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# WOOD RELATED CHEMICAL MARKERS OF AGED WINE BRANDIES

## MARCADORES QUÍMICOS DA MADEIRA EM AGUARDENTES VÍNICAS ENVELHECIDAS

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**SUMMARY**

Several low molecular weight phenolic compounds - phenolic acids, phenolic aldehydes, coumarins, acetovanillone and ethyl vanillin – were quantified by HPLC in Portuguese wine brandies aged four years in Portuguese oak (*Quercus pyrenaica* Willd.), Allier oak (*Quercus sessiliflora* Salisb.) and chestnut (*Castanea sativa* Mill.) wooden barrels (250 L), in order to identify those of chemical markers of the wood botanical species. The results obtained demonstrated that gallic acid, ellagic acid, ferulic acid, scopoletin, acetovanillone and ethyl vanillin play that role in the aged brandies. Gallic acid and acetovanillone present the highest contents in the brandies aged in chestnut, while ethyl vanillin exhibit the highest content in the brandies aged in oak woods. The differentiation of the brandies aged in oak woods is made by ellagic and ferulic acids, whose contents are higher in Portuguese oak, and by scopoletin that predominates in the brandies aged in Allier oak. Complementary, a practical and reproducible HPLC method was validated allowing a good separation and quantification of acetovanillone and ethyl vanillin in wine aged brandies.

**RESUMO**

Com o objectivo de identificar os compostos que podem desempenhar o papel de marcadores químicos associados à espécie botânica da madeira utilizada no envelhecimento, diversos compostos fenólicos de massa molecular baixa – ácidos fenólicos, aldeídos fenólicos, cumarinas, acetovanilona e etilvanilina – foram quantificados, por HPLC, em aguardentes vínicas portuguesas envelhecidas durante quarto anos em vasilhas (250 L) de carvalho português (*Quercus pyrenaica* Willd.), carvalho francês Allier (*Quercus sessiliflora* Salisb.) e castanheiro (*Castanea sativa* Mill.). Os resultados obtidos demonstraram que o ácido gálhico, o ácido elágico, o ácido ferúlico, a escopoletina, a acetovanilona e a etilvanilina presentes na aguardente envelhecida funcionam como marcadores químicos da madeira. As aguardentes envelhecidas em madeira de castanheiro apresentam os teores mais elevados de ácido gálhico e de acetovanilona, enquanto as aguardentes envelhecidas em madeira de carvalho exibem concentrações superiores de etilvanilina. A diferenciação das aguardentes envelhecidas em diferentes espécies de madeira de carvalho pode ser baseada nos ácidos elágico e ferúlico, cujos teores são superiores nas aguardentes envelhecidas em carvalho português, e na escopoletina, que predomina nas aguardentes envelhecidas em carvalho francês Allier. Complementarmente, um método cromatográfico (HPLC) prático e reprodutível foi validado, permitindo uma boa separação e quantificação da acetovanilona e da etilvanilina em aguardentes vínicas envelhecidas.

**Keywords:** Chemical markers, Phenolic compounds, Aged wine brandies, Wood botanical species, HPLC method.
**Palavras-Chave:** Marcadores químicos, Compostos fenólicos, Aguardentes vínicas envelhecidas, Espécies botânicas da madeira, Método HPLC.

## INTRODUCTION

The ageing process is of primary importance for the quality of the brandy. During the ageing period several low molecular weight compounds are extracted from wood to the distilled. Among them, phenolic compounds assume considerable significance due to their influence on the chemical characteristics (Puech *et al.*, 1985, Canas *et al.*, 1999; Canas *et al.*, 2000; Belchior *et al.*, 2001; Canas *et al.*, 2002; Belchior *et al.*, 2003; Patrício *et al.*, 2005), sensory properties (Singleton, 1995; Canas *et al.*, 2000a; Belchior *et al.*, 2001; Caldeira *et al.*, 2002) and nutraceutical quality (Goldberg *et al.*, 1999; Da Porto *et al.*, 2000; Umar *et al.*, 2003) of the aged brandy.

The phenolic compounds contents in the brandy are determined by the technological process, mainly the ageing conditions, including the wooden barrel characteristics, such as the wood botanical species (Lavergne *et al.*, 1991; Canas *et al.*, 1999; Canas *et al.*, 2000b; Snakkers *et al.*, 2000; Belchior *et al.*, 2001; Prida *et al.*, 2006), the toasting level (Artajona *et al.*, 1991; Rabier and Moutounet, 1991; Cantagrel *et al.*, 1992a; Viriot *et al.*, 1993; Canas *et al.*, 1999; Belchior *et al.*, 2001) and the barrel size (Belchior *et al.*, 2005; Canas *et al.*, 2008), as well as the cellar environment (Philp, 1989; Cantagrel *et al.*, 1992b; Singleton, 1995).

Concerning the wood botanical species, French oak is the most used in the ageing of brandies all over the world. However, in the Mediterranean countries other species are traditionally used for this purpose, namely *Quercus pyrenaica* Willd., *Quercus faginea* Lam. and *Castanea sativa* Mill.. Few studies have been made to compare the chemical composition of these species, but their results indicated that a number of phenolic compounds allow differentiating the

wood used in the barrel making, such as gallic acid, ellagic acid, vanillic acid, syringic acid, ferulic acid, vanillin, syringaldehyde, coniferaldehyde, sinapaldehyde, umbelliferone and scopoletin (Tricard *et al.*, 1987; Mattivi *et al.*, 1989; Canas *et al.*, 2000b; Férnandez de Simón *et al.*, 2006).
Our previous work also demonstrated significant differences in the phenolic composition between brandies aged in chestnut and oak wooden barrels (Canas *et al.*, 1999; Belchior *et al.*, 2001). In two-year-aged brandies in chestnut wooden barrels it was confirmed the role of gallic as chemical marker of this botanical species independently of the toasting level (Canas *et al.*, 1999).

Besides, there are two other phenolic compounds - acetovanillone and ethyl vanillin – that seem to be determining for the quality of brandies owing to their impact on their sensory properties. Acetovanillone was found in brandies (Nishimura *et al.*, 1983), and the corresponding descriptors are mainly "vanilla", "woody", "spicy", "molasses" and "clove-like" (Etiévant, 1991; Miranda Lopez *et al.,* 1992; Chatonnet, 1995; Cabaroglu *et al.,* 2002). Ethyl vanillin was found in brandies (Vlassov and Maruzhenkov, 1999), and is often used as an intensifier of the vanilla aroma in flavorings due to its high aromatic power (Sostaric *et al.*, 2000; Gerasimov *et al.*, 2002; Egawa *et al.*, 2006). In spite of their importance, acetovanillone and ethyl vanillin were not already used in quantitative analysis and their role as chemical markers of aged brandies was not investigated, since they were only identified by GC or GC-MS, which involves a delayed process of sample preparation, and without method validation.

So, the main objective of the present work was to study several low molecular weight phenolic compounds - phenolic acids, phenolic aldehydes, coumarins, acetovanillone and ethyl vanillin - performing the global analysis of their contents in the brandies aged in Portuguese oak, Allier oak and chestnut wood, in order to identify those of chemical markers of the botanical species. This will permit the assessment of wine brandies authenticity related to the kind of wood used in the ageing process, and would be a very useful tool for scientific research and for routine analysis. One HPLC method (Canas *et al.*, 2003) for the quantification of acetovanillone and ethyl vanillin in brandies was also in house validated.

## MATERIALS AND METHODS

### Materials

Brandies were sampled from two barrels of Portuguese oak wood (*Quercus pyrenaica* Willd.) - CN, two barrels of Allier oak wood (*Quercus sessiliflora* Salisb.) – CFA, and two barrels of chestnut wood (*Castanea sativa* Mill.) - CT, all with heavy toasting. The barrels (250 L) were produced at a cooperage industry - JM Gonçalves in the Northern of Portugal. These barrels were placed at "Adega Cooperativa da Lourinhã" in 1996 in similar cellar conditions and filled with the same *Lourinhã* brandy. Brandy sampling was made in the fourth year of ageing, and the six samples were identified as CN1, CN2, CFA1, CFA2, CT1, CT2. Two samples of each brandy were analyzed.

### Chemicals

Ellagic acid dihydrate, gallic acid monohydrate, vanillic acid, syringic acid, ferulic acid, vanillin, scopoletin, umbelliferone were purchased from Fluka (Buchs, Switzerland); syringaldehyde, coniferaldehyde, sinapaldehyde, acetovanillone, ethyl vanillin, and 4-hydroxibenzaldehyde were purchased from Aldrich (Steinheim, Germany). All of them were used as standards (purity > 97%) without further purification. The solutions were prepared fresh prior to use with ethanol/water (55:45 v/v). All solvents used were HPLC gradient grade purchased from Merck (Darmstadt, Germany).

### Equipment and chromatographic conditions

Chromatography was performed as described by Canas *et al.* (2003), with a HPLC Lachrom Merck Hitachi system (Merck, Darmstadt, Germany) equipped with a quaternary pump L-7100, a column oven L-7350, a UV-Vis detector L-7400, a fluorescence detector L-7480, and an autosampler L-7250, coupled to a HSM D-7000 software (Merck, Darmstadt, Germany) for management, acquisition and treatment of data. A 250 mm × 4 mm ID Lichrospher RP 18 (5μm) column (Merck, Darmstadt, Germany) was used. UV detection was 280 nm for phenolic acids, acetovanillone and ethyl vanillin, and 320 nm for phenolic aldehydes; fluorescence detection was 325 nm (excitation) and 454 nm (emission) for coumarins. Samples of brandies were added with an internal standard (20 mg/L of 4-hydroxybenzaldehyde), filtered through 0.45 μm membrane (Titan, Scientific Resources Ltd., Gloucester, UK) and analyzed by direct injection of 20 μL. The identification of chromatographic peaks was made by comparison of their relative retention times with those of external standards, as well as by their UV-Vis spectra. The chromatographic purity of the peaks and the UV-Vis spectra (200-400 nm) were performed using a Waters system equipped with a photodiode-array detector (Waters 996), with the same chromatographic conditions, managed by "Millennium 2010" software (Waters, Milford, USA).

### Statistical analysis

The analysis of variance and the calculation of least significant difference (LSD) that was applied for

comparison of the different averages were performed using Statistica vs '98 edition (Statsoft Inc., Tulsa, USA). The regression analysis was performed using Statistica vs '98 edition (Statsoft Inc., Tulsa, USA). The clustering analysis was performed using NTSYSpc vs 2.10q (Exeter software, New York, USA).

## RESULTS AND DISCUSSION

### Method validation

In spite of the matrix complexity and structural similarity of phenolic compounds present in the brandy, the HPLC method used (Canas *et al.*, 2003) allows good separation between all the studied phenolic compounds, without sample preparation - Figure 1.

This method was already validated for the analysis of phenolic acids, phenolic aldehydes and coumarins in aged brandies. Regarding acetovanillone and ethyl vanillin, the results obtained in the present study also show the good performance of the method:

### Linearity

The study of linearity (ISO 8466/1) was performed by the analysis of seven and six standard solutions in duplicate for acetovanillone and ethyl vanillin, respectively. The results obtained for slope, intercept, standard deviations of the slope and intercept, and correlation coefficient are presented in Table I. As for the analyzed compounds, linear regression is the best model for establishing a relationship between the peak area and the concentration, based on the statistical criteria (ISO 8466/1; Monteiro and Bertrand, 1990).

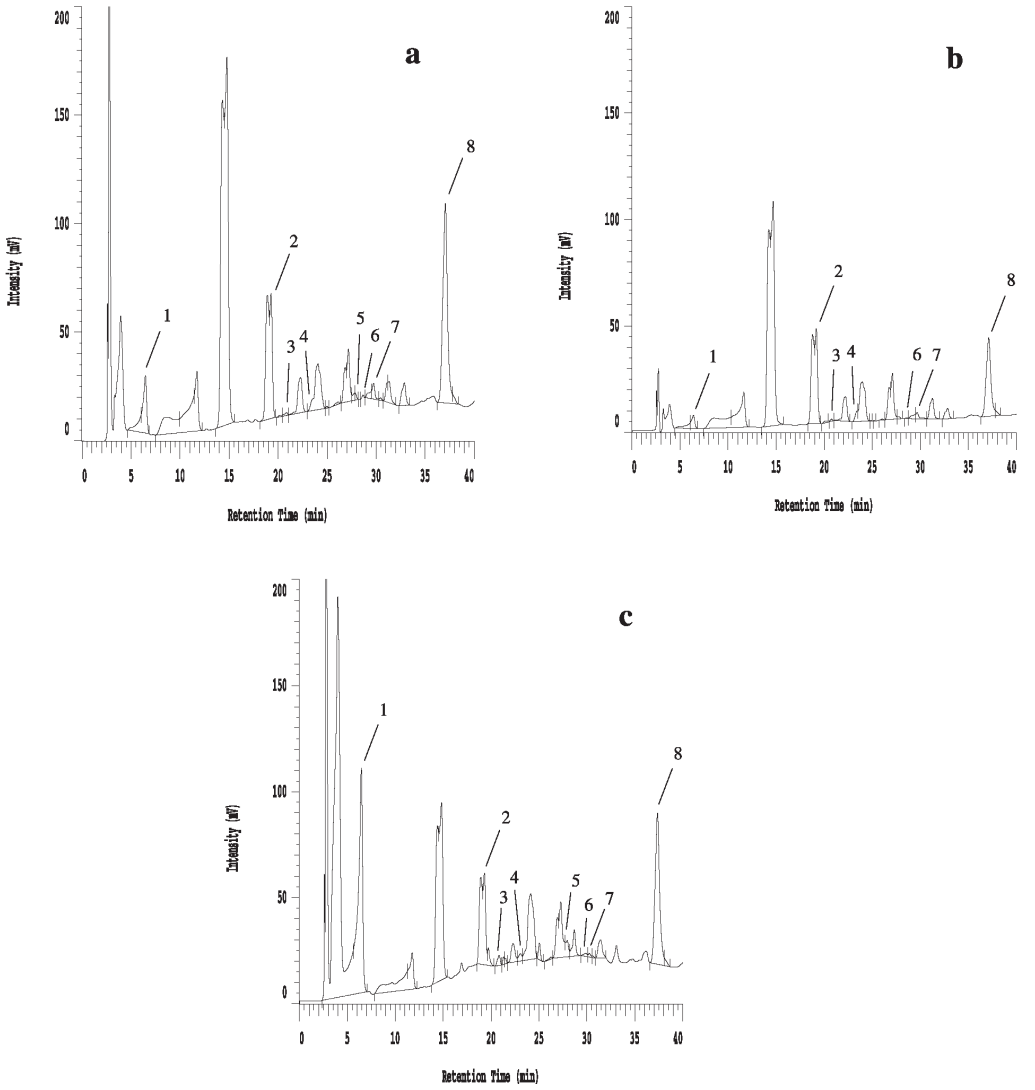


**Fig. 1 -** HPLC chromatograms of four-year-aged brandies in Portuguese oak wood (a), Allier oak wood (b) and chestnut wood (c), detected at 280 nm. 1 – gallic acid; 2 – 4-hydroxybenzaldehyde; 3 – vanillic acid; 4 – syringic acid; 5 – acetovanillone; 6 – ethyl vanillin; 7 – ferulic acid; 8 - ellagic acid.

*Cromatogramas HPLC de aguardentes com quarto anos de envelhecimento em carvalho português (a), carvalho Allier (b) e castanheiro (c), com detecção a 280 nm. 1 – ácido gálhico; 2 – 4-hidroxibenzaldeído; 3 – ácido vanílico; 4 – ácido siríngico; 5 – acetovanilona; 6 – etilvanilina; 7 – ácido ferúlico; 8 – ácido elágico.*

### Sensitivity

As regards to the linear model the sensitivity is expressed by the slope (Monteiro and Bertrand, 1990). This method presents high sensitivity for the two compounds, which indicates an excellent capacity to respond distinctly to small differences in compounds concentrations (Table I).

sample of brandy with each of six increasing concentrations of standard solutions within the concentration range, in duplicate (Monteiro and Bertrand, 1990), are presented in Table I. Considering the matrix complexity and the high number of compounds present in aged brandies, the recoveries achieved (between 90% and 101%) are highly satisfactory.

TABLE I

Validation data of the HPLC method for the quantification of acetovanillone and ethyl vanillin

*Dados de validação do método HPLC para quantificação da acetovanilona e etilvanilina*

| Validation criteria | | Parameters | Acetovanillone | Ethyl vanillin |
|---|---|---|---|---|
| Linearity | | Range (mg/L) | 0.73 - 200 | 0.84 – 20 |
| | | a [a] | 33558.62 | 2186.04 |
| | | SDa [b] | 13944.02 | 1996.69 |
| | | b [c] | 25911.78 | 22273.70 |
| | | SDb [d] | 329.58 | 218.68 |
| | | r [e] | 0.9990 | 0.9995 |
| Analytical limits | | LOD (mg/L) [f] | 0.73 | 0.84 |
| | | LOQ (mg/L) [g] | 2.42 | 2.79 |
| Sensitivity | | S (V s/mg $L^{-1}$) | 0.0259 | 0.0223 |
| Repeatability | Sample A | $\bar{x}$ (mg/L) [h] | 3.91 | 6.38 |
| | | SD (mg/L) [i] | 0.058 | 0.062 |
| | | r (mg/L) [j] | 0.18 | 0.20 |
| | | RSDr (%) [k] | 1.48 | 0.98 |
| | Sample B | $\bar{x}$ (mg/L) [h] | 13.60 | 3.86 |
| | | SD (mg/L) [i] | 0.056 | 0.042 |
| | | r (mg/L) [j] | 0.18 | 0.13 |
| | | RSDr (%) [k] | 0.41 | 1.08 |
| Selectivity and specificity | | Conc. added (mg/L) [l] | 30 – 180 | 2 - 12 |
| | | Recovery (%) | 100.8 | 91.9 |

[a] Intercept; [b] standard deviation of the intercept; [c] slope; [d] standard deviation of the slope; [e] correlation coefficient; [f] limit of detection; [g] limit of quantification; [h] mean concentration; [i] standard deviation ; [j] repeatability; [k] relative standard deviation of repeatability; [l] concentration range added

### Analytical limits

The determination of the analytical limits was carried out by the analysis of three blanks that consist in solutions of ethanol/water (75:25 v/v), in duplicate (Caporal_Gautier *et al.*, 1992). As shown in Table I, the values of the limit of detection (LOD) and the limit of quantification (LOQ) are quite low for the usual concentrations of acetovanillone and ethyl vanillin in aged brandies, so it has been proven reliable that the method could be used for their quantitative analysis.

### Repeatability

The repeatability was calculated from 10 replicate injections of two different four-year-old brandies (sample A and sample B) under constant operating conditions (laboratory, equipment, operator and method) over a short period of time (ISO 5725/2). The values of relative standard deviation, less than 1.5% throughout the concentration range, evidence the good precision of the method for the quantification of acetovanillone and ethyl vanillin in aged brandies (Table I).

### Specificity and selectivity

The recovery data, calculated by spiking the same

### Differentiation of aged brandies according to the botanical species of the wooden barrel

The results of variance analysis (Table II) show that the contents of ellagic acid, gallic acid, ferulic acid, scopoletin, acetovanillone and ethyl vanillin allowing to distinguishing very significantly the four-years-aged brandies according to the wood botanical species. This fact emphasizes the role of these phenolic compounds as chemical markers regarding the kind of wood used in the brandies ageing.

The brandies aged in chestnut wooden barrels present the highest content of gallic acid, and there is a homogeneous group formed by the brandies aged in Portuguese oak and Allier oak, as observed in the corresponding woods (Canas *et al.*, 1999; Canas *et al.*, 2000b).

Conversely, the brandies aged in Portuguese oak are the richest in ellagic acid, followed by the brandies aged in chestnut wood. The brandies aged in Allier oak presents the lowest content of ellagic acid. In the corresponding woods, our earlier study (Canas *et al.*, 2000b) revealed the existence of three clusters, by ordering them from the richest to the poorest: the Portuguese oak, the chestnut and the Allier oak. So, the proportion of ellagic acid in the brandies reflects the richness of the corresponding wood, i.e., the effect

TABLE II

Content of phenolic compounds in four-year-aged brandies in Portuguese oak, Allier oak and chestnut wooden barrels (mg/L of absolute ethanol)

*Teores de compostos fenólicos em aguardentes envelhecidas durante quarto anos em vasilhas de carvalho português, carvalho Allier e castanheiro (mg/L A.P.)*

| | *Effect* | CN | | CFA | | CT | |
|---|---|---|---|---|---|---|---|
| | | x | SD | x | SD | x | SD |
| **ellag** | ** | 158.39 ***c*** | 2.290 | 70.57 ***a*** | 2.486 | 119.95 ***b*** | 14.971 |
| **gall** | *** | 34.72 ***a*** | 1.628 | 17.86 ***a*** | 1.919 | 238.62 ***b*** | 18.539 |
| **van** | *ns* | 6.99 | 1.217 | 5.12 | 1.182 | 7.90 | 0.403 |
| **syrg** | *ns* | 13.28 | 2.295 | 8.80 | 3.226 | 22.46 | 11.169 |
| **ferul** | ** | 13.70 ***b*** | 1.295 | 4.23 ***a*** | 1.060 | 3.50 ***a*** | 1.779 |
| **vanil** | *ns* | 14.55 | 1.271 | 12.98 | 2.148 | 15.21 | 4.093 |
| **syrde** | *ns* | 37.95 | 4.359 | 30.06 | 7.309 | 28.16 | 8.920 |
| **cofde** | *ns* | 15.66 | 3.694 | 15.10 | 3.383 | 10.43 | 3.524 |
| **sipde** | *ns* | 39.66 | 14.071 | 28.40 | 6.683 | 12.18 | 4.687 |
| **umb** | *ns* | 0.025 | 0.0010 | 0.019 | 0.0044 | 0.017 | 0.0031 |
| **scop** | ** | 0.15 ***b*** | 0.020 | 0.27 ***c*** | 0.005 | 0.09 ***a*** | 0.011 |
| **acetov** | ** | (0.74) ***a*** | 0.538 | 0.00 ***a*** | 0.000 | 9.18 ***b*** | 0.110 |
| **ethylv** | ** | 9.41 ***c*** | 0.503 | 3.87 ***b*** | 0.163 | (2.49) ***a*** | 0.324 |

x = mean; SD = standard deviation; means followed by the same letter in a row are not significantly different at the 0.001*** or 0.01** level of significance; ns = without significant difference; 0.00 indicates value < limit of detection; parentheses indicate value < limit of quantification. ellag - ellagic acid; gall – gallic acid; van – vanillic acid; syrg – syringic acid; ferul – ferulic acid; vanil – vanillin; syrde – syringaldehyde; cofde – coniferaldehyde; sipde – sinapaldehyde; umb – umbelliferone; scop – scopoletin; ;acetov – acetovanillone; ethylv – ethyl vanillin;

of the botanical species prevailing over other factors that conditioning the content of ellagic acid in the aged brandies, namely the toasting of the barrels in cooperage (Rabier and Moutounet, 1991; Chatonnet, 1995; Canas *et al.*, 2007) and the hydrolysis of ellagitannins during the ageing process (Rabier and Moutounet, 1991; Viriot *et al.*, 1993; Viriot *et al.*, 1994).

Ferulic acid is also a discriminant compound for the wood botanical species used in the ageing of brandies, despite its low concentration. The brandies aged in Portuguese oak present higher content of this acid than the brandies aged in Allier oak and chestnut, and no significant differences between Allier oak and chestnut brandies were found. Comparing the ferulic acid contents of brandies with those of the corresponding woods - CN<CFA<CT (Canas *et al.*, 2000b) it is detected an inversion, likely by the oxidation of coniferaldehyde and the decarboxylation and degradation of ferulic acid under toasting effect (Chatonnet, 1995) and/or during the ageing process (Puech *et al.*, 1985), and also by the different rate of extraction in each kind of wood (Canas *et al.,* 2002).

In addition, scopoletin content permits the differentiation of the aged brandies in three groups, by ordering them from the richest to the poorest: the Allier oak, the Portuguese oak and the chestnut. It is interesting to point out that the ordination of the brandies is similar to the corresponding woods (Canas *et al.*, 2000b), but the differences between brandies are slighter than the differences between woods. To the best of our knowledge, the effects of toasting on coumarins and the phenomena involving coumarins during the ageing process have never been studied. Thus, further experiments on the mechanisms implicated in their formation and degradation should help explain this pattern.

Brandies aged in chestnut wood could be distinguished from those aged in oak wood by the highest concentration of acetovanillone and the lowest concentration of ethyl vanillin. The high concentration of ethyl vanillin in brandies aged in Portuguese oak wood allowed a significant discrimination between the former and those aged in Allier oak wood. It is interesting to note that these results are coherent with those obtained by Caldeira *et al.* (2006) with the corresponding woods. The results also indicate high variability in the content of both compounds in the four-year-aged brandies for each of the botanical species studied. As regards the ageing process, the existence of several mechanisms that could justify the diverse behavior of phenolic compounds in brandy can be considered. This suggests that besides acetovanillone formation by thermal degradation of lignin (Nishimura *et al*., 1983; Chatonnet *et al.*, 1989; Sarni *et al.*, 1990; Sarni *et al.*, 1991; Puech *et al.*, 1992) other pathways, like hydroalcoolisis of lignin (Puech and Sarni, 1990; Viriot *et al.,* 1993), condensation and degradation of polyphenols (Viriot *et al.,* 1993), and many other reactions such as oxidation or esterification (Nishimura *et al.*, 1983), could be determinant to the concentration of acetovanillone and ethyl vanillin during the ageing process. In fact, the mechanisms of formation and degradation of these compounds in aged brandies are

not clearly understood and require further investigation.

Furthermore, the higher odorant impact of ethyl vanillin than vanillin, and the concentration of ethyl vanillin found in the brandies aged in oak wood lead us to suggest that this phenolic ester could be the main responsible compound for vanilla aroma of these brandies.

In our previous studies phenolic aldehydes were reported as very discriminant compounds for the differentiation of the corresponding kinds of wood - chestnut and oak (Canas *et al.*, 1999; Canas *et al.*, 2000b). Nevertheless, the results obtained in the present work with aged brandies show that these compounds have not that discriminatory power. The influence of the toasting on lignin and its derivatives (Sarni *et al.*, 1990; Sarni *et al.*, 1991), the oxidative phenomenon occurred during ageing (Nishimura *et al.*, 1983; Puech *et al.*, 1985; Viriot *et al.,* 1993), and the different extraction kinetic in each kind of wood (Canas *et al.*, 2002) possibly account for the differing role of phenolic aldehydes between the wood and the corresponding brandy.

The multidimensional analysis permits a global evaluation of the data. Based on the results of the previous variance analysis, we had only considered the compounds with a very significant effect to the discrimination of the brandies according to the wood botanical species studied. The phenogram of UPGMA (Unweighted Pair-Group Method using Arithmetic) clustering (Figure 2) illustrates the existence of three clusters perfectly separated, although the variability between barrels (Towey and Waterhouse, 1996): the first one contains the brandies aged in chestnut barrels (CT), the second one includes the brandies aged in Portuguese oak barrels (CN), and the third one is constituted by the brandies aged in Allier oak barrels (CFA).

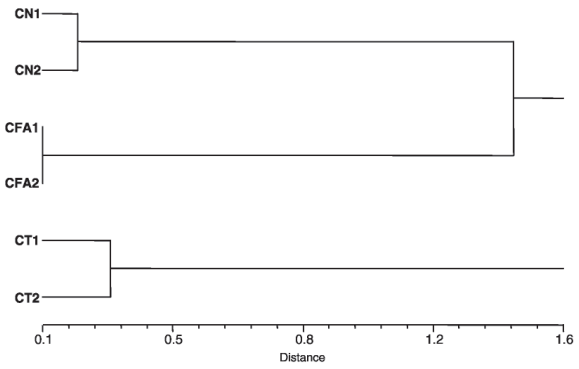


**Fig. 2 -** Phenogram of UPGMA clustering of four-year-aged brandies in Portuguese oak, Allier oak and chestnut barrels according to low molecular weight phenolic compounds contents.

Fenograma UPGMA *de aguardentes com quarto anos de envelhecimento em carvalho português, carvalho Allier e castanheiro em função dos teores de compostos fenólicos de massa molecular baixa.*

## CONCLUSIONS

In the experimental conditions, it is reliable that gallic acid, ellagic acid, ferulic acid, scopoletin, acetovanillone and ethyl vanillin are chemical markers of the botanical species used in the ageing of wine brandies. These compounds are of remarkable importance in the assessment of brandies authenticity related to the kind of wood used in the ageing process, and can be a very useful tool for scientific research and for routine analysis.

As far as it is known, this is the first time a HPLC method is validated and applied for the quantification of acetovanillone and ethyl vanillin in brandies. The method showed good performance, with very satisfactory analytical parameters. It allows the separation and quantification of the compounds and revealed to be practical and simple without sample preparation.

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