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## Analysis of old brandy and oak extracts by capillary electrophoresis

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### Abstract

Polyphenolic compounds in spirits and ethanolic oak extracts were studied by capillary electrophoresis and results compared with reversed-phase HPLC analysis. Different conditions of analysis were tried in order to set up the best separation conditions, and reproducibility of the migration times was studied with standard mixtures and tested for samples. Compounds usually studied in these samples were well separated with 0.1 M borate buffer with 5% ethanol, pH 9.2. Calibration curves (2.5–50 ppm) were established for some standard compounds. Capillary electrophoresis is a simple and useful method for the analysis of oak extracts and old brandies.

**Keywords:** Wood; Brandy; Food analysis; Polyphenols; Tannins; Organic acids

### 1. Introduction

Polyphenolic compounds are widely distributed in nature as they are important components of plants. These compounds have a large variety of structures and may be involved in various reactions: acid–base, oxidation–reduction, complexation equilibria, oligomerization and precipitation [1]. The formation of precipitates with proteins may explain the astringent taste of compounds from this group [2].

In wines, polyphenolic compounds are important components as they exist in grapes, accounting for the red colour of grapes and red wines [1]. Some wines and distilled drinks must be aged for a certain period in wood barrels (usually oak) and the aging of spirits in wood barrels involves several processes [3]: complex phenolic substances as tannins are extracted from wood; structural molecules (lignin and hemicellulose) are depolymerized and extracted to the dis-

tillate; and reactions may occur between components of wood and distillate. These chemical reactions occurring during the aging process of brandies and whiskies are very important for the quality of the final products whose characteristics of taste, flavour and colour appreciated by consumers depend on the composition of wood (origin of tree) [4], different treatments used in the manufacture of oak barrels (charring level of wood) [5], and history of the oak barrel (how often was it used and for how long) [6,7].

Tannins are classified in two groups: proanthocyanidins (condensed tannins) and hydrolysable tannins like gallotannins and ellagitannins (also known as hexahydroxydiphenyl esters) which by acid hydrolysis produce gallic acid and ellagic acid. Hydrolysable tannins are the most important in oak wood. Heartwood of *Quercus robur* L. may contain up to 10% (w/w) of ellagitannins [8]. The main compounds are castalagin, and vescalagin which were first isolated and identified by Mayer [9]. Other

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compounds (aldehydes, cinnamic and benzoic acids) are degradation products of lignin. Coumarins, especially the presence of scopoletin in a brandy is considered [10] as a proof of maturation in oak barrels.

Separation methods, with emphasis on chromatographic techniques [11,12], have been widely used for the chemical analysis of phenolic compounds in brandies, wines and wood extracts and separation and purification of eight ellagitannins (castalagin, vescalagin, roburin A, B, C, D, E and grandinin) has been reported [8]. There have been claims that the presence of these compounds is important for the taste, colour and oxidability of wines and brandies [13].

Capillary zone electrophoresis, a high-resolution technique, has been applied successfully in the analysis of phenolic compounds [14] of a large variety of samples (honey [15], plant extracts [16], liquid biomass hydrolysis eluates [17], sugarcane [18], wines [19]), requiring only small amounts of sample and buffer, and short times of analysis.

Some of the disadvantages of this technique are the low reproducibility of migration times, and less sensitive detection than in HPLC. In attempts to overcome the low reproducibility, different capillary silica surface conditionings have been developed depending on characteristics of samples.

This work is part of a study of brandies produced in Lourinha, an Appellation Control Region recognized in Portugal for production of brandy. Capillary electrophoresis is shown to be an adequate analytical technique for the characterization of phenolic compounds in oak wood extracts and brandies.

## 2. Experimental

### 2.1. Chemicals

All reagents were reagent grade. Derivatives from cinnamic acid, benzoic acid and aldehydes (listed in Table 1, see also Fig. 1, structure I) were obtained from Sigma (St. Louis, MO, USA) or from Aldrich (Steinheim, Germany). Other standards such as acetone (analytical-reagent grade) and rutin from Merck (Darmstadt, Germany), naphthol (Analar,

BDH, UK), furfural, 5-hydroxymethylfurfural, and ellagic acid from Aldrich, were used.

Standard solutions were prepared by dissolving compounds in analytical-reagent grade methanol from Riedel-de Haen (Seelze, Germany).

Buffer electrolyte solutions were prepared from analytical-reagent grade sodium tetraborate decahydrate (Aldrich) at a concentration of 10 mM pH 9.2, analytical-reagent grade sodium dihydrogenphosphate-1-hydrate (Merck) at a concentration of 10 mM pH 9.2 (adjusted by the addition of 1 M NaOH), sodium lauryl sulphate (Sigma) to a concentration of 100 mM, 5% ethanol (Riedel-de Haen).

Water used in all experiments was purified with a Milli-Q apparatus (Millipore, Bedford, MA, USA) with a conductivity 18 M $\Omega$ .

Acetonitrile and methanol used in HPLC analysis were HPLC grade from Lab-Scan (Dublin, Ireland). Orthophosphoric acid 85% was from Riedel-de Haen. Solvents used in HPLC were degassed with helium.

### 2.2. Equipment

#### Capillary electrophoresis analysis

Prince capillary electrophoresis equipment from Lauerlabs (Emmen, Netherlands) with a UV detector operating at fixed wavelength and an uncoated fused-silica capillary [100 cm (effective length 85 cm $\times$ 75  $\mu$ m I.D. $\times$ 340  $\mu$ m O.D.)] were used. Electropherograms were recorded with an integrator CR4A-Chromatopac from Shimadzu (Kyoto, Japan).

#### HPLC analysis

HPLC equipment from Unicam (Cambridge, UK) consisted of an automatic sampler injector, a Crystal 200 pump, a Crystal 240 diode array detector. Data were analysed with equipment software from Unicam. The column used was a Spherisorb S50DS2 (Hichrom, UK) 20 cm $\times$ 4.6 mm I.D. with a guard column.

### 2.3. Conditions

#### Capillary electrophoresis analysis

Standard solutions and samples were introduced into the capillary applying a 10 mbar differential pressure for 12 s. Separations were carried out at

Table 1  
List of compounds used as standards

|    | R1       | R2 | R3               | R4 | R5               | R6 | Compound name                 |
|----|----------|----|------------------|----|------------------|----|-------------------------------|
| 1  | CHO      | OH |                  |    |                  |    | Salicylaldehyde               |
| 2  | CHO      | OH |                  |    | OH               |    | Genitaldehyde                 |
| 3  | CHO      |    | OH               |    |                  |    | 3-Hydroxybenzaldehyde         |
| 4  | CHO      |    | OCH <sub>3</sub> | OH |                  |    | Vanillin                      |
| 5  | CHO      |    | OCH <sub>3</sub> | OH | OCH <sub>3</sub> |    | Syringaldehyde                |
| 6  | CHCHCHO  |    | OCH <sub>3</sub> | OH |                  |    | Coniferaldehyde               |
| 7  | CHCHCHO  |    | OCH <sub>3</sub> | OH | OCH <sub>3</sub> |    | Sinapinaldehyde               |
| 8  | COOH     |    |                  |    |                  |    | Benzoic acid                  |
| 9  | COOH     | OH |                  |    |                  |    | Salicylic acid                |
| 10 | COOH     |    | OH               |    |                  |    | <i>m</i> -Hydroxybenzoic acid |
| 11 | COOH     |    |                  | OH |                  |    | <i>p</i> -Hydroxybenzoic acid |
| 12 | COOH     | OH | OH               |    |                  |    | 2,3-Dihydroxybenzoic acid     |
| 13 | COOH     | OH |                  | OH |                  |    | $\beta$ -Resorcylic acid      |
| 14 | COOH     | OH |                  |    | OH               |    | Gentisic acid                 |
| 15 | COOH     | OH |                  |    |                  | OH | $\gamma$ -Resorcylic acid     |
| 16 | COOH     |    | OH               | OH |                  |    | Protocatechuic acid           |
| 17 | COOH     |    | OH               |    | OH               |    | $\alpha$ -Resorcylic acid     |
| 18 | COOH     |    | OH               | OH | OH               |    | Gallic acid                   |
| 19 | COOH     |    | OCH <sub>3</sub> | OH |                  |    | Vanillic acid                 |
| 20 | COOH     |    | OCH <sub>3</sub> | OH | OCH <sub>3</sub> |    | Syringic acid                 |
| 21 | CHCHCOOH |    |                  |    |                  |    | Cinnamic acid                 |
| 22 | CHCHCOOH |    |                  |    | OH               |    | Coumaric acid                 |
| 23 | CHCHCOOH |    | OH               | OH |                  |    | Cafeic acid                   |
| 24 | CHCHCOOH |    | OCH <sub>3</sub> | OH |                  |    | Ferulic acid                  |
| 25 | CHCHCOOH |    | OCH <sub>3</sub> | OH | OCH <sub>3</sub> |    | Sinapic acid                  |

The basic structural unit is shown in Fig. 1(I).

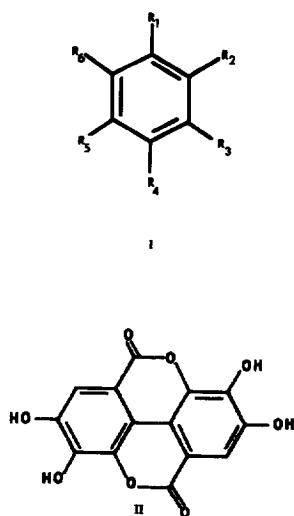


Fig. 1. (I) Basic structural unit of some of the phenolic compounds analysed and listed in Table 1. (II) Structure of ellagic acid (31).

35°C, applying a voltage of 25 kV. Detection was at 280 nm. Buffer solutions consisted of 10 mM sodium tetraborate decahydrate, pH 9.2, 10 mM sodium tetraborate decahydrate with 5% ethanol added, pH 9.2, 10 mM sodium tetraborate decahydrate with 100 mM SDS, pH 9.2, 10 mM sodium dihydrogenphosphate-1-hydrate, pH 9.2. Data sampling rate was 50 ms.

#### Capillary conditioning

In the beginning of a work day, the capillary was washed for 30 min with 1 M NaOH, followed by 0.2 M for 10 min, water and buffer solution for 5 min. The first analysis run after capillary conditioning was always neglected. Between samples, capillary was washed with 0.2 M NaOH (1 min), followed by standard buffer, 1 min at 1000 mbar and buffer, 12 s at 20 kV. The capillary was kept in 0.2 M NaOH solution.

### HPLC analysis

Standard solutions and samples were injected using a 20  $\mu$ l loop. The mobile phase consisted of eluent A: orthophosphoric acid 0.15%, eluent B: orthophosphoric acid–acetonitrile–water (1:400:599, v/v/v). Separations were carried out with a flow-rate of 700  $\mu$ l/min at a column temperature of 35°C. The analyses were performed by the following gradient of eluents: 0–15 min, 0 to 20% eluent B; 10 min with 20% eluent B; 25–70 min, 20 to 70% eluent B; 70–75 min, with 70% eluent B; 75–85 min, from 70 to 100% eluent B; 85–90 min, with 100% eluent B.

### 2.4. Samples and sample preparation

Oak wood (*Q. robur*) samples were taken from staves dried at room temperature.

Old brandies were obtained from a cellar situated in an Appellation Control Region in Portugal (Lourinha).

Samples of brandies and extracts were filtered through 0.45  $\mu$ m membrane from Millipore (Ireland) before analysis.

#### 2.4.1. Extraction of phenolic compounds from wood

In order to simulate the extraction of phenolic compounds from wood samples, three model solutions of brandies were tested: 100 ml of ethanolic solutions (45%, 55%, 75% and pH 4.2, adjusted with tartaric acid) were used to extract 5 g of oak wood shavings. The extract obtained with the 55% ethanolic solution had a higher phenolic content and therefore it was used to test three different extraction procedures:

(1) stirring at room temperature for 11 h; samples were collected every 1 h;

(2) stirring at room temperature for 2 h followed by ultrasonication for 20 min; samples were collected every 5 min;

(3) ultrasonication for 20 min; samples were collected every 5 min.

Total phenolic compound content in the extracts obtained was evaluated with a spectrophotometer measuring absorbance at 280 nm, using the extraction solution as reference.

Stirring at room temperature seemed to be the most effective process of extraction, and after 7 h,

extraction seemed completed. A time of 3 h stirring, and ultrasonication for 20 min are equivalent to 2 h of stirring followed by ultrasonication during 5 min. Ultrasonication was not so effective as stirring. Longer periods of ultrasonication were not used as temperature tends to rise and may be responsible for changes in sample composition.

Powdered wood was used instead of shavings and the extraction was almost complete with 60 min stirring.

## 3. Results and discussion

### 3.1. Choice of conditions for analysis by capillary electrophoresis

The optimisation of the analytical method by capillary electrophoresis was carried out using solutions containing compounds listed in Table 1. Some of these compounds are natural components of brandies, acetone was used as an electroosmotic flow marker, and naphthol seemed to have an adequate structure to interact with micelles in analysis with buffer containing SDS. A typical electropherogram is presented in Fig. 2.

High pH values are the most adequate for the separation of phenolic compounds as at pH 9.2 most phenolic compounds are negatively charged and present migration times longer than neutral compounds.

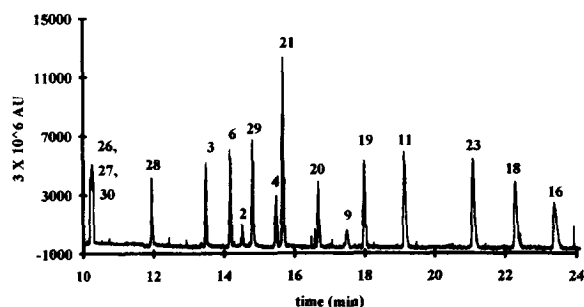


Fig. 2. Electropherogram obtained in the analysis of a standard mixture with a borate buffer (10 mM sodium tetraborate solution with 5% ethanol pH 9.2). Other conditions of analysis as in Section 2. Peak identification: 26, acetone; 27, furfural; 28, naphthol; 29, rutin; 30, 5-HMF. Other compound identification as in Table 1.

Both phosphate, borate and mixtures of phosphate and borate buffers have been used in the separation of phenolic compounds. When complex samples such as brandies are analysed it may be helpful to analyse them with buffers of different composition and compare the results. When borate buffers are used, hydroxylic compounds may form borate complexes which have higher negative charges than the original compounds: therefore they have higher migration times and the resolution of separation may be improved. In order to establish the best conditions of analysis, two different buffers (borate and phosphate at the same pH 9.2) were used.

The behaviours of standard compounds in these two buffers were compared: the values of electrophoretic velocities were represented in the form  $(1/t_{m_{ac}} - 1/t_m)$ , where  $t_{m_{ac}}$  is the migration time of acetone (used as electroosmotic flow marker), and  $t_m$  is the migration time of the compound. The figure illustrates how formation of borate complexes may affect the migration times of compounds with similar structures. Apparently, compounds are grouped along three straight lines (see Fig. 3):

(1) The migration times of compounds that do not form complexes with borate (benzoic acid (8) and cinnamic acid (21) for instance) will be the same in both buffers (line A corresponding to slope equal to 1).

(2) Benzoic and cinnamic acids with a hydroxyl

group in position 4 ( $R_4 = OH$  in Fig. 1, structure I) have migration times in borate longer than in phosphate and therefore are forming complexes with borate (there is a group along line B with slope 1.33).

(3) Another group of compounds on line C (with slope 1.67) correspond to compounds having two hydroxyl groups in positions 4 and 3. The slope is lower than 2 because although there is a charge increase due to complex formation, the size of complex is also larger than the single ion.

Comparing the monosubstituted hydroxybenzoic acids (9,10,11), the behaviour of salicylic acid (9) is different from the others. This effect may be explained as due to hydrogen bonding between the adjacent carboxylic and hydroxy groups: a similar behaviour is observed for 2,6-dihydroxybenzoic acid (15).

Ellagic acid (31), (see Fig. 1, structure II) which is an important component of samples studied, also deviates from line A. The molecule has four OH groups in its structure and in a borate buffer solution it must react and give borate complexes. However, in these conditions there will be no gain in negative charge and therefore it does not migrate faster: it has a lower mobility in borate due to the larger size of the complex ion.

Cinnamic acids have smaller velocities than corresponding benzoic acids and the introduction of

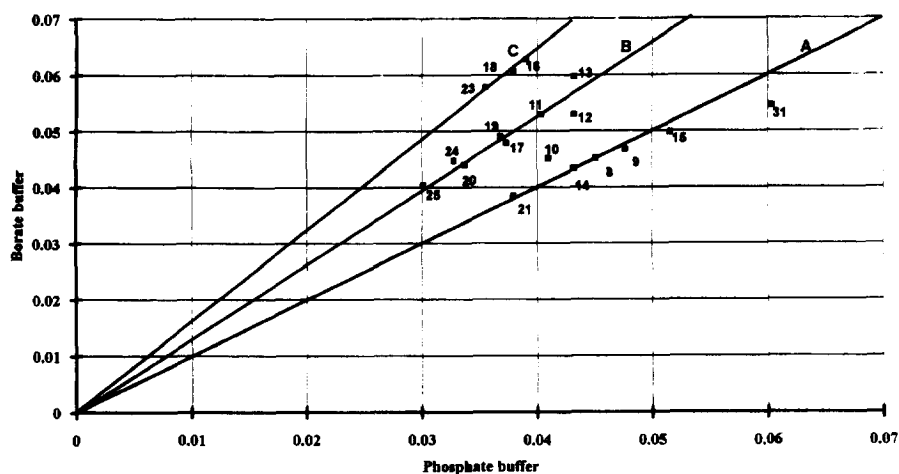


Fig. 3. Comparison between migration times of standards in a 10 mM sodium dihydrogenphosphate-1-hydrate pH=9.2 buffer and a 10 mM sodium tetraborate buffer pH 9.2. Other conditions of analysis as in Section 2. Compound identification as in Table 1 and Fig. 2.

methoxy groups in the structure of these acids will increase the size of ions and cause lower mobility for 4-hydroxybenzoic and 4-hydroxycinnamic acids.

Borate buffer gave a better separation of standard compounds and it was usually preferred in analysis of samples.

In order to improve separation, additives (5% ethanol and 0.1 M SDS) were added to the borate buffer. A buffer containing 5% ethanol presented a better separation of standards. The use of surfactant made possible the separation of furfural which is an important component of brandies and is a neutral compound.

### 3.2. Reproducibility

To test the repeatability of migration times, a sequence of 20 analyses of the same standard mixture was run. At the end of these 20 analyses, a change of 0.2 units was observed in the pH value of the running buffer. The relative standard deviations (R.S.D.s) for migration times were calculated and ranged between 0.075–0.896% for borate buffer, 0.0572–0.667% for borate buffer with 5% ethanol, 0.18–3.8% for borate buffer with 0.1 M SDS. If a correction of migration times was considered in order to compensate this regular variation due to change in pH, R.S.D. values were reduced for naphthol, 3-hydroxybenzaldehyde, gentisaldehyde,

syringic acid, vanillic acid, *p*-hydroxybenzoic acid, and gallic acid, about ten times.

Reproducibility with micelles seemed very poor compared with the other buffers but correcting the results for variation of pH, the R.S.D. values were acceptable (0.029–0.246%).

Reproducibility of analysis of an oak extract was evaluated in a similar way (20 injections of the same sample): R.S.D. values calculated for 15 peaks ranged between 0.141–0.385% without correction.

Calibration curves were determined for standards listed in Table 2 with concentrations in the range 2.5–50 ppm. Peak areas and peak heights were measured and results for peak areas are presented. Compounds like gallic acid (18) and protocatechuic acid (16) have better correlation measuring peak areas than peak heights. This is explained by the shape of peaks that get wider when migration times are higher.

### 3.3. Separation of a standard mixture by CE and HPLC

As HPLC has been a well established technique for the analysis of plant extracts (including wines and brandies), it may be useful to compare the results obtained by CE analysis with the chromatograms of the same samples.

Table 2

Calibration of a standard mixture analysed by CE with borate buffer with 5% ethanol, at four different concentrations ranging from 2.5–50 ppm, each run was repeated three times

| Compound name                      | Slope |       | Intercept |       | <i>r</i> | Standard error |
|------------------------------------|-------|-------|-----------|-------|----------|----------------|
|                                    | Value | S.D.  | Value     | S.D.  |          |                |
| Naphthol (28)                      | 157.8 | 5.171 | –31.14    | 160.8 | 0.9957   | 291.2          |
| 3-Hydroxybenzaldehyde (3)          | 229.0 | 7.208 | –407.9    | 205.2 | 0.9951   | 448.6          |
| Coniferaldehyde (6)                | 256.8 | 3.635 | –85.51    | 99.44 | 0.9989   | 236.3          |
| Gentisaldehyde (2)                 | 112.5 | 2.945 | 124.1     | 83.84 | 0.9966   | 183.3          |
| Rutin (29)                         | 540.8 | 13.22 | –370.5    | 348.7 | 0.9964   | 889.9          |
| Vanillin (4)                       | 151.1 | 1.864 | –54.69    | 51.02 | 0.9992   | 121.2          |
| Syringic acid (20)                 | 356.0 | 8.181 | –207.0    | 215.8 | 0.9968   | 550.6          |
| Ferulic acid (24)                  | 510.4 | 5.715 | –136.0    | 150.7 | 0.9992   | 384.6          |
| Vanillic acid (19)                 | 553.5 | 2.961 | 195.9     | 78.08 | 0.9998   | 199.3          |
| <i>p</i> -Hydroxybenzoic acid (11) | 585.9 | 4.407 | –213.8    | 116.2 | 0.9997   | 296.6          |
| Gallic acid (18)                   | 657.9 | 9.661 | –661.6    | 254.8 | 0.9987   | 651.0          |
| Protocatechuic acid (16)           | 450.3 | 3.827 | –174.2    | 100.9 | 0.9996   | 257.6          |

Peak area were used for evaluation; *r* = correlation coefficient.

In order to compare the two separation techniques, the mixture containing standard components was analysed and the retention times (HPLC) and migration times (CE) are presented in Fig. 4.

As expected, points are well scattered because the mechanisms and conditions of separation are different in HPLC and CE.

However, some general trends can be pointed out:

(1) At low pH, used in HPLC, most of these compounds are in neutral form and reversed-phase HPLC separates compounds according to differences in polarity. Some highly polar compounds (with small retention times in HPLC), in the alkaline buffer of electrophoresis will ionise and have longer migration times as, for instance, gallic (18) and protocatechuic (16) acids.

(2) Cinnamic acids have longer retention times than benzoic acids, while in CE they present shorter migration times than the corresponding benzoic acid (compare in Fig. 4, *p*-hydroxybenzoic acid (11), vanillic acid (19), syringic acid (20) with corresponding coumaric acid (22), ferulic acid (24) and sinapic acid (25)).

(3) Substitution of acids with methoxy groups will cause an increase in retention time, and substitution with hydroxy groups will decrease retention time (compounds are more polar). The opposite occurs for the corresponding migration times observed in CE.

(4) Aldehydes in general have longer retention

times than the corresponding acids in HPLC while in CE they present shorter migration times.

In Fig. 4, some groups of compounds relevant for the analysis of brandies are shown: neutral compounds, aldehydes with free OH groups and acids. In general, compounds that have longer migration times in CE have low retention times in HPLC.

### 3.4. Analysis of samples by CE and HPLC

The phenolic compounds must be extracted from oak wood samples before analysis. The extraction solution used to study oak wood should be similar to a brandy. When brandy is stored in the oak barrel its ethanol content is about 75%, this value decreases during maturation and when the brandy is commercialised its ethanol content is about 55%.

Fig. 5 illustrates the study of an oak extract: the chromatogram and electropherogram are shown and peaks were identified by spiking samples with standard solutions.

When comparing a chromatogram and an electropherogram it is important to bear in mind that separations are carried out at different pH. As separation conditions are not the same in both techniques absorption spectra of some compounds are not the same. Spectra from an extract diluted with eluent from HPLC analysis (orthophosphoric acid 0.15%) and buffer from CE (10 mM sodium

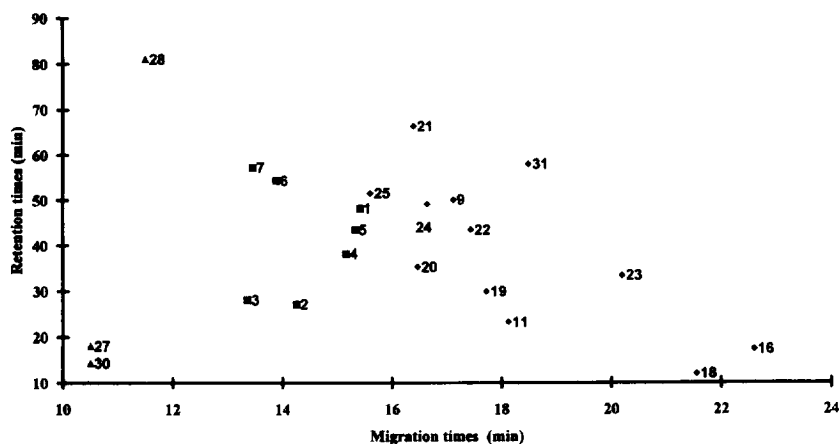


Fig. 4. Comparison between analysis of standards by CE and reversed-phase HPLC. Conditions of analysis as in Section 2 and compound identification as in Table 1 and Fig. 2. ■ phenolic aldehydes; ♦ phenolic acids; ▲ other compounds.

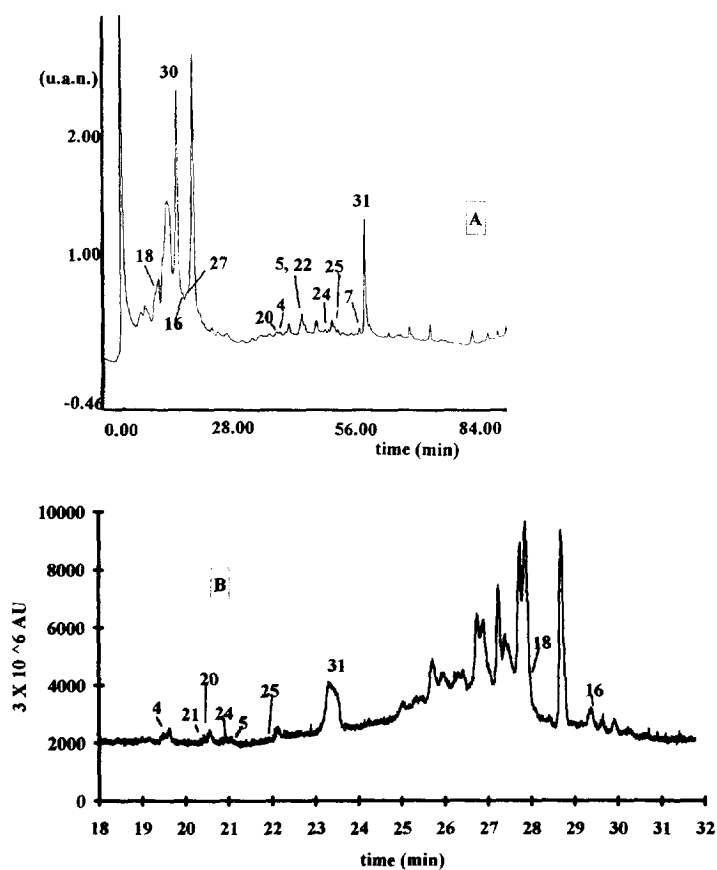


Fig. 5. Analysis of oak wood extracted with a solution 55% ethanol, pH 4.2 (adjusted with tartaric acid). (A) Analysis by reversed-phase HPLC; (B) analysis by capillary electrophoresis. Conditions of analysis as in Section 2 and compound identification as in Table 1 and Fig. 2.

tetraborate decahydrate pH 9.2) were compared. As buffer pH value in CE is higher, spectrum seems to be displaced to higher wavelengths with increase in absorption between 250–450 nm.

Electropherograms show ellagitannins having longer migration times and a good separation of these compounds is obtained in CE. The complex structure and large number of hydroxyl groups may explain the migration times obtained similar to gallic acid (18) and protocatechuic acid (16).

Comparing the electropherograms of an oak extract and an old brandy, ellagitannins zone was less important in brandies and ellagic acid content was more important (Fig. 6A and B). Although an electropherogram does not present a stable baseline, the same occurs in HPLC analysis, and compounds

like tannins with a very complex structure must be responsible for this apparent baseline variation.

CE is a high-resolution technique but compounds do not look well separated. However, as shown by enlarging a segment of the electropherogram it was possible to identify peaks by spiking with standards (Fig. 6C).

There are industrial processes for the artificial accelerated ageing of spirits by adding caramel, vanillin and/or plant extracts in order to get colour and taste similar to genuine old brandies. Therefore, detailed information on the chemical composition of products is useful for detection of non-genuine old brandies and may be helpful for relating the quality of aged brandy with the origin of oak wood and charring level of wood used in the making of barrels.



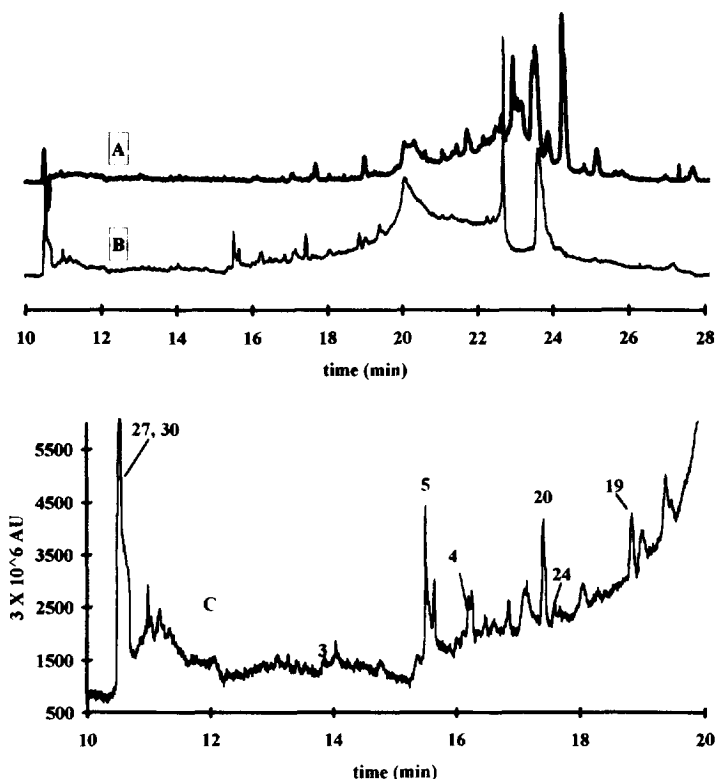


Fig. 6. Analysis by CE of (A) an oak extract; (B) an old brandy; (C) segment of the old brandy electropherogram. Conditions of analysis as in Section 2 and compound identification as in Table 1 and Fig. 2.

#### 4. Conclusions

Although a full characterisation of different wood extracts and brandies will require more experimental work, some conclusions can be pointed out: (1) Capillary electrophoresis is a simple and useful method for the analysis of wood extracts and brandies. (2) The results obtained with this method may be used for comparison with complementary HPLC in order to get a full characterisation of these samples. (3) Characterisation by this method may contribute to the study of the quality and authenticity of brandies.

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