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Analysis of phenolic compounds in Muscatel wines produced in Portugal

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Abstract

A liquid chromatography method associated with mass spectrometry and diode array, fluorescence and electrochemical detectors was used in order to study phenolic composition of Muscatel sweet wines from Setúbal region in Portugal. Samples were collected during winemaking production at different representative producers of this region. Total phenolic contents of samples were also determined using the Folin–Ciocalteu method. Mass spectrometry results show that atmospheric pressure chemical ionisation (APCI) in negative mode presents higher sensitivity for the majority of the compounds studied. Some phenolic acids, stilbenes as resveratrol and piceid, and flavonols as quercetin and quercetin glycosides were identified in these Muscatel wines. For resveratrol, piceid, gallic acid, protocatechuic acid, catechin and quercetin, fluorescence and electrochemical properties were used as complementary or alternative methods of detection. Differences in phenolic composition and total phenolic contents were found among samples collected.

Keywords: Muscatel wine; Phenolic compounds; Fluorescence detector; Electrochemical detector; Mass spectrometry; Folin–Ciocalteu

1. Introduction

Phenolic compounds play an important role in colour and flavour of foods and beverages and its regular consumption on a diet has been associated with beneficial effects for human health [1]. Some phenolic compounds found in wines are antioxidants contributing to a reduction in the risk of cardiovascular diseases, others such as resveratrol, gallic acid and quercetin have been claimed to have activity against allergies, inflammation, hypertension, arthritis and carcinogens [2–6]. White wines, with a lower phenolic content than red wines, have lower antioxidant activity, although some phenolic compounds present in these wines are more effective in the in vitro inhibition of LDL oxidation process [7].

The type and concentration of phenolic compounds in wines is influenced by the chemical composition of the raw materials (grapes) which are influenced by the variety, ripening stage, atmospheric conditions during ripening and type of soil. The techniques used during the winemaking process of the wine and ageing conditions [8–10] are also important. Phenolic aldehydes (e.g. vanillin), benzoic acids (e.g. gallic acid), hydroxycinnamic acids (e.g. caffeic, ferulic and *p*-coumaric acids) and their esters obtained by condensation with tartaric acid (hydroxycinnamoyltartaric acids), flavanols, flavonols (e.g. quercetin) and anthocyanins are extracted from grapes during the winemaking process. Also flavan-3-ols as catechins present in the grape as monomers or polymerized to form proanthocyanidins and hydrolysable tannins [11,12] and stilbenes, as resveratrol or its glycoside form (piceid) occur in wine [13,14].

Some phenolic compounds can be extracted from wood during the ageing stage and oxidation reactions may also occur increasing the stability of the wine and its pleasant sensorial characteristics.

Phenolic compounds have been analysed by liquid chromatography (LC) with diode array (DAD), fluorescence (FD) [10,14,15] and electrochemical (ED) detection [16,17]. Liquid chromatography with mass spectrometry (MS) using atmospheric pressure ionisation (electrospray or chemical ionisation) has also been used [18] in order to identify their chemical structures.

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In Portugal, there are significant productions of dessert Muscatel wines in Setúbal and Douro regions. These wines are produced from white or red grapes of Muscatel vine varieties: the strong and characteristic bouquet of these grapes is usually much appreciated by consumers.

After harvest, Muscatel grapes are fermented and when sugar content of the must is about 90–100 g L^{-1}, spirit is added in order to stop the fermentation process. Flavour and phenolic compounds from grape pulp and skin are extracted in a maceration process lasting for several months. The wine is then separated from the pomace and the liquid from pressing the pomace is added to the wine; afterwards the wine is transferred into wooden barrels where it stays for at least 24 months [19].

The aim of this work was to identify compounds and monitor the changes in the phenolic composition during the winemaking process of Muscatel wines from different producers in the Setúbal region. Some phenolic compounds detected in the samples were identified by LC–MS^n and the analysis were also carried out with a LC-DAD-FD-ED system. Results obtained from chromatographic profiles were compared with the total phenolic content measured by Folin–Ciocalteu method.

2. Experimental

2.1. Reagents

Acetonitrile (LC–MS and gradient grade) and methanol (HPLC grade) used were from Lab-Scan (Dublin, Ireland). The *o*-phosphoric acid 85% and formic acid (analytical reagent grade) were respectively from Riedel-deHaën (Seelze, Germany) and Panreac (Barcelona, Spain).

Deionised water with 0.050 μS cm^{-1} conductivity, prepared with a Mili-Q system (Millipore, Molsheim, France), was used in all experiments.

The standards as gallic acid, 5-hydroxymethylfurfural (5-HMF), protocatechuic acid, furfural, *p*-hydroxybenzoic acid, catechin, caffeic acid, vanillin, ferulic acid, *trans*-piceid, *trans*-resveratrol were obtained from Aldrich (Steinheim, Germany) and epicatechin, quercetin-3-glucoside and quercetin were obtained from Extrasynthése (Genay, France).

Standard stock solutions (at 10.0 g L^{-1}) were prepared by dissolving the compounds in methanol and stored in the darkness at 4 °C. Working solutions were prepared by dilution of the standard stock solutions with deionised water.

Folin–Ciocalteu reagent was obtained from Sigma (Steinheim, Germany) and sodium carbonate from Riedel-de Haën (Seelze, Germany).

2.2. Sampling

Muscatel Setúbal wines from the 2004 vintage were produced in three wineries identified as producers A, B and C. After spirit was added to stop fermentation, samples were collected during the maceration process (M) corresponding to wine in contact with the seeds and the skins: for 7 months ($1M_A$–$7M_A$) for producer A, for 5 months ($1M_B$–$5M_B$) for producer B and for 8 months ($2M_C$–$9M_C$) for producer C. The pomace was than pressed, the liquid obtained (LP) was added to the wine and a maturation stage (ST) was started. Samples were collected monthly: for 4 months ($1ST_A$–$4ST_A$) for producer A, 6 months ($1ST_B$–$6ST_B$) for producer B and 2 months for producer C ($1ST_C$–$2ST_C$).

2.3. Sample preparation

Ten millilitre of wine were extracted four times with 7 mL of ethyl acetate. The ethyl acetate extracts were combined and evaporated to dryness under vacuum. The residue was redissolved in 250 μL of methanol/water (6:4, v/v) [13] and analysed by liquid chromatography with diode array and mass spectrometry.

Wines were filtered with Acrodisc® Syringe Filter 0.45 (m HT Tuffryn® Membrane from Pall Corporation (Ann Arbor, USA) and analysed by liquid chromatography with diode array, fluorescence and electrochemical detection.

One millilitre of wine was diluted with 4 mL of deionised water for determination of the total phenolic content using the Folin–Ciolcalteu method.

2.4. Equipments and conditions of analysis

2.4.1. Liquid chromatography with mass spectrometry

Analyses by LC were performed with a Surveyor equipment from Thermo Finnigan. The mass spectrometry system was an LCQ ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) sources. The LC–MS system was run by Xcalibur version 1.3 software (Thermo Finnigan—Surveyor, San Jose, USA).

Separations were performed at 35 °C with a LiChrospher C18 (5 μm, 250 mm × 4 mm i.d.) column from Merck with a guard column of the same type. The samples were injected using a 20 μL loop.

The separations were carried out with a flow rate of 700 μL min^{-1} and the mobile phase consisted of a gradient mixture of eluent A (formic acid 0.5%) and eluent B (formic acid–acetonitrile–water 5:400:595, v/v/v). The following gradient of eluents was used: 0–15 min from 0 until 20% eluent B; 10 min with 20% eluent B; 25–70 min, from 20 until 70% eluent B; 70–75 min, with 70% eluent B; 75–85 min from 70 until 100% eluent B; 85–90 min, with 100% eluent B.

The following conditions were used for the mass spectrometer experiments:

- *ESI source*: temperature of the heated capillary 280 °C; electrospray voltage 3.7 kV in positive mode and 3.0 kV in negative mode;
- *APCI source*: vaporizer temperature 465 °C; discharge current 5 μA; temperature of the heated capillary 250 °C.

Nitrogen was used as sheath gas and auxiliary gas in the experiments performed by ESI and APCI. The sheath and auxiliary gas flow rates were 80 and 20 arbitrary units, respectively.

LC–MS was performed in the full scan mode from *m/z* 80 to 2000. The collision energies used in MS^2 fragmentation experiments conducted by LC–MS were established from preliminary MS^2 assays done by direct injection of individual standard solutions of phenolic compounds studied. All the fragmentation experiments were done using 35% collision energy [20].

2.4.2. Liquid chromatography with diode array, fluorescence and electrochemical detection

A Surveyor equipment from Thermo Finnigan with a diode array detector (Thermo Finnigan—Surveyor, San Jose, CA, USA), a fluorescence detector (Shimadzu, RF-535) and an electrochemical detector (Dionex, ED40) with a vitreous carbon electrode were used. Conditions of analysis by LC were the same described in Section 2.4.1 but replacing formic acid (0.5%) by phosphoric acid (0.1%).

Diode array detection (DAD) was performed between 200 and 800 nm. For fluorescence detection several $\lambda_{Ex}/\lambda_{Em}$ were used: 280/320, 260/400 and 300/390 nm. These wavelengths were chosen as they have been described as adequate for the analysis of phenolic compounds using fluorescence detection [14,15].

Electrochemical detection was programmed for a linear variation −1.0 V at 1.0 V in 1.00 s (detection by integrated voltametry using a cyclic variation of the potential). The measurements were taken with a 50 Hz frequency with an analogic/digital converter.

The data acquisition systems were the Chromquest version 4.0 (Thermo Finnigan—Surveyor, San Jose, CA, USA) for the diode array detector and the software 4880 (Unicam) for the electrochemical and fluorescence detectors.

2.4.3. Analysis by Folin–Ciocalteu method

Total phenolic content was determined according to Curvelo-Garcia [21]. A calibration curve was obtained with gallic acid solutions (concentration range 0.4–5 mg L^{-1}) and the results are expressed as grams of gallic acid per litre of wine. Standard and samples were analysed in triplicate.

Absorbance at $\lambda = 750$ nm using 1 cm glass cells was measured with the spectrophotometer (DU-70, Beckman Instruments, Inc., Fullerton, USA).

Data analysis in comparisons of total peak areas from LC analysis (absorption at 280 nm) with total phenolic content measured by Folin–Ciocalteu method were performed using Excel (Microsoft), Version 2002: the correlation coefficients and standard errors were determined using regression statistics.

2.4.4. Measurement of pH

When necessary, the pH of wine was measured by the usual method [21] using Crison-micropH 2002 pH meter with a combined electrode Mettler Toledo type U402-S7/120 and a probe of temperature (CRISON).

3. Results and discussion

3.1. Qualitative analysis of phenolic compounds

A qualitative study of the phenolic compounds present in wine samples was performed by LC–MS. In order to optimize the conditions of analysis by MS, results obtained from electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) in positive and negative modes were compared. For the majority of the compounds, a higher sensitivity was obtained using APCI in negative mode as mentioned in a previous paper

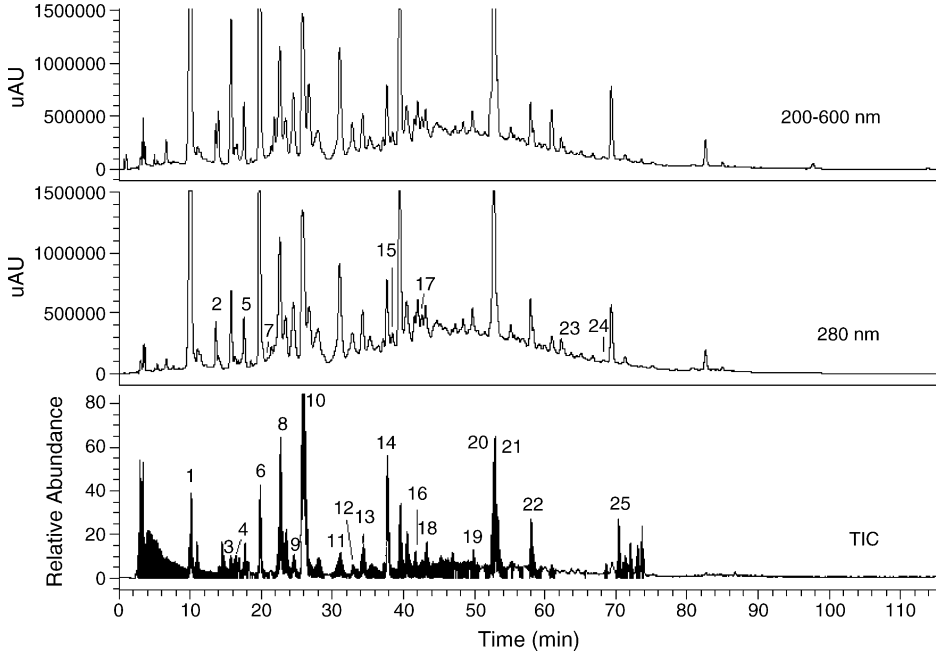

Fig. 1. Chromatograms at λ_{max} (range 200–600 nm), 280 nm and TIC (*m/z* 80–2000) of an extract of Muscatel wine from producer C after 5 months of maceration ($5M_C$).

[20], however detection of resveratrol was only achieved in positive polarity mode. For this compound the analytical conditions used were not the most suitable and more recent experiments [22] showed that lower flows in LC favored the detection of resveratrol using APCI in negative mode.

Chromatograms presented in Fig. 1 were obtained for an extract of Muscatel wine from producer C after 5 months of maceration ($5M_C$) using detection by UV–vis absorption (280 nm and at λ_{max} in the range 200–600 nm) and MS (total ion current).

Chromatograms at 280 nm are widely used to study phenolic compounds because absorption at this wavelength is suitable to detect a large number of such compounds [19].

Mass spectrometry was used to confirm the identifications of gallic acid (peak 1), protocatechuic acid (peak 3), caffeic acid (peak 11), catechin (peak 10), epicatechin (peak 14), *trans*-piceid (peak 19) and quercetin glycosides in Muscatel wines. Compounds were identified comparing *m/z* values obtained by MS and MS^2 with the mass spectra from standards tested in the same conditions of analysis and by comparison with data reported in literature (see Table 1) [2,7,12,18,23–29].

The caftaric (peak 6), *cis*-coutaric (peak 9) and fertaric (peak 12) acids were identified by their $[M-H]^-$ ions at *m/z* 311, 295 and 325, respectively. MS^2 experiments yielded the corresponding $[M-H]^-$ ions of caffeic, coumaric and ferulic acids by neutral loss of tartaric acid moiety (132 mass units) [23].

Full MS spectrum of catechin (peak 10) is illustrated in Fig. 2: ions at *m/z* 289 and 335, correspond to the deprotonated form, $[M-H]^-$, and to the formate adduct, $[M+HCOO]^-$, respectively. The formate adduct is due to the presence of formic acid in the mobile phase. MS^2 of precursor ion at *m/z* 289 originated several fragments: *m/z* 179, 205, 245 and 271 (Fig. 3). The fragment at *m/z* 205 is probably due to a loss of flavonoid A-ring, the ion at *m/z* 245 could result from the loss of a CO_2 group or the loss of a $-CH_2-CHOH-$ group and fragment at *m/z* 271 results from a neutral loss of 18 mass units equivalent to a molecule of water [29]. The fragment at *m/z* 179 may be due to the loss of the B-ring from the flavonoid.

The peaks 8, 13, 16 and 18 in TIC chromatogram (Fig. 1) may correspond to oligomers of catechin and epicatechin (procyanidins). Dimers have a molecular mass of 578 Da and therefore, the ions detected in mass spectrum at *m/z* 577 correspond to the $[M-H]^-$ ions observed in the spectra of the above-mentioned peaks.

Quercetin glycosides as quercetin-3-glucuronide (peak 20) and quercetin-3-glucoside (peak 21) were also identified. Quercetin-3-glucuronide is characterized by the $[M-H]^-$ ion at *m/z* 477; MS^2 yields the fragment at *m/z* 301, by the neutral loss of a glucuronide moiety (176). The ion at *m/z* 463, is a

Table 1
Characterization of phenolic compounds identified in Setúbal Muscatel wines

Peak no.	Compounds	t_r (min)	$[M-H]^-$ *m/z* (MS)	Fragments *m/z* (MS^2)	λ_{max} (nm)	Electrochemical properties	Fluorescence properties
1	Gallic acid	10.23	169	125	256	Yes	Yes[a]
2	5-HMF	13.66	–	–	280	No	No
3	Protocatechuic acid	16.01	153	109	258	Yes	Yes[a]
4	Epigallocatechin	16.77	305	179, 219, 221, 261	258	Yes	Yes[a]
5	Furfural	17.25	–	–	278	No	No
6	Caftaric acid	20.23	311	179	322	Yes	Yes[a]
7	*p*-Hydroxybenzoic acid	20.27	137	93	280	No	Yes[a]
8	Procyanidin dimer	23.27	577	289, 407, 425	278	Yes	Yes[a]
9	*cis*-Coutaric acid	25.29	295	149, 163	310	No	No
10	Catechin	26.48	289	205, 245	276	Yes	Yes[a]
11	Caffeic acid	31.93	179	135	322	Yes	Yes[a]
12	Fertaric acid	33.68	325	193	282	No	No
13	Procyanidin dimer	34.85	577	289, 407, 425	278	Yes	Yes[a]
14	Epicatechin	38.20	289	205, 245	278	Yes	Yes[a]
15	Vanillin	38.45	–	–	274	No	No
16	Procyanidin dimer	42.08	577	289, 407, 425	280	Yes	Yes[b]
17	Ferulic acid	42.58	193	134	278	No	Yes[c]
18	Procyanidin dimer	43.63	577	289, 407, 425	278	Yes	Yes[a]
19	*trans*-Piceid	49.72	389	227	282	Yes	Yes[b]
20	Quercetin-3-glucuronide	52.88	477	301	256	Yes	Yes[a,b,c]
21	Quercetin-3-glucoside	53.32	463	301	256	Yes	Yes[a,b,c]
22	*cis*-Piceid	58.10	389	227	282	Yes	Yes[c]
22	Kaempferol-3-glucoside	58.10	447	285	262	Yes	–
22	Quercetin-3-rutinoside	58.10	447	301	–	Yes	–
23	*trans*-Resveratrol	62.36	229[d]	135	284	Yes	Yes[b]
24	*cis*-Resveratrol	68.23	229[d]	135	284	Yes	Yes[c]
25	Quercetin	70.43	301	151, 179	254	Yes	Yes[c]

[a] 280/320 nm.
[b] 300/390 nm.
[c] 260/400 nm.
[d] $[M+H]^+$ positive mode.

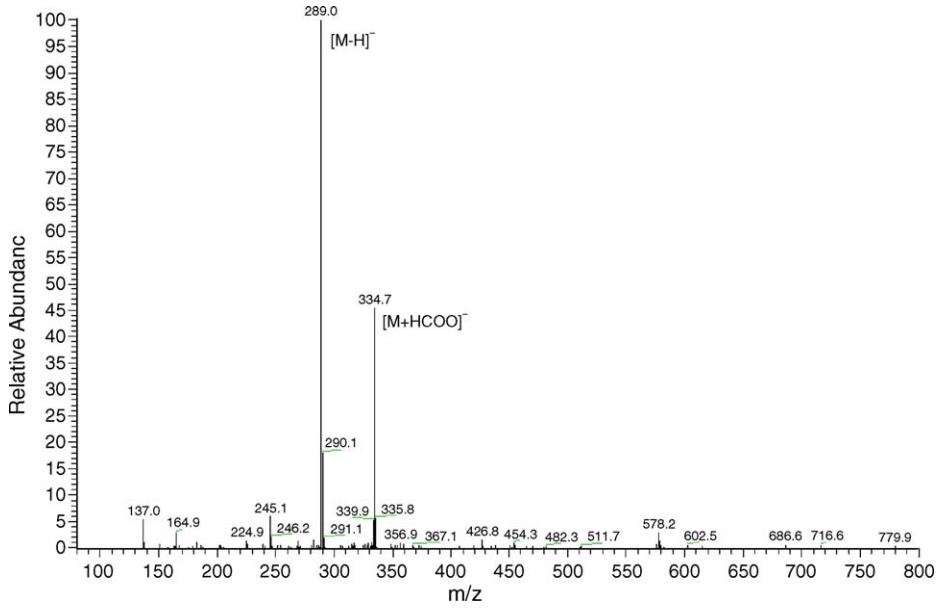

Fig. 2. Full mass spectrum of catechin (peak 10).

deprotonated form, $[M-H]^-$, of quercetin-3-glucoside. Fragmentation of that ion by means of MS^2 results in the aglycon at *m/z* 301. As expected, quercetin (peak 25) was detected at a higher retention time, since the aglycone elutes after the corresponding glycoside. The identification of quercetin was also confirmed by comparison with LC–MS data obtained with a standard solution.

As producer B stopped the maceration stage earlier than producers A and C, samples of Muscatel wines collected after 5 months maceration ($5M_A$, $5M_B$ and $5M_C$) are compared: TIC chromatograms obtained in analyses are presented in Fig. 4. Comparison of chromatographic profiles shows that the same compounds are present in all wines. For wine $5M_A$ the concentrations are much lower for compounds with retention times below 40 min. At about 70–75 min, a group of compounds is detected in the chromatograms of the three wines: peak 25 was identified as quercetin but the other compounds were not identified yet: these peaks are not well resolved and may be divided in two groups characterized by *m/z* values of 447 and 461.

The experimental conditions were not adequate for ionization of some compounds as shown by comparison with chromatograms obtained with the DAD detector used in tandem with the mass spectrometer: various peaks present in the chromatogram obtained by UV absorption were not detected by MS.

The chromatograms presented in Fig. 5 show that the lower content of phenolic compounds in wine from producer A ($5M_A$) is mainly related with a lower content of the gallic acid (peak

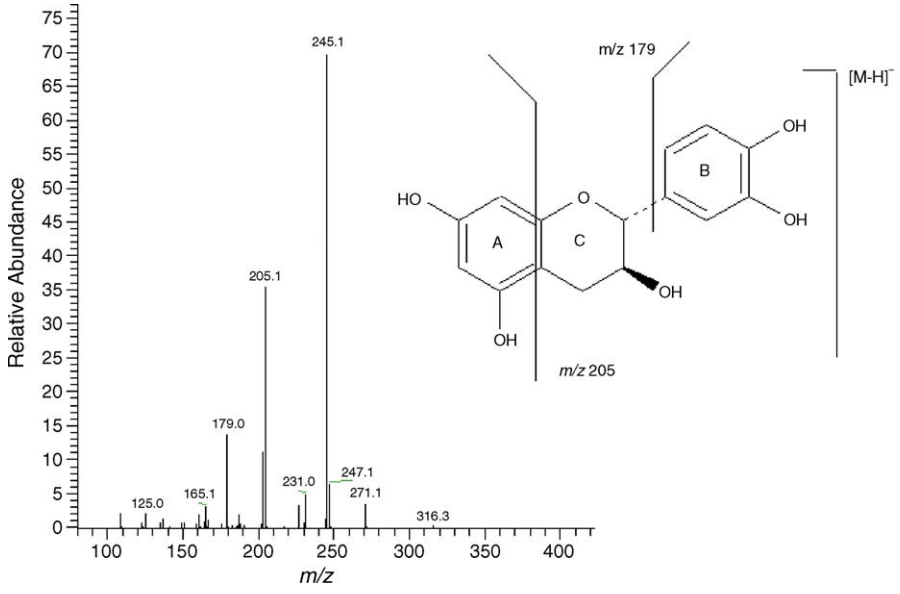

Fig. 3. MS^2 spectrum from the precursor ion *m/z* 289 and the proposed fragmentation scheme.

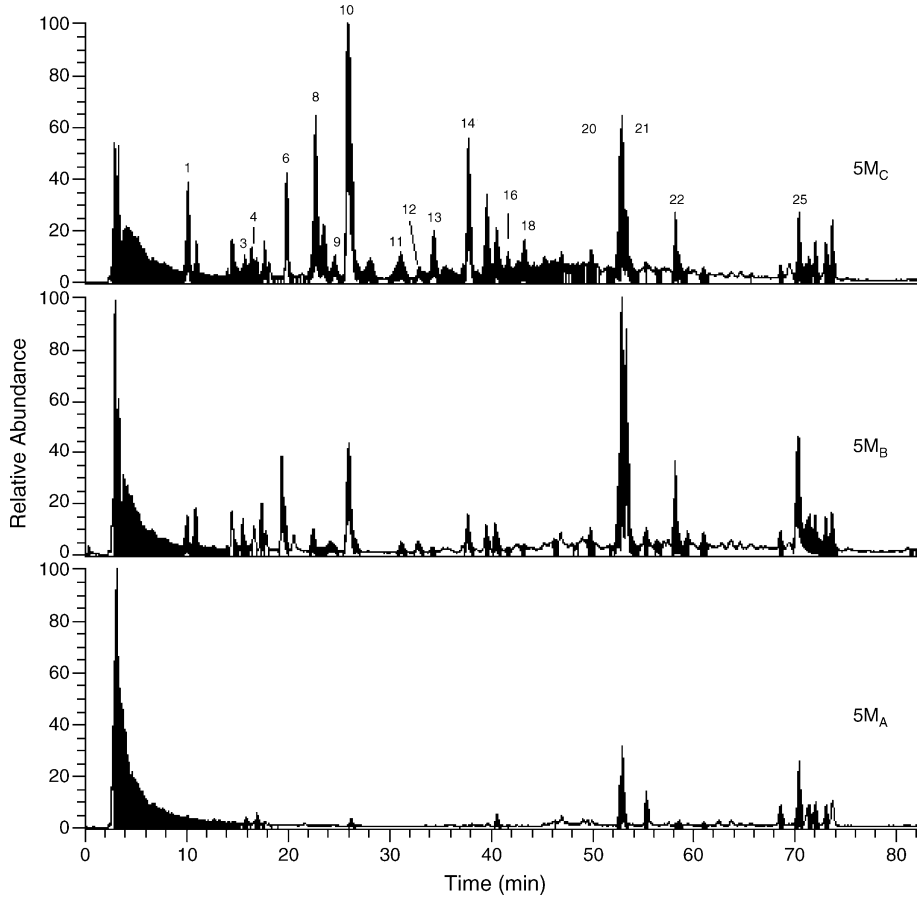

Fig. 4. Chromatogramas of total ionic current of extracts of Muscatel Setúbal wine from producers A (5M$_A$), B (5M$_B$) and C (5M$_C$) after 5 months of maceration.

1), caftaric acid (peak 6), catechin (peak 10) and caffeic acid (peak 11). The main sources of gallic acid (peak 1) and catechin (peak 10) are grape seeds and it is known [30] that higher concentrations of these compounds in wines may be due to some practices that increase seed extraction such as extended macerations, higher temperatures or aggressive pressing. The caftaric acid (peak 6) is an ester of cafeic and tartaric acids found in grape skins and pulp but not in seeds: both the ester and free cafeic acid are found in wine. Caftaric acid is readily oxidized during processing and fermentation [9,30].

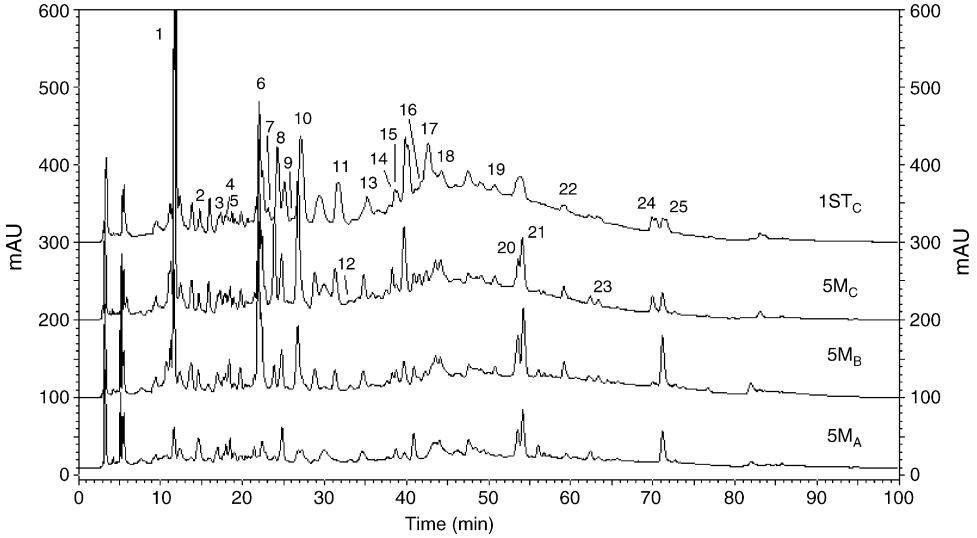

Fig. 5. Chromatograms at 280 nm of Muscatel Setúbal wine from producers A (5M$_A$), B (5M$_B$) and C (5M$_C$) after 5 months of maceration and after adding the liquid from pressing (1ST$_C$).

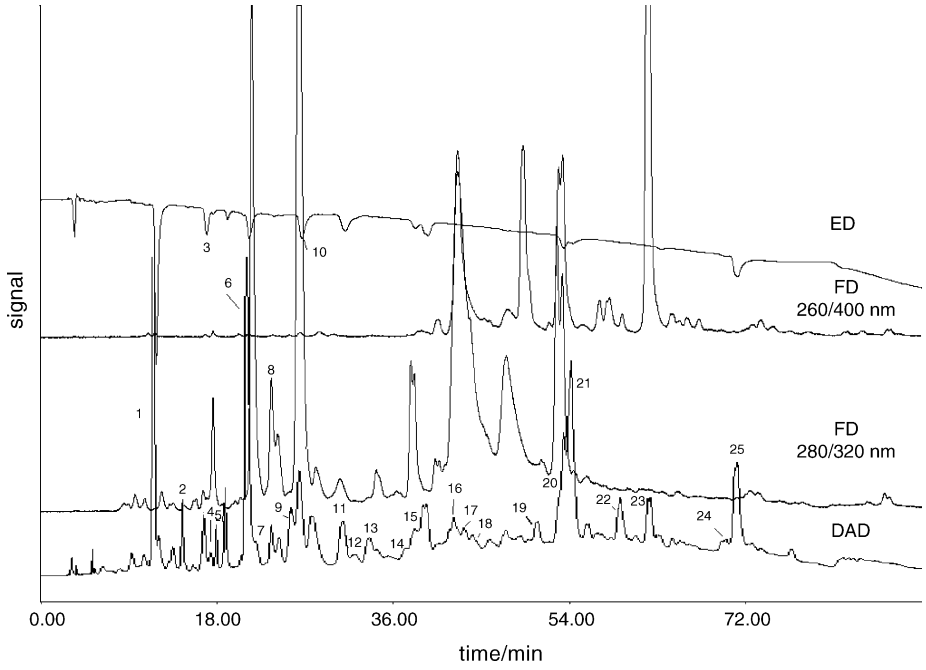

Fig. 6. Chromatograms of Setúbal Muscatel wine from producer B after 5 months of maceration ($5M_B$) using diode array (280 nm), fluorescence ($\lambda_{Ex}/\lambda_{Em}$ = 280/320 and 260/400 nm) and electrochemical (−1 V, +1 V) detectors in tandem.

On pressing the pomace, more phenolic compounds can be extracted and there are changes in phenolic composition after adding liquid from pressing of pomace to the wine as can be seen in Fig. 5 looking at chromatogram of sample $1ST_C$: the content of gallic acid (peak 1), caffeic acid (peak 11), ferulic acid (peak 17) increased, but for quercetin (peak 25) and quercetin glycosides (peaks 20 and 21) reactions may have occurred leading to lower concentrations of these compounds in the wine sample analysed.

Comparison of chromatographic profiles obtained when liquid chromatography with diode array detector was used in tandem with fluorescence and electrochemical detection (Fig. 6) showed that electrochemical detection was useful for identification of compounds such as gallic acid (peak 1), protocatechuic acid (peak 3), caftaric acid (peak 6), catechin (peak 10), caffeic acid (peak 11) and quercetin (peak 25) as these compounds could be easily detected without interference from other compounds eluting at near retention times. Catechin was easily detected with fluorescence detection at λ_{Ex} = 280 nm and λ_{Em} = 320 nm: there were no interfering co-eluting peaks and the signal/noise ratio was much higher than for the peak detected by UV absorption. The same occurred for quercetin-3-glucoronide (peak 20) and quercetin-3-glucoside (peak 21).

Trans-piceid (19) and *trans*-resveratrol (23) were detected by fluorescence at λ_{Ex} = 300 nm and λ_{Em} = 390 nm, and for *cis*-piceid (22), and *cis*-resveratrol (24) the optimal excitation and emission wavelengths were 260 and 400 nm, respectively. These stilbenes are present in low concentrations in wines but they are important compounds due to their antioxidant properties. Production of stilbenes is induced in grapes under stress conditions (attack from pathogens, UV-C light or lesion) [28].

In the chromatogram obtained at 280 nm with DAD, 25 compounds were identified. For 5-HMF (peak 2), furfural (peak 5), *p*-hydroxybenzoic acid (peak 7), vanillin (peak 15), ferulic acid (peak 17), identification was confirmed by analysis of samples spiked with these standards using the LC-DAD-FD-ED system.

In Table 1, are summarized data obtained concerning compounds identified using the several analytical methods discussed in this paper: retention times, *m/z* of $[M-H]^-$, the MS^2 fragments corresponding to each phenolic compound detected by LC–MS with APCI, λ_{max} and information concerning detection with electrochemical and fluorescence detectors.

3.2. Quantitative analysis of phenolic compounds

Chromatograms at 280 nm for all samples have no horizontal straight baselines as illustrated in Fig. 5: these baseline drifts are even larger after adding the liquid from pressing of the pomace to the wine ($1ST_C$). These variations observed in the baseline correspond to the presence of various polymeric compounds not separated in these conditions and eluting between 25 and 75 min. The total content of these unresolved compounds, measured as the sum of peak areas considering a horizontal baseline (total peak areas at 280 nm), can contribute to the total phenolic content measured by the Folin–Ciocalteu method.

The total phenolic contents of wines from the different producers at different times of winemaking process were measured. The standard error for the Folin–Ciocalteu method and for the peak areas was about 5%. An attempt to relate the total peak areas at 280 nm with total phenolic content is presented in Fig. 7. The *r*-value obtained (0.914) confirms that there is a linear relationship although some dispersion of points is observed specially for wines from producer C.

The lower total phenolic contents obtained for the wines from producer A are in accordance with the chromatogram shown in

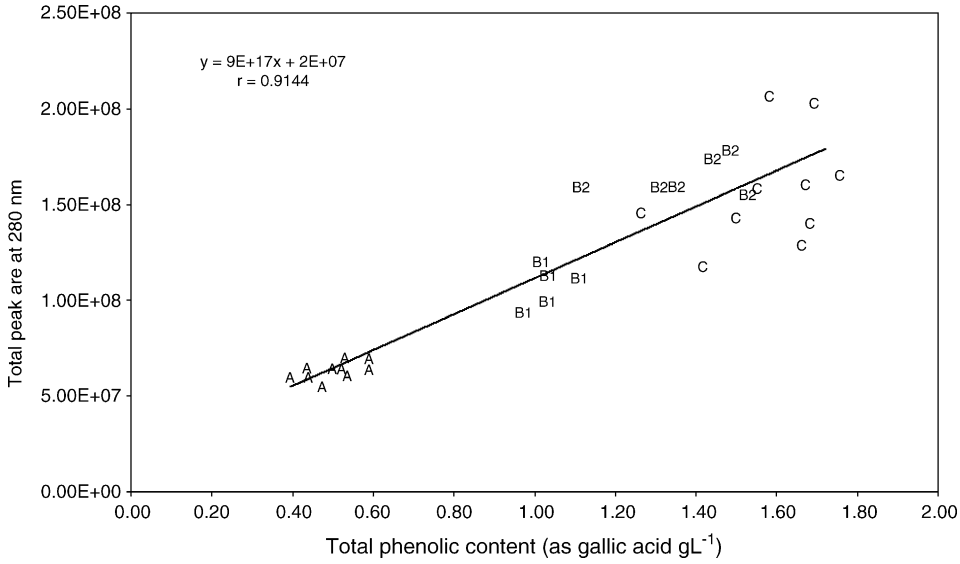

Fig. 7. Total phenolic content vs. LC total areas at 280 nm (peak areas + compounds not separated corresponding to the variation of the baseline) of Setúbal Muscatel wines from producers A, B and C.

Fig. 5. During the maceration, there were no important changes in phenolic composition for this producer.

In wines from producers A and C the total phenolic contents were not strongly influenced by the addition of the liquids from pressing of pomace (LP). On the contrary, an increase in the phenolic content was observed in wine of producer B when this liquid was added: samples identified in Fig. 7 as B1 correspond to wines before the addition of LP and B2 correspond to wines after the addition of LP. The phenolic composition of wines of sub-group B2 became similar to wines from producer C.

When sums of peak areas detected at 280 nm (without imposing a horizontal baseline) were compared with phenolic contents, the *r*-value obtained was 0.938. The lower dispersion observed in Fig. 8 can indicate that the above mentioned polymeric compounds responsible for shifts in the baseline may react in a different way with Folin–Ciocalteu reagent leading to a worse correlation.

One sample ($6M_C$) had peak areas and total phenolic content remarkably lower than samples collected at the same producer (C) in the previous month ($5M_C$) and 1 month later ($7M_C$): sample ($6M_C$) corresponds to the outlier observed in Fig. 8 but we have no explanation for this apparent change in composition.

Differences observed among wines are usually explained by the pH, ethanol content of wines, temperature and time of fermentation, maceration process [9]. The pressing conditions of the pomace are also important because many phenolic compounds are present in the grape (skin and seeds) and the pressing process favours their transfer into the wine.

The pH of samples from producers A, B and C were 4.0, 3.7 and 3.5, respectively, according to their informations: these

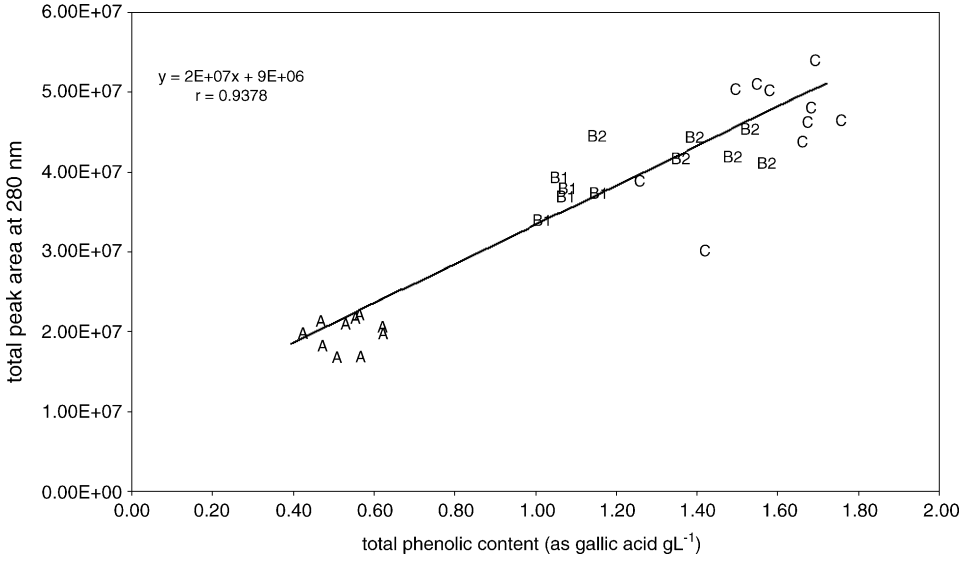

Fig. 8. Total phenolic content vs. LC peak areas at 280 nm of Setúbal Muscatel wines from producers A, B and C.

values were confirmed by measurement in our laboratory. The ethanol content of wines after adding spirit was about 23% for wines from producers A and B and about 20% for the wine of producer C. Therefore major differences in concentration of phenolic compounds could not be attributed to ethanol contents. Other possible explanation for observed differences may be the different times of harvest: the wines of producer C were made with grapes harvested at an earlier time and the strip off the grapes from the stalks was not so complete. Differences observed in wine may also be due to reservoirs used for keeping the wine in contact with pomace at the maceracion stage: stainless steel (producer A) or concrete (producers B and C).

4. Conclusions

This work shows that LC–MS using APCI in negative mode is a valuable tool for qualitative analysis of a large number of phenolic compounds in Muscatel wines. Identification of compounds as phenolic acids (gallic, protocatechuic and caffeic), catechin, epicatechin, piceid and quercetin glycosides was confirmed in these wines by MS. However, these APCI conditions were not adequate for the detection of some phenolic acids like ferulic acid and aldehydes such as vanillin: some complementary work with MS will be necessary in order to optimize the conditions for analysis of these compounds.

The same wine samples were analysed using a tandem of detectors (LC-DAD-FD-ED) to assess the possibility of carrying out analysis of relevant phenolic compounds with simpler LC detectors: compounds such as gallic acid, protocatechuic acid, catechin and quercetin were detected with ED taking advantage of the electrochemically active behaviour of these antioxidant substances. Diode array and fluorescence detectors allow discrimination of fluorescent and non-fluorescent overlapping peaks and they were used to identify the fluorescent compounds present in wine like resveratrol and piceid.

Wines from different producers representative of Setúbal Muscatel wine region were analysed: similar chromatographic patterns were found although significant differences in concentrations of phenolic compounds were observed. Phenolic composition during the maceration processes of wines for each producer had similar evolutions but when the liquid from pressing of pomace was added to the wine an increase in phenolic compounds was observed specially for one producer.

More work will be necessary to identify some compounds already detected in the chromatograms and assess their properties including potential health benefits.

Some further validation work will be set up in order to develop a quantitative method for the analysis of the phenolic compounds in samples.

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References

[1] E. Haslam, Pratical Polyphenolics: from Structure to Molecular Recognition and Physiological Action, Cambridge University Press, Cambridge, 1998.
[2] Á.M. Alonso Borbalan, L. Zorro, D.A. Guillén, C.G. Barroso, J. Chromatogr. A 1012 (2003) 31.
[3] J. Kanner, E. Frankel, R. Granit, B. German, J.E. Kinsella, J. Agric. Food Chem. 42 (1994) 64.
[4] M. Lopez, F. Martinez, C. Del Valle, C. Orte, M. Miró, J. Chromatogr. A 922 (2001) 359.
[5] C. Domínguez, D.A. Guillén, C.G. Barroso, J. Chromatogr. A 918 (2001) 303.
[6] M.S. Fernández-Pachón, D. Villaño, M.C. Garcia-Parrila, A.M. Troncoso, Anal. Chim. Acta 513 (2004) 113.
[7] B. Baderschneider, P. Winterhalter, J. Agric. Food Chem. 49 (2001) 2788.
[8] N. Vivas de Gaulejac, N. Vivas, M.F. Nonier, C. Absalon, G. Bourgeois, J. Sci. Food Agric. 81 (2001) 1172.
[9] R.S. Jackson, Wine Science: Principals and Applications, Academic Press, Toronto, 1994.
[10] P. Viñas, C. López-Erroz, J.J. Marín-Hernández, M. Hernández-Cordoba, J. Chromatogr. A 871 (2000) 85.
[11] M.R. Bronze, L. Vilas Boas, A. Belchior, J. Chromatogr. A 768 (1997) 143.
[12] M. Monagas, C. Gómez-Cordovés, B. Bartolomé, O. Laureano, J.M. Ricardo da Silva, J. Agric. Food Chem. 51 (2003) 6475.
[13] Y. Wang, F. Catana, Y. Yang, R. Roderick, R.B. Van Breemen, J. Agric. Food Chem. 50 (2002) 431.
[14] X. Vitrac, J.P. Monti, J. Vercauteren, G. Deffieux, J.M. Mérillon, Anal. Chim. Acta 458 (2002) 103.
[15] M.A. Rodriguez-Delgado, S. Malovaná, J.P. Pérez, T. Borges, F.J.G. Montelongo, J. Chromatogr. A 912 (2001) 249.
[16] C. Bocchi, M. Careri, F. Groppi, A. Mangia, P. Manini, G. Mori, J. Chromatogr. A 753 (1996) 157.
[17] I. Kolouchová-Honzlíková, K. Melzoch, V. Filip, J. Šmidrkal, Food Chem. 87 (2004) 151.
[18] F. Riccardo, Mass Spectrom. Rev. 22 (2003) 218.
[19] M.N. Matos, Chemical and Sensory Characterization of Fortified Muscatel Wines, M.Sc.Thesis, Faculty of Pharmacy, University of Lisbon, 2001.
[20] S. Silva, A.A. Matias, A. Nunes, C. Duarte, A.V. Coelho, M.R. Bronze, Ciência Téc. Vitiv. 20 (2005) 17.
[21] A.S. Curvelo-Garcia, Controlo de Qualidade dos Vinhos: Química Enológica-Métodos Analíticos, Instituto da Vinha e do Vinho, Lisboa, 1988.
[22] S. Silva, M.L. Gomes, L. Vilas Boas, A. Matias, A. Nunes, C. Duarte, A.V. Coelho, M.R. Bronze, Abstract Book of 29th International Symposium on High Performance Liquid Phase Separations and Related Techniques, Stockholm, 2005.
[23] D. Kammerer, A. Claus, R. Carle, A. Schieber, J. Agric. Food Chem. 52 (2004) 4360.
[24] K. Ku, P. Chang, Y. Cheng, C. Lien, J. Agric. Food Chem. 53 (2005) 3877.
[25] W. Andlauer, M.J. Martena, P. Fürst, J. Chromatogr. A 849 (1999) 341.
[26] Q. Wu, M. Wang, J.E. Simon, Anal. Chem. 75 (2003) 2440.
[27] J. Vial, M. Hennion, A. Fernandez-Alba, A. Agüera, J. Chromatogr. A 937 (2001) 21.
[28] E. Cantos, J.C. Espín, F.A. Tomás-Barberán, J. Agric. Food Chem. 50 (2002) 5691.
[29] W.M. Stoggl, C.W. Huck, G.K. Bonn, J. Sep. Sci. 27 (2004) 524.
[30] On-line, The ETS Red Wine Phenolic Report in http://www.etslabs.com/scripts/ets/pagetemplate/blank.asp?pageid=298.