneous chemical class. Many different monomers contribute to their structure (Wong, 1973). Depending on different formulas, substituents, or positional isomerism, such monomers may exhibit different properties, such as color (Wong, 1973) and redox potentials (Chiavari et al., 1988). Consequently, tannin properties should be highly specific, and analytical methods to investigate the molecular composition of tannins would be valuable.

Routine tannin assays are generally based on colorimetric tests, which provide little or no information about tannin molecular structure (AOAC, 1990). On the other hand, fast atom bombardment mass spectrometry (Galletti and Self, 1985; Self et al., 1986) and nuclear magnetic resonance (Gujer et al., 1986) have been used for tannin structural analysis, but such techniques are expensive and require specifically trained operators and their applications have been limited to oligomers of relatively low molecular weight.

Compared to the above-mentioned spectroscopic techniques, pyrolysis/gas chromatography/mass spectrometry (PY/GC/MS) is relatively inexpensive, easy to operate, and based on an instrument, i.e. GC/MS, which is nowadays conventional equipment in many analytical laboratories. PY/GC/MS is well established for the study of lignin, a plant structural polyphenol (Ralph and Hatfield, 1991; Galletti et al., 1993), and has been recently tested for tannins (Galletti and Reeves, 1992; Galletti and Antonelli, 1993; Helleur and Abrajano, 1994). Phenolics of low mass are produced by pyrolysis of polyphenols at 500–1000 °C in an inert atmosphere. Such a pool of pyrolysis fragments is analyzed by GC/MS and allows a chemical characterization of the original sample. When samples are pyrolyzed in the presence of tetramethylammonium hydroxide (TMAH), a so-called thermally assisted hydrolysis–methylation (THM) occurs (Challinor, 1994). By THM, formerly called simultaneous pyrolysis methylation (Challinor, 1991), hydroxyl groups are methylated, thus improving the gas chromatographic behavior of the more polar compounds. The thermal fragmentation pathways may be different in THM compared to regular pyrolysis (Challinor, 1994). To our knowledge, little is known about THM/GC/MS of tannins (Galletti and Antonelli, 1993) and no practical application of PY/GC/MS to compare different wine tannins has been reported.

As a part of research aimed at upgrading wines from Calabria, Italy, by means of aging in oak wood barrels, the present paper reports on PY/GC/MS and THM/GC/MS of wine tannins (i) to characterize their molecular composition both qualitatively and quantitatively and (ii) to check whether analytical pyrolysis can be used to show changes in tannin molecular composition due to aging in wood barrels.

EXPERIMENTAL PROCEDURES

A total of four samples (1A, 1B, 2A, and 2B) from two red wines of Calabria were analyzed: two control samples collected from 2 m^3 batches of red wine after fermentation prior to transferring into wood barrels (1A and 2A) and the corresponding two samples aged in 225 L oak wood barrels for 7 and 15 months (1B and 2B, respectively). Wine samples (100 mL) were rotary-evaporated under reduced pressure at temperature not exceeding 35 °C. Tannins were isolated by gel filtration (Galletti and Self, 1985) of the viscous wine residue (approximately 1 g) through a column packed with Sephadex LH-20 (15 × 1.5 cm) (Pharmacia, Uppsala, Sweden), which was eluted first with water (250 mL) to remove glycerol, second with ethanol 95% (250 mL) to remove colored compounds, and third with acetone/water (70/30 v/v, 200 mL) to collect the brown tannic substances retained on the top of the column. The solvent was rotary-evaporated, and aliquots (approximately 0.1 mg) of dry, brown tannin were subjected to pyrolysis as such (PY/GC/MS) or after addition of TMAH (25% aqueous, 5 μL) on the sample in the pyrolysis quartz sample holder (THM/GC/MS). Pyrolyses were performed at 600 °C for 5 s

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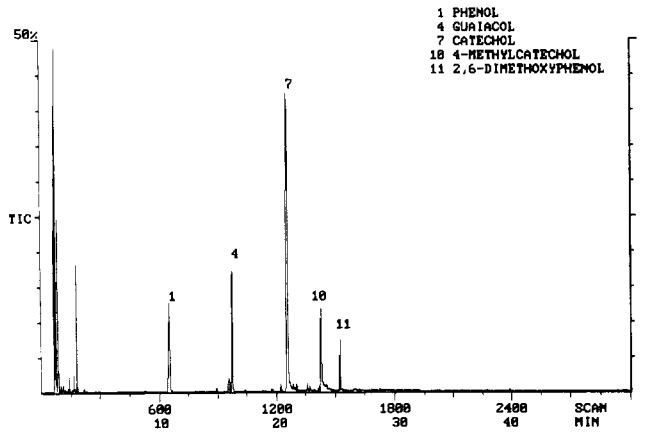


Figure 1. PY/GC/MS chromatogram of wine tannin (sample 1A). For peak identification, see Table 1.

with a Pt heated-filament pyrolyzer Pyroprobe 1000 (Chemical Data System, Oxford, PA) using a quartz sample holder. The gas chromatograph Varian 3400 (Varian, Walnut Creek, CA) was equipped with a Supelco SPB-5 column (30 m × 0.32 mm i.d., film thickness 0.25 μm) (Supelco, Bellefonte, PA), which was heated from 50 to 300 °C at 5 °C/min, holding the initial temperature for 10 min. The PY/GC interface was at 200 °C for PY/GC/MS and at 70 °C for THM/GC/MS. The injector was at 250 °C in the split mode (split ratio 1/100). The Finnigan MAT Model 800 ion trap mass spectrometer with upgraded short transfer line (Finnigan MAT, Bremen, Germany) was operated under electron impact at 70 eV. Mass spectra were recorded in the range m/z 40–400 (1 scan/s). Peak identification was based on mass spectra, on PY/GC/MS of standard compounds, and on published collections of PY/GC/MS data of phenolic compounds (Ralph and Hatfield, 1991; Galletti and Reeves, 1992; Galletti et al., 1993). Quantifications are expressed as peak area percentages. Peaks with retention times shorter than 300 scans were omitted. PY/GC/MS analyses were done in triplicate. A t-test was used to compare control (A) and aged (B) samples.

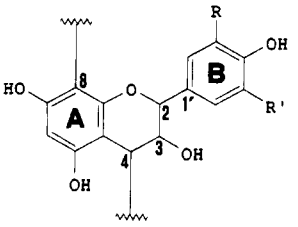
RESULTS AND DISCUSSION

PY/GC/MS. Figure 1 shows the PY/GC/MS chromatogram of sample 1A as a representative tannin pyrogram. Peak identification, area percentage, and mass spectra are reported in Table 1. The peaks with retention times shorter than 300 scans are not discussed because the chromatographic separation at the beginning of the chromatogram is poor. A change of chromatographic conditions to improve peak separation is worthless, since such peaks correspond to the smallest fragments obtainable by pyrolysis, e.g. low-boiling alcohols and acids, which are of no diagnostic value.

According to Galletti and Antonelli (1993), pyrolysis of standard flavanoids yields the arylic groups "B" originated by the cleavage of the C(2)–C(1′) bond in the flavanoid (Chart 1). Therefore, the presence of phenol, guaiacol, and catechol as main pyrolysis fragments (peaks 1, 4, and 7 in Figure 1 and Table 1) can be explained as due to the presence of pelargonidin-, peonidin-, and cyanidin-like moieties, respectively, in the original tannic substance (Chart 1). Likewise, smaller amounts of 3-methoxycatechol and 2,6-dimethoxyphenol (peaks 9 and 11 in Figure 1 and Table 1) can be ascribed to petunidin and malvidin, respectively (Chart 1). Relatively smaller amounts of 4-alkyl-substituted phenolics, namely 4-methylphenol, 4-ethylphenol, 4-vinylphenol, 4-methylguaiacol, and 4-methylcatechol (peaks 3, 5, 8, 6, and 10, respectively, in Figure 1 and Table 1), suggest that fission of the heterocyclic ring occurred, although such a process is less important compared to the cleavage of the C(2)–C(1′) bond. The pyrogram showed no evidence of fragments belonging to rest of the flavanoid molecule.

Apart from a small amount of 3-methylphenol (peak 2 in Figure 1) previously identified in the PY/GC/MS analysis of lignins (Ralph and Hatfield, 1991), pyrograms showed a unique pattern not resembling those of lignins (Ralph and Hatfield, 1991; Galletti et al., 1993) and of tannins isolated from sorghum grain and grape skins (Galletti and Reeves, 1992). In particular, unlike the experiments previously published by Galletti and Antonelli (1993), there was no evidence of sugar dehydration products and of contaminants from the column used for the purification step. This observation suggests that Sephadex LH-20 should be preferred to polyvinylpolypyrrolidone (PVPP) for tannin purification.

Chart 1. Proposed Origin of the Tannin Pyrolysis Fragments



GENERAL STRUCTURE OF A CONDENSED TANNIN MONOMERIC UNIT

Parent anthocyanin	"B" ring substituents	Pyrolysis fragment	Peak #
Pelargonidin	R=R′=H	Phenol	1
Peonidin	R=OCH_3, R′=H	Guaiacol	4
Cyanidin	R=OH, R′=H	Catechol	7
Petunidin	R=OH, R′=OCH_3	3-Methoxycatechol	9
Malvidin	R=R′=OCH_3	2,6-Dimethoxyphenol	11

Table 1. Proposed Identification and Eight-Peak Mass Spectra of the Main Pyrolysis Fragments Found in the PY/GC/MS Analysis of Wine Tannins Purified by Sephadex LH-20 (Sample 1A)[a]

peak no.	scan	MW	m/z (%)		compd	area % (SD)
1	636	94	94, 66, 65, 40, 63, 50, 55, 95	(100, 46, 33, 11, 9, 8, 7, 6)	phenol	11.95 (0.73)
2	868	108	108, 107, 79, 77, 44, 50, 51, 94	(100, 94, 70, 63, 51, 40, 39, 33)	3-methylphenol	0.62 (0.05)
3	924	108	107, 108, 77, 79, 51, 80, 50, 53	(100, 68, 41, 38, 23, 17, 17, 10)	4-methylphenol	1.49 (0.51)
4	939	124	109, 81, 124, 53, 51, 52, 50, 63	(100, 98, 71, 48, 24, 18, 18, 11)	guaiacol	10.69 (0.53)
5	1133	122	107, 77, 122, 44, 51, 53, 50, 65	(100, 36, 32, 18, 14, 12, 11, 11)	4-ethylphenol	0.13 (0.04)
6	1175	138	123, 138, 67, 95, 77, 55, 65, 51	(100, 85, 68, 64, 33, 31, 26, 24)	4-methylguaiacol	0.47 (0.04)
7	1214	110	110, 64, 63, 53, 81, 82, 50, 51	(100, 32, 31, 23, 21, 21, 13, 13)	catechol	50.37 (1.91)
8	1250	120	120, 91, 119, 65, 94, 66, 40, 89	(100, 80, 43, 23, 14, 10, 10, 8)	4-vinylphenol	1.12 (0.10)
9	1322	140	97, 125, 140, 51, 53, 79, 50, 52	(100, 89, 88, 51, 31, 23, 20, 16)	3-methoxycatechol	1.77 (0.20)
10	1390	124	124, 78, 123, 51, 77, 66, 50, 67	(100, 74, 60, 32, 28, 23, 20, 18)	4-methylcatechol	11.60 (2.23)
11	1486	154	154, 139, 93, 65, 111, 96, 53, 51	(100, 53, 46, 42, 35, 34, 23, 23)	2,6-dimethoxyphenol	9.65 (0.68)

[a] Numbers as in Figure 1. Peak area percentage, mean of three replicates (± standard deviation).

Table 2. Main Pyrolysis Products in the PY/GC/MS Analyses of Tannins from Control (A) and Aged (B) Wines[a]

peak no.	compd	1A	SD	1B	SD	*t*-test	2A	SD	2B	SD	*t*-test
1	phenol	11.95	0.7	9.72	0.5	*	16.47	0.5	21.40	0.5	**
4	guaiacol	10.69	0.5	9.45	0.3	*	13.80	0.7	16.27	1.8	*
7	catechol	50.37	1.9	49.58	2.8	ns	53.13	0.2	46.27	0.1	**
9	3-methoxycatechol	1.77	0.2	2.60	0.3	*	1.16	0.1	0.87	0.3	ns
10	4-methylcatechol	11.60	2.2	14.68	2.8	ns	10.30	0.5	10.03	1.7	ns
11	2,6-dimethoxycatechol	9.65	0.7	10.63	0.6	ns	5.23	0.2	5.17	0.6	ns

[a] Sample 1B, 7-month aging; sample 2B, 15-month aging. Figures are means of three replicates (SD, ± standard deviation). *t*-Test: ** = significant differences at $P < 0.01$; * = significant differences at $P < 0.05$; ns, nonsignificant differences.

Table 3. Proposed Identification and Eight-Peak Mass Spectra of the Main Phenolic Fragments Found in the THM/GC/MS Analysis of Wine Tannins Purified by Sephadex LH-20[a]

peak no.	scan	MW	*m/z* (%)	compd[b]
1	357	108	108, 64, 44, 78, 43, 42, 45, 59 (100, 89, 85, 84, 58, 52, 44, 41)	methoxybenzene (1)
2	1105	138	138, 95, 77, 123, 65, 52, 51, 41 (100, 53, 40, 34, 27, 24, 21, 17)	1,2-dimethoxybenzene (2)
3	1297	152	152, 109, 137, 79, 81, 91, 77, 65 (100, 60, 49, 44, 39, 34, 34, 26)	3,4-dimethoxytoluene (3)
4	1438	168	168, 153, 110, 125, 95, 93, 65, 51 (100, 77, 57, 47, 45, 41, 23, 21)	1,2,3-trimethoxybenzene (4)
5	1597	168	168, 139, 169, 109, 125, 140, 69, 167 (100, 70, 23, 19, 17, 13, 13, 10)	1,3,5-trimethoxybenzene
6	1720	182	182, 151, 153, 167, 181, 121, 139, 183 (100, 30, 26, 22, 21, 19, 16, 16)	2,4,6-trimethoxytoluene
7	1775	196	196, 165, 181, 77, 79, 91, 153, 121 (100, 44, 43, 25, 20, 20, 18, 18)	2,4,6-trimethoxyethylbenzene
9	1879	196	165, 196, 79, 51, 77, 166, 121, 50 (100, 92, 34, 28, 17, 15, 15, 14)	3,4-dimethoxybenzoic acid methyl ester
10	1887	192	177, 192, 43, 149, 41, 69, 74, 121 (100, 85, 58, 32, 23, 21, 20, 20)	4-methoxycinnamic acid methyl ester
11	1926	194	194, 151, 179, 91, 107, 77, 65, 119 (100, 70, 60, 52, 25, 24, 24, 20)	cis-1-(2-methoxy-ethenyl)-3,4-dimethoxybenzene[c]
12	1944	194	194, 179, 91, 151, 65, 119, 77, 51 (100, 67, 60, 46, 24, 23, 23, 22)	trans-1-(2-methoxy-ethenyl)-3,4-dimethoxybenzene
13	2060	226	226, 211, 155, 53, 195, 66, 151, 227 (100, 54, 36, 19, 18, 17, 16, 15)	3,4,5-trimethoxybenzoic acid methyl ester
16	2226	222	209, 222, 41, 191, 43, 91, 69, 55 (100, 83, 58, 41, 40, 40, 38, 37)	3,4-dimethoxycinnamic acid methyl ester

[a] Numbers as in Figure 2. [b] Corresponding underivatized phenolics in PY/GC/MS: (1) phenol; (2) guaiacol and catechol; (3) 4-methylguaiacol and 4-methylcatechol; (4) 3-methoxycatechol and 2,6-dimethoxyphenol. [c] Impure.

Triplicate sequential PY/GC/MS analyses showed relative standard deviations ranging from 3.8 to 7.0% for the main peaks originating from the C(2)−C(1′) cleavage, i.e. peaks 1, 4, 7, and 11 (peak areas ranging from 9.65 to 50.37%). 3-Methoxycatechol (peak 9) showed a somewhat larger standard deviation (11.3%), but its area was only 1.77%. Relative standard deviations of 8.1−34.2% were found for the 4-alkyl-substituted phenolics. Such a larger range of standard deviations can be explained as due to either the small peak areas (all fragments were less than 1.5% with the exception of 4-methylcatechol) or to a less reproducible thermal fission. In fact, such fragments are derived from the heterocyclic ring by cleavage of two or more bonds with multiple proton rearrangement as opposed to the single C(2)−C(1′) cleavage of the main pyrolysis products.

Finally, Table 2 shows the comparison between control and aged samples as obtained by comparing the peak areas of the five main phenolics originated by the C(2)−C(1′) cleavage of the flavanoid (i.e. peaks 1, 4, 7, 9, and 11) along with 4-methylcatechol (peak 10), the main 4-alkyl-substituted phenolic. Significant changes ($P < 0.01$) were found for the two main peaks, i.e. catechol and phenol, between control and 15-month-aged samples, whereas the differences were nonsignificant or significant at $P < 0.05$ between control and 7-month-aged samples. A decrease in catechol and a corresponding increase in phenol concentration may be related with the large difference in the oxidation potentials of the two compounds, namely 0.61 and 0.98 V vs standard calomel electrode at 20 mV s^{-1}, respectively (Chiavari et al., 1988). Such observations suggest that a 15-month aging in oak wood barrels did affect tannin molecular composition in the analyzed samples, when the main pyrolysis peaks were considered. A shorter aging period (7 months) or other less abundant pyrolysis fragments resulted in changes nonsignificant or significant to a low degree of probability ($P < 0.05$).

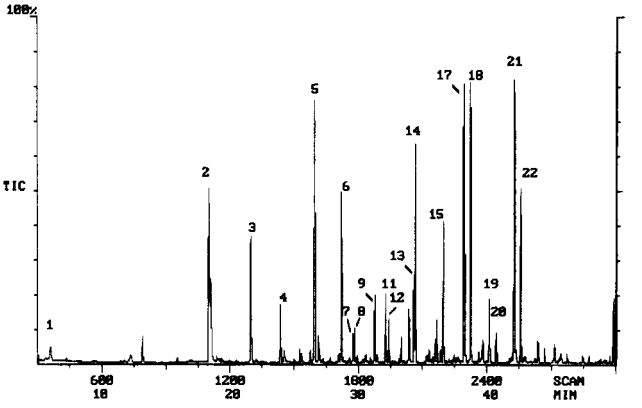


Figure 2. THM/GC/MS chromatogram of wine tannin (sample 1A). For peak identification, see Table 3. Peaks 8, 14, 15, and 17−22 are fatty acid methyl esters (see mass chromatograms in Figure 3).

THM/GC/MS. Figure 2 shows the pyrogram of THM/GC/MS analysis of sample 1A. The pyrogram looks much more complicated than that of regular PY/GC/MS. In fact, more phenolics were visible as methyl derivatives than in the underivatized form (Table 3). Moreover, phenolic acids and fatty acids were eluted in their permethylated forms. Peak areas are not reported since THM/GC/MS was used only to complement PY/GC/MS with qualitative information on tannin pyrolysis products.

Methoxybenzene, 1,2-dimethoxybenzene, 3,4-dimethoxytoluene, and 1,2,3-trimethoxybenzene (peaks 1−4 in Figure 2 and Table 3) are the methyl ether derivatives of phenolics identified in the previous PY/GC/MS analysis. Their presence in the THM/GC/MS trace (i) corroborates previous pyrolysis fragments and (ii) suggests that the pyrolysis fragmentation pathway leading to such molecules was not affected by the addition of TMAH and that a simple methylation of free hydroxyls occurred. However, such methoxy derivatives are of

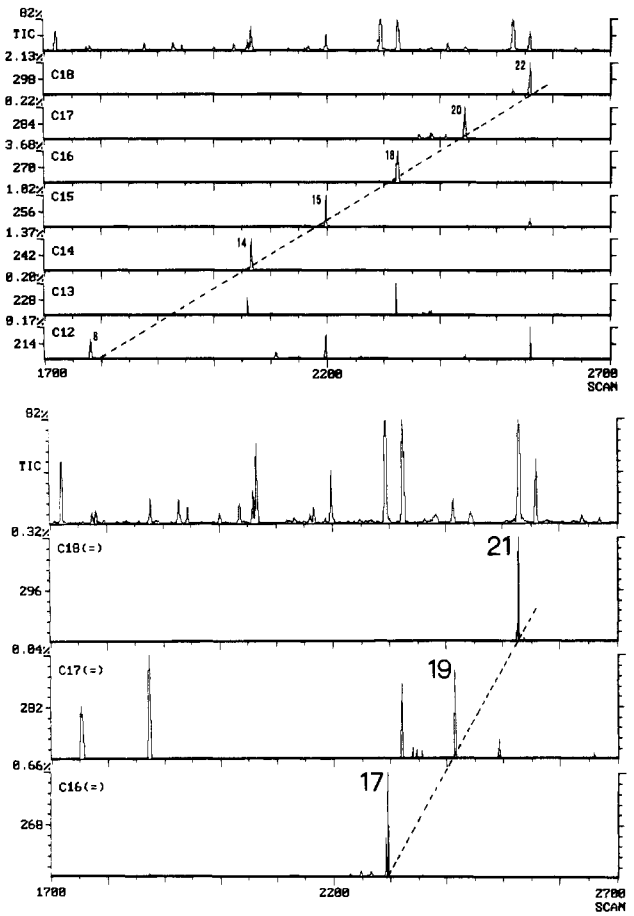


Figure 3. Mass chromatograms (M^+, molecular ion) of (a, top) saturated and (b, bottom) unsaturated fatty acid methyl esters found in the THM/GC/MS chromatogram of wine tannin. Peak numbers are as in Figure 2.

little analytical value, since each of them can be ascribed to two underivatized phenolics, except methoxybenzene (Table 3). Peaks 5–7 (Figure 2 and Table 3) are more interesting, because they represent the A moiety of the flavanoid molecule which was missing from PY/GC/MS traces. Peaks 11 and 12 (Figure 2 and Table 3) show that the heterocyclic ring was cleaved with the hydroxyl remaining on C(3). The corresponding underivatized enol/keto fragments were never observed in PY/GC/MS of tannins, but such enol-methyl derivatives are reminiscent of the enol-trimethylsilyl derivatives which can be obtained by silylation of molecules with a keto/enol equilibrium, such as sugars (Poole, 1978). Such peaks were confirmed by THM/GC/MS analysis of catechin.

Permethylated phenolic acids have been observed by Galletti and Antonelli (1993) in the simultaneous pyrolysis methylation of tannins. Phenolic acids can be released by grape skins and can be esterified with flavanoids (Liao et al., 1992). The presence of the methyl esters of 3,4-dimethoxybenzoic acid, 3,4,5-trimethoxybenzoic acid, 4-methoxycinnamic acid, and 3,4-dimethoxycinnamic acid, though at trace levels in the present THM/GC/MS tannin analysis, confirms the previous reports (peaks 9, 13, 10, and 16, respectively, in Figure 2 and Table 3). The very low amount of 4-methoxycinnamic acid makes negligible its possible contribution to the formation of 4-ethylphenol and 4-vinylphenol by decarboxylation during PY/GC/MS experiments.

A homologous series of saturated fatty acid methyl esters from C_{12} to C_{18} (except C_{13}) was detected by THM/GC/MS. Unsaturated C_{16}–C_{18} fatty acid methyl esters were also present. Figure 3 shows relevant mass chromatograms. Dodecanoic, tetradecanoic, and a trace of pentadecanoic acid have been found previously in the PY/GC/MS analysis of wine tannins (Galletti and Antonelli, 1993). It is known that grape seeds contain fats. Tannins also originate from seeds. It is therefore possible that tannins (i) trap fatty acids in their free form or as triglycerides or (ii) are esterified to some extent with fatty acids, the latter occurrence having important effect on tannic properties such as astringence. Further studies are necessary to confirm or dismantle such hypotheses. THM/GC/MS made possible the detection of fatty acids in tannic substances, by means of their esterification or transesterification into methyl esters.

Conclusion. Tannin is a complex polymer elusive of accurate characterization. Analytical pyrolysis provided information about the molecular composition of tannic substances isolated by gel filtration with practically no sample workup. On the basis of catechol and phenol PY/GC/MS data, a significant change in tannin composition occurred after wine aging in wood barrels for 15 months. Although a larger set of samples will be necessary to confirm such results, the important observation here is that PY/GC/MS is sufficiently reproducible to show differences in tannin composition, if they exist. Such a remark is not trivial, since many misconceptions about the reproducibility of analytical pyrolysis still exist (Wampler, 1994). THM/GC/MS allowed the determination of more polar phenolic moieties, otherwise undetectable by PY/GC/MS. Phenolic acids and fatty acids, which are included in the tannic fraction, were evidenced by THM/GC/MS as permethylated derivatives.

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