

# Atraric acid, a marker for epiphytic lichens in the wood used in cooperage: Identification and quantification by GC/MS/(MS)

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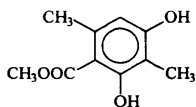
**Abstract.** A phenolic derivative, atraric acid, was identified by GC/MS and quantified by GC/MS/MS in the outer parts of wood from oaks specifically colonised by lichens. This compound was correlated to the presence of a bitter depside, atranorin, which is a natural metabolite of the lichen species belonging to the genus *Parmelia*.

**Key words.** Lichens – atranorin – atraric acid – oak wood – ion trap mass spectrometry – quantification.

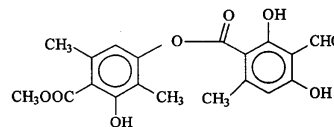
## Introduction

Wine or spirits are aged in barrels made with stave wood coming from the heartwood, mainly of two oak species: *Quercus petraea* (Mattusch.) Liebl. and *Quercus robur* L. Oaks used for cooperage activities are grown in regularly maintained forests in the centre of France and are felled between 150 and 250 years of age [1]. The bark of oaks is a support for different corticolous species, which have been listed [2]. Among the listed species, lichens are known to produce bitter depsides which could contaminate the bark and possibly the heartwood. Contaminated heartwood could induce off-flavour effects in the wines or spirits aged in such barrels and this is the reason for the interest of the cooperage industry in an estimate of the hazards related to the occurrence of bitter-producing organisms on the oak bark.

GC/MS analysis of acetone extracts from contaminated bark samples allowed us to identify the atraric acid **1**. GC/MS/MS analysis permits estimation of the quantity of compound **1**, the amounts of which decreased from the bark (not used in cooperage) to the outer heartwood normally used to make staves. Derivative **1** has been related to the well known lichen product **2** (atranorin), identified twenty years ago [3] in two of the lichen species that we have found on the bark of some trees.



**1**, atraric acid,  $M = 196$   
 benzoic acid, 2,4-dihydroxy-3,6-dimethyl-, methyl ester.



**2**, atranorin,  $M = 374$   
 benzoic acid, 3-formyl-2,4-dihydroxy-6-methyl-,  
 3-hydroxy-4-(methoxycarbonyl)-2,5-dimethylphenyl ester.

## Materials and methods

### Preparation of the extracts

Outer part of the bark was carefully scraped to remove all the plants and especially lichens. Samples (1g dry weight) of cork, phloem, sapwood or heartwood were powdered to fine sawdust (60 Mesh) and extracted for 4 hours with 200 mL acetone using a "Soxhlet" apparatus. The solvent was evaporated under reduced pressure at 50 °C and the residue was recovered with 0.1 mL acetone. To quantify, 10  $\mu$ L acetone solution containing 1 g L<sup>-1</sup> naphthalene were added to the wood powder, so that the final extract contained 10 ppm naphthalene.

### Hydrolysis of atranorin

A solution of 1 mg atranorin (A-6652 Sigma Aldrich Chemie, 89552-Steinheim, Germany) in 1 mL methanol was added to 1 mL water acidified (pH 2) by HCl and heated one day at 60 °C. The reaction mixture was extracted by

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# Original articles

diethyl ether. The extracted solution was dried over anhydrous sodium carbonate and directly injected into the GC/MS system.

## Identification of atraric acid, **1**, in extracts from the bark samples contaminated by lichens (Fig. 1) and in hydrolysate of atranorin (Fig. 2)

Compound **1** was identified in GC/MS analysis by comparison of its spectrum with the reference spectrum of atraric acid listed in the NIST library [4], using a "SATURN 4D" (VARIAN INC. - Palo Alto - CA94304 - USA) ion trap mass spectrometer coupled to a VARIAN "Star 3400CX" gas chromatograph fitted with a "split-splitless" injector.

**GC conditions:** "DB XLB" (J&W Scientific - Folsom - CA 95630 - USA, 30 m × 0.25 mm, film 0.25 μm) WCOT capillary column. Split mode. 1 μL injection. Split rate: 1/50. Temperature programmed from 50 °C to 300 °C (4 °C min<sup>-1</sup>). The DB XLB phase was chosen in reason of its low bleeding and its ability to separate a wide range of molecules of different boiling points.

**MS conditions:** Electron Impact 70 eV. Manifold temperature 220 °C. Mass range 35-450 Daltons (100 scans min<sup>-1</sup>).

## Detection and quantification of atraric acid (Fig. 3)

**GC conditions:** DB XLB J&W, 30 m × 0.25 mm, film μm capillary column. Splitless mode. 1 μl injection. Temperature programmed from 50 °C (1 min) to 150 °C (50 °C min<sup>-1</sup>) and from 150 °C to 250 °C (12 °C min<sup>-1</sup>).

**MS conditions:** mass spectra and mass chromatograms were successively recorded, during the same analysis, using the ion trap spectrometer in two different modes (Fig. 3):

- detection of the internal standard (naphthalene) performed by GC/MS in single ion monitoring of the molecular ion  $m/z$  128.
- detection of the atraric acid performed by GC/MS/MS to improve the selectivity [5]. The molecular ion ( $m/z$  196) was isolated and fragmented by non-resonant collision induced dissociation (excitation amplitude 45 V - isolation time 5 ms). The intense daughter  $m/z$  164, resulting from the elimination of one molecule of methanol from the molecular ion  $m/z$  196 was monitored during the chromatography.

The quantitative estimation of atraric acid was performed by comparison of the areas of the peaks corresponding to the daughter  $m/z$  164 in MS/MS mode and the peak corresponding to the molecular ion  $m/z$  128 of the naphthalene in direct mode. The calibration was done by running four reference acetone solutions, each containing 100 mg L<sup>-1</sup> naphthalene and in addition 0.1, 1, 10 or 100 mg L<sup>-1</sup> atraric acid.

## Results and discussion

A compound of molecular weight 196 was detected by GC/MS exclusively in the cork or in the sapwood of trunks

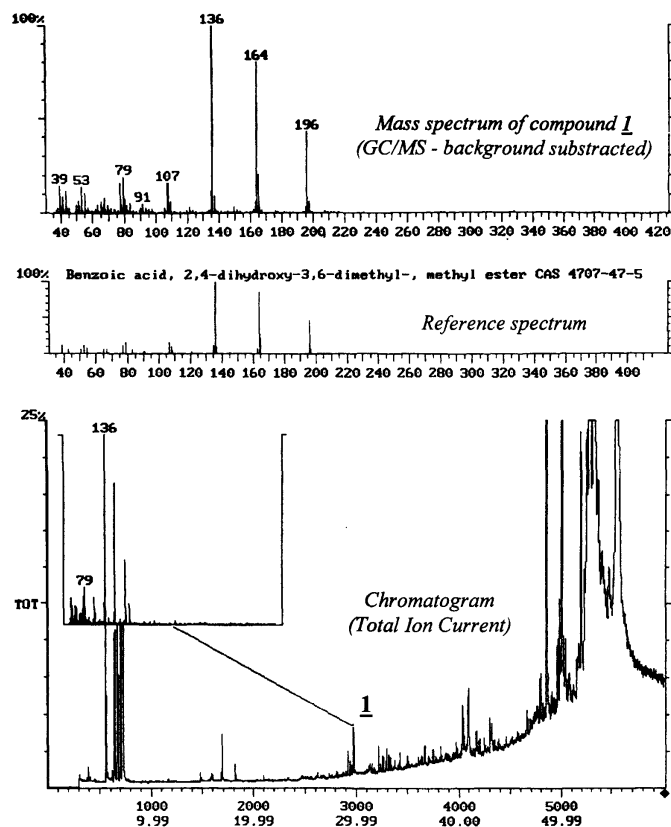


Fig. 1. Identification of atraric acid, **1**, in the extract from a bark sample contaminated by *Parmelia olivetorum* and *P. perlata*.

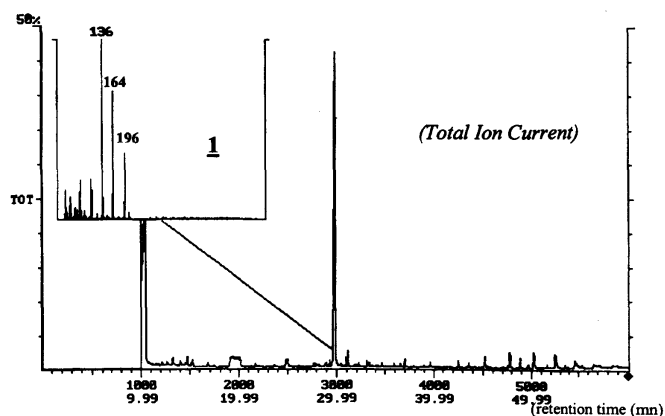


Fig. 2. Identification of atraric acid, **1**, in the hydrolysate of atranorin, **2**, by GC/MS (total ion current and mass spectrum).

colonised by one at least of the two *Parmelia* species previously identified [2]: *Parmelia olivetorum* (Ach.) Nyl. and *Parmelia perlata* (Huds.) Ach. Both species as many others classified in the genus *Parmelia* or in some allied genera, produce the depside, atranorin, **2** [3].

The mass spectrum obtained by GC/MS was in good agreement with the reference spectrum of atraric acid, **1**, in the NIST library (Fig. 1) and with the spectrum obtained at the same retention time, by hydrolysis of atranorin, **2** (Fig. 2). Atraric acid mass spectrum has three intense characteristic peaks: the molecular ion ( $m/z$  196), the peak resulting of the loss of methanol ( $m/z$  164) and the peak ( $m/z$  136) resulting from elimination of CO from the last ion. Significant concentrations of **1** were detected by GC/MS/MS (Fig. 3) only in cork (10 – 100  $\mu\text{g g}^{-1}$  of sample) and in sapwood (1  $\mu\text{g g}^{-1}$ ). In heartwood, the concentration in atraric acid did not exceed 0.001  $\mu\text{g g}^{-1}$  (Fig. 4), which is the limit of detection. Atraric acid was not detected in cork samples taken off trunks without lichen or those with very small populations of lichens. Presence of atraric acid in the extracts of sapwood can obviously be correlated both to the occurrence of *Parmelia olivetorum* and/or *P. perlata* on the trunk of the tree and to the hydrolysis of the natural bitter depside atranorin: the tasteless derivative **1** is an internal marker within the oak wood for the epiphytic *Parmelia* species.

The atraric acid or its precursor, the atranorin, did not diffuse deeper than the very outer heartwood (Fig. 4) and therefore did not significantly contaminate more than the outer staves in the case of an unusual strong colonisation of the bark by lichens; even in this unfavourable eventuality, few staves are likely to be contaminated and only very slightly. Such a contamination is presumed undetectable in the aged products.

## Conclusion

Atraric acid, the hydrolysis product from the bitter atranorin, was found only in low concentrations in the heartwood used for staves and therefore its depside does not influence significantly the gustatory properties of wines or spirits aged in oak barrels. Nevertheless, it is advisable as an extra precaution, to avoid using for cooperage trees which are strongly colonised by *Parmelia olivetorum* and/or *P. perlata*, especially in the middle part of the trunk from which stave wood is taken.

## Acknowledgements

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## References

- Vivas, N. *J. Cooperage Sci. Tech.* **1995**, *1*, 33-48.
- Suire, C.; Vivas, N.; Bourgeois, G. *J. Cooperage Sci. Tech.* **1996**, *2*, 113-119.
- Taylor, C. J. *Ohio Biol. Surv., Biol. Notes* (1967) In: Culberson C.F. *The Bryologist* **1970**, *73*, 177-377.
- NIST/EPA/NIH Mass Spectral Database, Version 4.0, US Department of Commerce, Gaithersburg, USA (1992).
- David, F.; De Clercq, C.; Sandra, P. VARIAN INC. Palo Alto, USA, Application note N° 52.

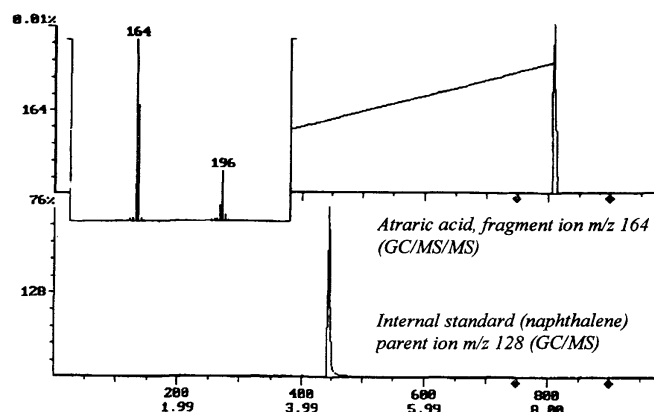


Fig. 3. Quantification of atraric acid, **1** (concentration measured: 0.9  $\mu\text{g g}^{-1}$  of sapwood) by GC/MS/MS, using the naphthalene as internal standard.

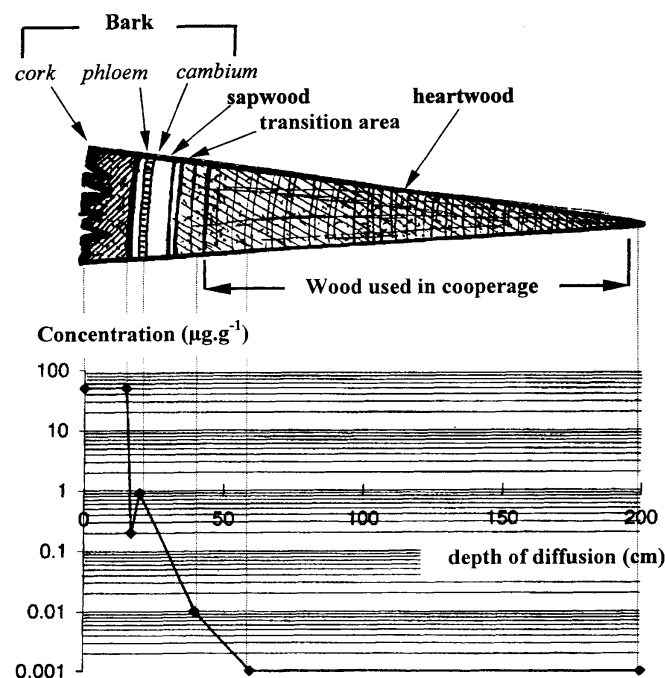


Fig. 4. Plot of the concentration in atraric acid, **1**, according to the diffusion depth in the wood.

# Separation and quantitation of orange juices using liquid chromatography of polymethoxylated flavones

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**Abstract.** A rapid method of extraction and quantitation of polymethoxylated flavones (PMF) using isocratic liquid chromatography is described. With this method the content of six PMF: sinensetin, hexamethoxyflavone, nobiletin, heptamethoxyflavone, tetra-O-methyl scutellarein and tangeretin from 60 commercial orange juices have been determined. The PMF extraction method is mainly established on sample preparations using C<sub>18</sub> cartridge extractions with water/acetonitrile (60/40, v/v) as mobile phase. PMF compositions of orange commercial juices show a quantitative difference on population samples which are linked to the PMF contents and varieties. By these rapid quantitative method, the PMF values observed were in accordance with literature. This technique present correct characteristics of repeatability and reproducibility (4.5% and 6.6% respectively).

**Key words.** Polymethoxylated flavones – extraction – determinations – liquid chromatography – orange juice.

## Introduction

Various polymethoxylated flavones (PMF) which can be found in oranges, have been isolated and identified some years ago [1-3]. For example, nobiletin (NOB) and sinensetin (SIN) were isolated from sweet orange flavedo (*Citrus sinensis*) or, tangeretin (TAN) isolated from tangerine (*C. reticulata*) [1-3] (Fig. 1). Six PMF were isolated from orange essential oil [4,5] and the PMF identified are characterized by the absence of a hydroxyl group. Their methoxylation level, heptamethoxyflavone (HEP) containing the highest methoxylation level, differentiated the PMF. The PMF are mainly located in the flavedo (external part of the citrus peel) and therefore they are found in essential oil of citrus. During the extraction process a part of the essential oil is mixed with the juice. The composition study of PMF in essential oils and in orange and mandarin juices has been largely investigated using thin layer chromatography [6], and five PMF have been identified: SIN, NOB, HEP, tetramethylscutellarein (SCU) and TAN in oranges juices and essential oils. Then, separation techniques using gas chromatography [7] and liquid chromatography (isocratic and gradient elution) have been developed [8-10], allowing a rapid and precise determination. Nevertheless, due to the low content in PMF found in mandarin and orange juices compared to the PMF content in essential oils of this citrus (from 200 to 500 fold lower in juices), many extraction techniques have been developed. One of them consist in a liquid/liquid extraction using organic solvents such as benzene or chloroform [10-12]. Another technique used for PMF extraction,

the concentration on C<sub>18</sub> cartridges after juice filtration on celite, have significantly simplified the sample preparation [9]. In this paper, we have used a rapid method of separation and quantitation of the six main PMF occurring in orange juices [SIN, hexamethoxyflavone (HEX), NOB, SCU, HEP and TAN] using an isocratic liquid chromatographic method with a C<sub>18</sub> cartridge extraction system, to separate and quantify PMF. This method was applied for the PMF content determination in commercial orange juices and the validation of this method was achieved.

## Materials and methods

### Samples

The quantitative analysis of PMF content was carried out on 60 commercial orange juices composed of pure fresh juices (10 samples), pure pasteurized juices (30 samples) and juices made with concentrates (20 samples), from different varieties and origins. A ring test has been carried out on three pure valencia orange juices, 2 made from Spain (A and B) and one from Cuba (C).

### Standards

Three standards (SIN, SCU and TAN), analytical grade, were obtained from Extrasynthese (Genay, France). The mother solutions of SIN, SCU and TAN were prepared at 100 mg L<sup>-1</sup> in methanol (Carlo Erba) and were stable for

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