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Classification of Boal, Malvazia, Sercial and Verdelho wines based on terpenoid patterns

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Abstract

Thirty-six Madeira wine samples from Boal, Malvazia, Sercial and Verdelho white grape varieties were analyzed in order to estimate the free fraction of monoterpenols and C_{13} norisoprenoids (terpenoid compounds) using dynamic headspace solid phase micro-extraction (HS-SPME) technique coupled with gas chromatography–mass spectrometry (GC–MS). The average values from three vintages (1998–2000) show that these wines have characteristic profiles of terpenoid compounds. Malvazia wines exhibits the highest values of total free monoterpenols, contrary to Verdelho wines which had the lowest levels of terpenoids but produced the highest concentration of farnesol. The use of multivariate analysis techniques allows establishing relations between the compounds and the varieties under investigation. Principal component analysis (PCA) and linear discriminant analysis (LDA) were applied to the obtained matrix data. A good separation and classification power between the four groups as a function of their varietal origin was observed.

Keywords: Free terpenoids; Headspace analysis; Solid-phase micro-extraction; Multivariate analysis

1. Introduction

Madeira wines, made from the four noble white grapes varieties: Boal, Malvazia, Sercial and Verdelho, are characterized by a typical vinification and aging processes. One of these processes includes *fortification*, which results in the ethanol content of 18% (v/v). This process is followed by a baking process known as ''estufagem'', during which the wine is submitted to rather high temperatures (45–50 °C) for three months. The full knowledge of Madeira wine grape varieties, namely their aromatic characterization, becomes important as it may serve to discriminate these varieties and to better explore their own potential to produce high quality wines.

A typical cultivars bouquet in wine can be attributed to the aroma of the corresponding grape cultivars and is caused by some compounds which are typical of the grape variety that is transferred from the grape to the wine without being affected by the fermentation process and therefore can be used for variety characterization (Marais, 1983; Rapp & Mandery, 1986). These compounds include monoterpenes, C_{13} norisoprenoids (Falqué, Fernández, & Dubourdieu, 2002; Oliveira, Araújo, Pereira, Maia, & Amaral, 2004), thiols (Darriet, Tominaga, Demole, & Dubourdieu, 1993; Tominaga & Dubourdieu, 2000; Tominaga, Guyot, Peyrot des Gachons, & Dubourdieu, 2000) and methoxypyrazines (Allen, Lacey, & Boyd, 1994; Allen, Lacey, Harris, & Brown, 1991; Sala, Mestres, Marti, Busto, & Guasch, 2002).

At present about 50 monoterpene compounds are known from which the most prominent occurring in grapes and wines are linalool, nerol, geraniol and α-terpineol. These compounds are responsible for the aroma profile of the Muscat varieties, but some of non-Muscat grape varieties such as Riesling, Sylvanner and Gewürztraminer also contain higher levels of monoterpenes (Guth, 1997).

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Some experiments (Günata, Bayonove, Baumes, & Cordonnier, 1985a, 1985b) have demonstrated the presence of two forms of monoterpenes: free and glycosidically conjugated forms (Günata, Bayonove, Baumes, & Cordonnier, 1985b). The free aroma compounds (hydrocarbons, aldehydes and alcohols) are interesting for their flowery odors being most of them related to wine quality. The glycosidically fraction (polyols or glycosides) is, quantitatively, the most important one, although it does not have a direct contribution on wine aroma. Due to its potential role in the aroma characteristics of wine, its quantification could be a useful index for winemakers to determine. The analysis of the optimal maturity of grapes would allow choosing between the most suitable winemaking processes for their maximal valorization.

Terpenoid compounds concentration in must and wines would obviously depend on several factors specially cultivars, region and wine making techniques (Castro, Pérez-Coello, & Cabezudo, 2002; Sánchez-Palomo, Díaz-Maroto, González Viñas, & Pérez-Coello, 2005). Many wines show terpenoids above the threshold levels, so they are active components of the wine aroma. Once the winemaking process starts, all forms of monoterpenols undergo various types of reactions: acid and enzyme catalyzed hydrolysis, isomerization and cyclization (Günata, Bitteur, Brillouet, Bayonove, & Cordonnier, 1998). Catalyzed hydrolysis reactions cleave the sugar moiety from the base terpenols, forming either an odorless polyol or aromatic free terpenols. Polyols can directly form free terpenols through acid hydrolysis (Williams, Strauss, & Wilson, 1981).

Although monoterpenes biosynthesis has been characterized in some organisms such as yeasts and aromatic plants, nothing has been described for grapes so far. The similarity of metabolic pathways between the studied samples and those organisms, allows us postulate that in the grapes the mechanism is quite similar. According to Rohmer (1999), monoterpenes biosynthesis is a multi-step process (Fig. 1). The first corresponds to the formation of (*R*)-(+)-mevalonic acid (MVA) from glucose via acetyl-CoA. After a sequence of phosphorylation, decarboxylation and dehydration reactions, the MVA form isopentenyl pyrophosphate (IPP) is isomerized to 3,3-dimethylallyl pyrophosphate (DMAPP) by the action of *isopentenyl pyrophosphate isomerase*. These are the two basic unities from which the monoterpenes are formed.

This is the same pathway that makes cholesterol in humans and animals (Elson & Yu, 1994). Earlier on, cancer researchers realized that some aspects of cholesterol metabolism were involved in the tumor growth. They then discovered that plant monoterpenes interfered with animal cholesterol synthesis, thereby reducing cholesterol levels and reducing tumor formation in animals (Elson & Yu, 1994). Monoterpenes also increase the levels of liver enzymes involved in the detoxification of carcinogens; an effect that decreases the possibility of carcinogens causing the cellular damage. In addition, monoterpenes stimulate apoptosis, a cellular self-destruction mechanism triggered when a cell's DNA is badly damaged. The generation of C_{13} norisoprenoid compounds involves the enzymatic degradation of carotenoids by regiospecific oxygenases (Mills, 1995).

Due to the low levels of the terpenoid compounds in wines, a suitable extraction/concentration step is usually required. Microwave assisted extraction (Razungles, Günata, Baumes, Pinatel, & Bayonove, 1993), supercritical fluid extraction (Blanch, Reglero, & Herraiz, 1995), solid phase extraction (López, Aznar, Cacho, & Ferreira, 2002;

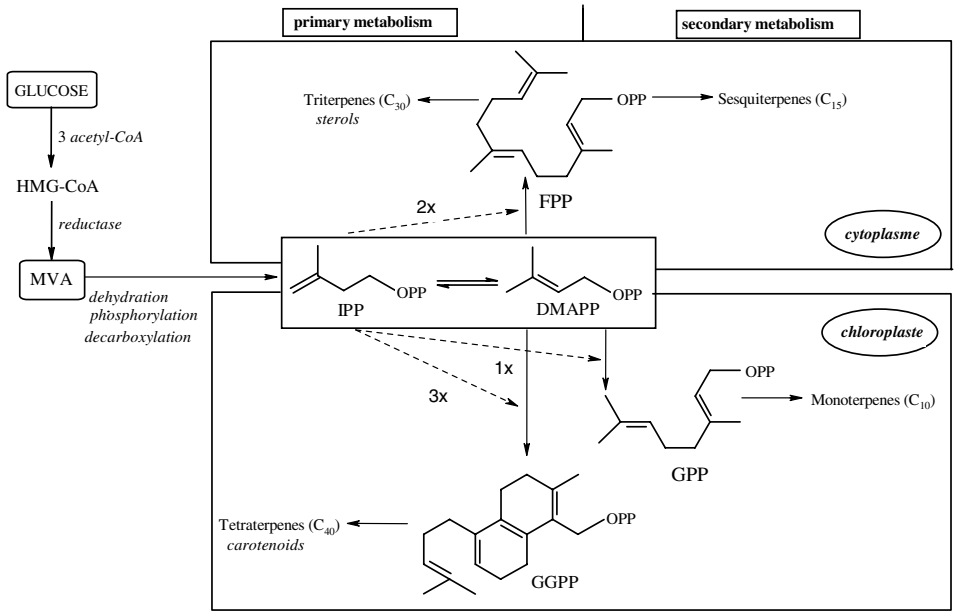

Fig. 1. Biosynthesis of terpenols in plants (16) (HMG-Co-A: β-hydroxy-β-mehylglutaryl coenzyme A; MVA: (*R*)-(+)-mevalonic acid; IPP: isopentenyl pyrophosphate; OPP: *ortho*-pyrophosphate; DMAPP: 3,3-dimethylallyl pyrophosphate; FPP: farnesyl pyrophosphate; GPP: geranyl pyrophosphate; GGPP: geranyl geranyl pyrophosphate).

Piñeiro, Palma, & Barroso, 2004) and solid phase micro-extraction (SPME) (Bencomo-Rodríguez, Conde, Rodríguez-Delgado, García-Montelongo, & Pérez-Trujillo, 2002; Câmara, Herbert, Marques, & Alves, 2004; Marengo, Aceto, & Maurino, 2001; Sánchez-Palomo, Díaz-Maroto, & Pérez-Coello, 2005) has recently been used in replacement of the classical methods such as liquid–liquid extraction (Ferreira, López, Escudero, & Cacho, 1998; Ortega, López, Cacho, & Ferreira, 2001; Wada & Shibamoto, 1997). Compared to traditional techniques, SPME offers many advantages such as high sensitivity and reproducibility, does not require solvent and combines extraction and pre-concentration in a single step without pre-treatment of samples. Moreover it is fast, inexpensive, requires low sample volumes and can be easily automated (De la Calle Garcia, Magnaghi, Reichenbacher, & Danzer, 1996; Demyttenaere et al., 2003; Vás, Gál, Harangi, Dobó, & Vékey, 1998). This technique has been successfully been used in wine samples (De la Calle Garcia, Magnaghi, Reichenbacher, & Danzer, 1998; De la Calle Garcia, Reichenbacher, Danzer, Bartzch, & Feller, 1999).

The present study analyses the composition of monovarietal white wines produced from Boal, Malvazia, Sercial and Verdelho grapes obtained over three consecutive vintages (1998–2000), with the objective of finding typical profiles of free monoterpenols and C_{13} norisoprenoids and of differentiating wines according to the variety using multivariate analysis techniques. Before addressing these questions, the validity of the chosen method was verified. A preliminary study was made on the influence of the matrix effect on extraction, accuracy of the method, limits of detection and quantification, linearity and compound recoveries (Câmara, Alves, & Marques, 2006). The terpenoid compounds were quantified in the 36 wine samples of the four grape varieties and these were characterized according to the variety and vintage year. Multivariate techniques of data analysis – principal component analysis (PCA) and linear discriminant analysis (LDA) – were employed, to establish differentiation criteria as a function of the varieties from which the wines are made.

Because the four grape varieties were from the same vineyard, they should have been subject to similar environmental factors such as soil characteristics, climate, amongst others. Therefore, any difference that might be found in varietal composition of their wines should be due exclusively to the variety used for winemaking.

2. Experimental

2.1. Chemicals

All chemicals were of analytical grade. The terpenic compounds were obtained from Sigma–Aldrich (Sigma Chemical, Co., St. Louis, MO, USA). HPLC grade methanol, ethanol and hydrochloric acid were obtained from Merck (Darmstadt, Gremany). Ultrapure water from the Milli-Q system, was used in all cases.

2.2. Sample wines

Grapes of *Vitis vinifera* cultivars Boal, Malvazia, Sercial and Verdelho, supplied by the *Instituto do Vinho Madeira* (IVM), collected at the final stage of ripening were used. This study was carried out over three vintages (1998–2000). The vinification of all varieties was carried out with the same technologic processes. The musts were fermented in oak casks (8000–10,000 l) with spontaneous yeast and stopped by the addition of natural grape spirit containing 95% (v/v) ethyl alcohol (EU N°. 3111, 1993), when the appropriate amount of natural grape sugars has been fermented according to the wine type to obtain. The 36 wine samples were collected eight month after fermentation and stored at −28 °C until analysis. All the analysis was carried out in triplicate.

2.3. Sample extraction conditions

A SPME polyacrylate (PA) 85-μm film thickness coated fused-silica fiber from Supelco, Inc. (Bellefonte, PA, USA) was used in order to extract sample components. Prior to the first extraction, the fiber was conditioned in the GC injector port at 300 °C for 2 h according to the manufacturer's recommendations.

Free monoterpenols and C_{13} norisoprenoids were extracted by headspace solid phase micro-extraction (HS-SPME) after optimization of the major parameters that influence the extraction processes (Câmara et al., 2006). Fifty milliliter of the wine samples were spiked with 0.422 μg/l of octan-3-ol (Sigma–Aldrich, Barcelona, Spain), which was used as internal standard (50 μl of alcoholic solution at 422 mg/l). Optimal conditions for extraction were obtained using the following procedure: 2.4 ml of sample were transferred to a 4 ml vial (headspace volume was 1.6, according to the phase ratio $1/\beta = 0.6$) (De la Calle Garcia et al., 1996), the ionic strength was adjusted to 30% with NaCl and the pH was maintained at 3.3–3.5 (pH of the wine). The vial was sealed and headspace extraction was performed for 120 min at 40 °C with 85-μm PA fiber, keeping the sample under continuous stirring (1250 rpm). After extraction, the SPME fiber was withdrawn into the needle, removed from the vial and inserted into the hot injector port (260 °C) of the GC–MS system for 6 min, where the extracted chemicals were desorbed thermally and transferred directly to the analytical column.

2.4. Gas chromatography–mass spectrometry (GC–MS) conditions

The wine extracts were analyzed by GC–MS using a Varian STAR 3400Cx series II gas chromatograph (Varian, Inc. Corporate Headquarters, Palo Alto, CA, USA), equipped with a 30 m × 0.25 mm ID, with a 0.25 μm film thickness, *Stabilwax* fused silica capillary column (JW Scientific, Folsom, CA, USA), connected to a Varian Saturn III mass selective detector, according to the method

described by Câmara, Herbert, Marques, and Alves (2003). Splitless injections were used. The initial oven temperature was set to 40 °C (for 1 min), then increased in three steps: 40–120 °C, at 1 °C/min; 120–180 °C at 1.7 °C/min and 180–220 °C, at 25 °C/min. Each step was preceded by a small period at constant temperature for 2, 1 and 10 min, respectively. The injector temperature was 260 °C and the transfer line was held at 220 °C. The carrier gas was Helium N60 (Air Liquid, Portugal) with a column-head pressure of 13 psi (1 psi = 6894.76 Pa). The detection was performed by a Saturn III mass spectrometer in the electronic impact (EI) mode (ionization energy, 70 eV; source temperature, 180 °C). The electron multiplier was set to the auto tune procedure. The acquisition was made in scanning mode (the mass-to-charge ratio range used was 30–300 m/z; 1.9 spectra/s).

The compounds were identified by comparison of mass spectra data obtained from the sample with that taken from pure commercially available standards injected in the same conditions. The Kováts indexes and the mass spectra were compared with those from the NIST library.

2.5. Quantification

Quantification was performed by GC–MS. Triplicate calibration graphs, at five concentrations levels, were constructed by least square linear regression using the results for the standard solution (18% hydro-alcoholic solution) submitted to the same procedure as the samples. The concentration ranges of the studied compounds were: linalool, 4.4–68.7 μg/l; α-terpineol, 3.0–18.1 μg/l; citronellol, 0.3–19.2 μg/l; nerol, 1.5–14.9 μg/l; β-damascenone, 1.4–10.5 μg/l; nerylacetone, 0.9–20.8 μg/l; α-ionone, 0.8–12.4 μg/l; geraniol, 1.4–17.0 μg/l; β-ionone, 1.9–15.1 μg/l; nerolidol, 2.4–20.5 μg/l. The calibration graphs were linear with r^2 values between 0.974 (linalool) and 0.998 (nerol). The pH was adjusted to 3.4 ± 0.1.

2.6. Statistical analysis

Principal component analysis (PCA) was used to examine the relationship among the composition and the wine variety. It is an unsupervised technique that reduces the dimensionality of the original data matrix retaining the maximum amount of variance. Linear discriminant analysis (LDA) is a supervised technique method used for classification purposes. Both methods were carried out using the SPSS Program, version 11.0 (SPSS Inc. Headquarters, Chicago, IL, USA). These techniques were applied to the normalized concentrations of free monoterpenols and C_{13} norisoprenoids. One-way analysis of variance (ANOVA) was employed to evaluate significant differences between cultivars and harvest years.

3. Results and discussion

Table 1 shows a summary of the average data from the physicochemical analysis, which characterizes Madeira wine samples studied according to variety. These parameters were studied because they are directly correlated with wine quality (volatile acidity and ethanol), wine stability (pH, titrable acidity and total SO_2) and very likely will be responsible for differences in the extraction potential of the wines. The acidic composition showed an average pH value quite similar for all the samples studied, which lies between 3.3 and 3.7 (20 °C). Sercial was the wine variety with higher total acidity and lower pH values. The dry extract and density (20 °C) increased with the sugar content, as expected.

The dynamic HS-SPME/GC–MS method was found to be fully suitable for the analysis of free terpenols and C_{13} norisoprenoids in wine, due to its selectivity and sensitivity. The repeatability of the method was estimated by the relative standard deviation (RSD) of the concentrations for six consecutive extractions of a hydro-alcoholic (18%, v/v) standard solution. The values obtained for this parameter ranged from 4.3% for citronellol to 14.2% for nerolidol, with an average of about 8.3% for all analytes considered which is acceptable for this type of analysis. The limits of detection (LOD) were estimated from the area corresponding to three fold the system noise. The values obtained ranged from 0.4 μg/l for β-damascenone to 3.0 μg/l for linalool (Câmara et al., 2006).

The study was conducted in four different grape varieties: Boal, Malvazia, Sercial and Verdelho collected in three consecutive years (1998–2000). Fig. 2 shows a typical SIM chromatogram (Selected Ion Monitoring – SIM) obtained from HS-SPME/GC–MS analysis of a wine sample. The monoterpene content of a wine is considered to be a positive quality factor. This is because they contribute to its varietal aroma, serve to differentiate it from other varieties, and supply sensorial nuances to the wine. The most important monoterpene compounds present in grape must and wines are shown in Fig. 3. The concentration values of free monoterpenols and C_{13} norisoprenoids found in the Boal, Malvazia, Sercial and Verdelho wine samples over the three

Table 1
Average ($n = 3$) data obtained for the analytical characteristics of 2000 Madeira wines samples according to the variety

Wines	Density (g/ml, 20 °C)	pH	Alcohol (%, v/v)	SO_2 (mg/l)		Acidity (g/l)			Sugars (g/l)		Dry extract (g/l)
				Free	Total	Volatile	Fix	Total	Reducing	Total	
Boal	1.0144	3.7	16.8	3.7	10.9	0.4	4.5	4.8	34.0	72.6	98.8
Malvazia	1.0094	3.4	19.2	3.8	11.1	0.3	5.7	6.1	28.7	64.7	90.5
Sercial	0.9852	3.3	16.9	3.7	8.6	0.4	7.1	7.7	24.8	53.1	31.0
Verdelho	1.0028	3.5	16.9	3.9	10.6	0.9	4.7	5.7	28.7	43.8	67.2

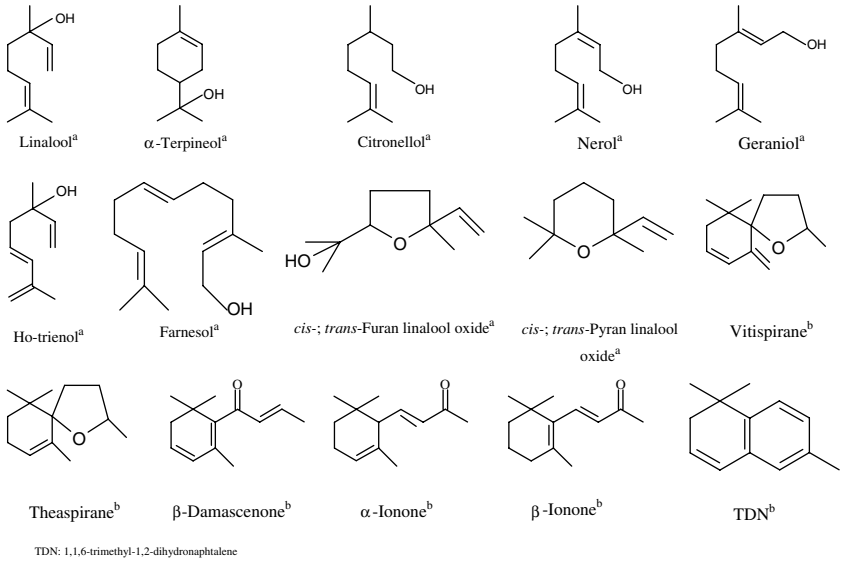

Fig. 2. Chemical structure of main (a) volatile monoterpenes in wines, and metabolites (b) from higher terpenes as grape aroma constituents.

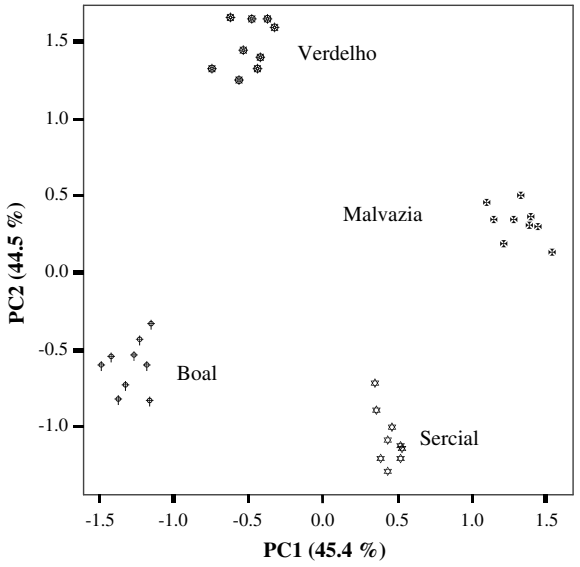

Fig. 3. Principal component analysis for all wines differentiating the varieties projected in the plane defined by the first two factors.

vintages studied (1998–2000) are shown in Table 2. The ANOVA treatments of the data showed significant differences between cultivars.

For the three years of study, the major monoterpenols found in the analyzed wines were linalool, α-terpineol and citronellol. As shown in Table 2, Malvazia is the wine variety with the highest values of total free monoterpenols followed by Boal wines, in opposition to Sercial and Verdelho wines. However, the levels of free terpenoid compounds of the four wine varieties are below the olfactive perception threshold.

The results showed that α-terpineol, linalool and citronellol are among the most relevant monoterpenols in Malvazia wines (Table 2). However, these compounds are present at levels lower than its perception threshold (50, 110 and 18 μg/l (Dugelay, Günata, Sapis, & Bayonove, 1992; Miller, Amon, & Simpson, 1987) in wines with an alcoholic content of 11–12% (v/v)) and, probably, do not contribute to the flowery aroma of this wine. In Boal wines citronellol and linalool are the main monoterpenols identified and represent 45.8% of the total free monoterpenic fraction. Citronellol is also present in Boal wine samples at concentrations near its perception threshold of 18 μg/l (Ribéreau-Gayon, Boidron, & Terrier, 1975) and will certainly also contribute to the aroma of these wines. Linalool and geraniol are the predominant monoterpenols present in the Sercial wines (53.7% from the free monoterpenic fraction). Verdelho is the variety that exhibits the lowest content of free monoterpenols, being α-terpineol and farnesol present at higher levels than the other varietal compounds. The wines from the Verdelho variety have much higher concentrations of farnesol than the other wine samples studied. Geraniol and nerolidol were present in similar concentrations in all the analyzed Madeira wines.

In general, the presence of C_{13} norisoprenoids is also considered to be a quality factor and typical from each variety, as they supply an agreeable scent of tobacco, fruits and tea. Also, although usually present in very low amounts (a few μg/l), as their perception threshold is very low, they play an important part in the aroma. Among the norisoprenoids that were identified (Table 2), β-damascenone is the most abundant. Its content is above the olfactive perception threshold (45 ng/l according to Ribéreau-Gayon, Glories, Maujean, & Dubourdieu (2000)), hence it may be considered as a possible odorant to the aroma of Madeira wines. Its descriptor is "violets", "exotic fruit" and/or "exotic flowers". Malvazia is the wine variety with the highest concentration of this compound in opposition to the Sercial wines variety.

The content of terpenoids remained relatively constant throughout the three vintages studied (1998–2000). The total content of free monoterpenols and C_{13} norisoprenoids

Table 2
Mean (±standard deviation) concentrations (μg/l) of free monoterpenols and C_{13} norisoprenoids found in the wines produced by the four grape varieties

Wines	*t*linox[a]	*c*linox[a]	lin	ter	cit	ger	nero	far	vitisI[b]	vitisII[b]	TDN[b]	dam
1998												
VB	0.2 ± 0.06	n.d.	9.9 ± 1.1	2.9 ± 0.1	14.5 ± 0.9	2.4 ± 0.5	3.2 ± 0.4	4.6 ± 0.4	2.5 ± 0.3	1.8 ± 0.2	0.9 ± 0.1	6.2 ± 0.4
VM	1.5 ± 0.3	0.7 ± 0.2	8.7 ± 0.8	14.1 ± 0.8	9.2 ± 0.7	4.5 ± 0.4	n.d.	1.4 ± 0.5	0.7 ± 0.07	0.4 ± 0.04	0.2 ± 0.03	12.8 ± 1.0
VS	n.d.	n.d.	14.6 ± 1.3	4.5 ± 0.8	3.9 ± 0.2	6.3 ± 0.4	1.4 ± 0.4	0.3 ± 0.04	0.8 ± 0.1	0.5 ± 0.08	0.4 ± 0.05	5.8 ± 0.8
VV	0.2 ± 0.07	0.3 ± 0.1	5.5 ± 0.5	8.7 ± 1.3	0.9 ± 0.1	1.0 ± 0.3	n.d.	6.9 ± 1.1	0.7 ± 0.09	n.d.	0.7 ± 0.09	6.4 ± 0.8
1999												
VB	0.2 ± 0.06	n.d.	9.4 ± 0.6	3.0 ± 0.7	19.6 ± 1.1	2.3 ± 0.4	2.5 ± 0.6	3.8 ± 0.5	2.8 ± 0.5	2.3 ± 0.4	1.2 ± 0.2	6.8 ± 0.4
VM	1.7 ± 0.2	0.5 ± 0.08	9.8 ± 0.7	13.4 ± 1.3	7.9 ± 0.9	3.7 ± 0.5	n.d.	1.5 ± 0.1	0.8 ± 0.05	0.4 ± 0.02	0.3 ± 0.06	12.3 ± 0.4
VS	n.d.	n.d.	13.5 ± 0.9	5.4 ± 0.8	3.3 ± 0.3	5.7 ± 0.5	2.2 ± 0.4	0.2 ± 0.03	1.2 ± 0.4	0.7 ± 0.1	0.4 ± 0.08	4.3 ± 0.6
VV	0.2 ± 0.04	0.3 ± 0.05	5.9 ± 1.2	8.7 ± 0.7	1.0 ± 0.3	1.1 ± 0.4	n.d.	7.3 ± 0.9	0.8 ± 0.06	n.d.	0.7 ± 0.06	6.5 ± 1.2
2000												
VB	0.2 ± 0.05	n.d.	8.7 ± 1.5	3.6 ± 0.3	12.1 ± 0.9	2.4 ± 0.5	2.8 ± 0.4	3.5 ± 0.9	2.8 ± 1.0	2.1 ± 0.6	1.5 ± 0.2	7.1 ± 0.9
VM	1.3 ± 0.4	0.6 ± 0.06	8.0 ± 0.7	12.5 ± 0.9	7.9 ± 0.5	3.9 ± 0.5	n.d.	1.2 ± 0.2	0.8 ± 0.2	0.4 ± 0.07	0.2 ± 0.06	12.7 ± 0.8
VS	n.d.	0.04 ± 0.05	13.2 ± 0.6	4.0 ± 0.7	2.7 ± 0.3	5.8 ± 0.4	1.5 ± 0.1	0.2 ± 0.03	0.8 ± 0.09	0.5 ± 0.08	0.3 ± 0.05	5.4 ± 0.6
VV	0.2 ± 0.09	0.3 ± 0.08	5.4 ± 0.6	8.7 ± 0.8	0.9 ± 0.09	1.0 ± 0.2	n.d.	5.8 ± 1.5	0.7 ± 0.2	n.d.	0.6 ± 0.08	6.5 ± 0.8

*c*linox, *cis*-linalool oxide; *t*linox, *trans*-linalool oxide; lin, linalool; ter, α-terpineol; cit, citronellol; ger, geraniol; nero, nerolidol; far, farnesol; vitis I, vitispirane (isomer 1); vitis II, vitispirane (isomer 2); TDN, 1,1,6-trimethyl-1,2-dihydronaphthalene; dam, β-damascenone.
n.d. = not detected.
[a] Expressed in equivalents of linalool.
[b] Expressed in equivalents of β-damascenone.

was 43.3 ± 10.3, 41.3 ± 8.7 and 40.1 ± 9.4 μg/l for the 1998, 1999 and 2000 vintages, respectively. The one-way analysis of variance (ANOVA) test, shows that at the 95% level, there are no significant differences ($p > 0.05$) between the mean values of monoterpenols and C_{13} norisoprenoids of the four wine varieties harvested in different years, despite the fact that the 1998 vintage produced higher levels of these compounds (Table 3).

3.1. Principal component analysis

Grape variety and vintage year, in conjugation to winery (grapevine cultivars practices and winemaking methods) are the main sources of variation in the chemical composition of wines. Although the main purpose of this study was to test which varietal components could differentiate Madeira wines according to the grape variety, an attempt

Table 3
Results from ANOVA and LSD test for multiple comparisons between variety (casta) and harvest year (ano)

		Sum of squares	d.f.	Mean square	*F*	Sig.
ANOVA						
Between groups		0.225	3	0.075	0.101	0.959
Within groups		23.775	32	0.743		
Total		24.000	35			

(I) casta 1	(J) casta 1	Mean difference (I − J)	Std. error	Sig.	95% Confidence interval	
					Lower bound	Upper bound
Multiple comparisons						
LSD						
VB	VM	−0.2250	0.40886	0.586	−1.0578	0.6078
	VS	−0.1000	0.39604	0.802	−0.9067	0.7067
	VV	−0.1000	0.39604	0.802	−0.9067	0.7067
VM	VB	0.2250	0.40886	0.586	−0.6078	1.0578
	VS	0.1250	0.41884	0.767	−0.7281	0.9781
	VV	0.1250	0.41884	0.767	−0.7281	0.9781
VS	VB	0.1000	0.39604	0.802	−0.7067	0.9067
	VM	−0.1250	0.41884	0.767	−0.9781	0.7281
	VV	0.0000	0.40633	1.000	−0.8277	0.8277
VV	VB	0.1000	0.39604	0.802	−0.7067	0.9067
	VM	−0.1250	0.41884	0.767	−0.9781	0.7281
	VS	0.0000	0.40633	1.000	−0.8277	0.8277

Dependent variable: ano.

was made to determine whether the variables selected for this purpose could also reveal other sources of distinction, such as harvesting year. In order to determine the causes of variability in the data sets, principal component analysis (PCA) from data matrix was performed.

By application of PCA to the normalized concentrations of the analytical variables (terpenoids) and 36 objects (wines), two principal components were extracted with eigenvalues higher than 1 (Kaiser's rule) that account for 82.1% of the total variance from the initial data set. The observation of the loading scores suggests that 10 variables, having coefficients magnitude higher than 0.8 – *trans*-linalool oxide (tlinox), linalool (lin), α-terpineol (ter), geraniol (ger), nerolidol (nero), farnesol (far), 1,1,6-trimethyl-1,2-dihydronaphtalene (TDN), vitispirane (vitis1), (*E*,*E*)-farnesal (efar) and β-damascenone (dam), may be enough to adequately describe the samples according to variety. This new variable's set explains 89.9% of the total variance.

Table 4 presents the total variance explained by the two first principal components. The first component, explains 45.4% of the variability in the initial data set and the second component explains 44.5%. In Fig. 3, the first principal component (PC1) of wine samples is plotted against the second principal component (PC2). The separations among different categories of wine samples from this PC1–PC2 scatter point plot are obvious. The first two principal components account for 89.9% of the total variance of data. Fig. 4. shows the corresponding loadings plot that establishes the relative importance of each variable and it is therefore useful for the study of relations among the terpenoid compounds and relations between terpenoid compounds and wines. The variables that most contribute to the first component and account for 45.5% of total variance of data set, are α-terpineol (0.96), nerolidol (−0.93), *trans*-furan linalool oxide (0.92) and to a minor extent vitispirane (−0.76) and β-damascenone (0.74). The second principal component (44.5% of total variance) is strongly correlated with geraniol (0.97), farnesol (−0.92) and linalool (0.89).

The Malvazia wines appear on the first quadrant of the plot of the 36 wines on the plane defined by those first two principal components. These samples are characterized by variables associated to positive values from the two first principal components – (*E*,*E*)-farnesal (0.76) and β-damascenone (0.74). Free terpenoids of Boal wines are related to the negative PC1 and PC2 side, being characterized, primarily, by vitispirane (−0.76), 1,1,6-trimethyl-1,2-dihydronaphtalene (−0.73) and farnesol (−0.92). According to PC1, α-terpineol (0.96), *trans*-furan linalool oxide (0.92) and β-damascenone (0.74) (positive PC1 and negative PC2) are the variables that most characterize Sercial wines. Verdelho samples are represented in the second quadrant (negative PC1 and positive PC2). Geraniol (0.97) and linalool (0.89) are the variables most related with this wine variety (Table 5).

Table 4
Eigenvalues, percentage of variance and cumulative percentage explained by the two first principal components

Principal component	Eigenvalue	Rotation sums of squared loadings	
		Variance (%)	Cumulative (%)
1	3.634	45.431	45.431
2	3.562	44.543	89.974

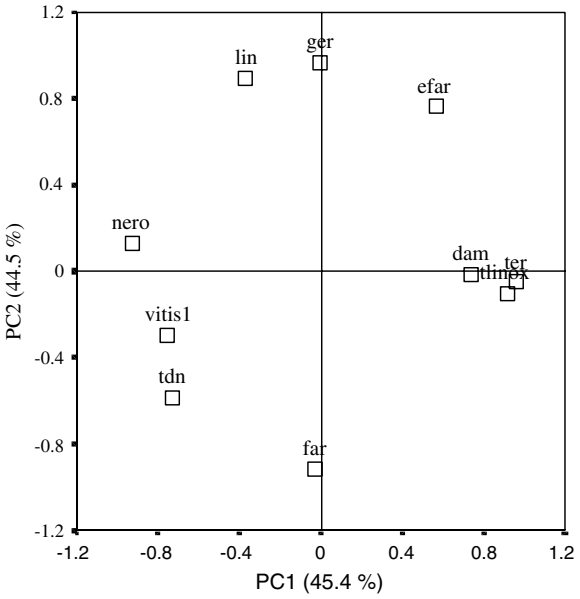

Fig. 4. Extracted principal components as a function of eight variables for the 36 samples of Madeira wines.

3.2. Linear discriminant analysis

This technique is a widespread parametric method used for classification purposes and assumes an a priori knowledge of the number of classes and sample class membership. The classification was performed according to the wine variety. Variables were selected according a wilk's Lambda criterion (Λ). Fig. 5. shows a projection of the

Table 5
Loadings of terpenes in the first two principal components (1-PC1 and 2-PC2; rotation method: Varimax with Kaiser normalization)

	Component	
	1	2
Rotated component matrix[a]		
ter	0.960	−0.052
nero	−0.926	0.132
tlinox	0.920	−0.104
vitis1	−0.756	−0.300
dam	0.735	−0.016
tdn	−0.731	−0.587
ger	−0.008	0.966
far	−0.033	−0.920
lin	−0.374	0.898
efar	0.568	0.762

[a] Rotation converged in three iterations.

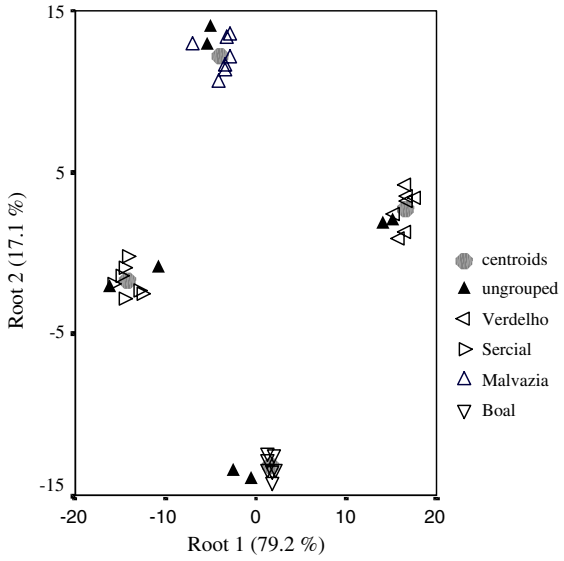

Fig. 5. Ungrouped cases incorporated in corresponding group.

wines in 2D space, with the ungrouped cases in the adequate cluster. Four groups representing each variety were clearly observed. The first two discriminant functions (roots) were effective in discriminating between wine varieties (Table 6). The variables that most contributed to discriminate between the four groups of wines were: linalool and geraniol (first root – 79.2%) and (*E*,*E*)-farnesal, α-terpineol and 1,1,6-trimethyl-1,2-dihydronaphtalene (second root – 17.1%).

The prediction capacity of the SLDA model was evaluated by the "leave-one-out" cross-validation. During this cross-validation test, ungrouped cases are removed to the initial matrix of data set. The classification model is rebuilt and the cases removed are classified in this new model. Table 7 summarizes the results of the classification matrix of the LDA model, obtained for all the samples and separated for variety, showing an average classification of 100%, which means that 8/8 of the objects were correctly classified (Table 5). Hence, the results can be considered satisfactory and acceptable being the selected variables useful to classify and differentiate these wines according to the variety. Due to the great importance of Madeira wines in the Madeira Island's economy, these results constitute a major contribution to investigating possible adulterations and falsifications.

Table 6
Pooled within-group correlations between discriminating variables and standardized canonical discriminant functions (variable ordered by absolute size of correlation within function)

	Function		
	1	2	3
Structure matrix			
ger	0.341[a]	0.234	0.051
lin	0.259[a]	0.033	−0.001
efar	0.147	0.421[a]	−0.349
ter	−0.203	0.398[a]	0.180
tdn	−0.043	−0.286[a]	0.099
nero	0.176	−0.260[a]	0.213
dam	−0.120	0.273	0.600[a]
vitis1	0.037	−0.288	0.447[a]
far	−0.306	−0.219	−0.311[a]
tlinox	−0.148	0.255	0.262[a]

[a] Largest absolute correlation between each variable and any discriminant function.

Table 7
Prediction abilities for the different Madeira wine varieties, using stepwise discriminant analysis

		casta1	Predicted group membership				Total
			VB	VM	VS	VV	
Classification results[b,c]							
Original	Count	VB	7	0	0	0	7
		VM	0	7	0	0	7
		VS	0	0	7	0	7
		VV	0	0	0	7	7
		Ungrouped cases	2	2	2	2	8
	%	VB	100.0	0.0	0.0	0.0	100.0
		VM	0.0	100.0	0.0	0.0	100.0
		VS	0.0	0.0	100.0	0.0	100.0
		VV	0.0	0.0	0.0	100.0	100.0
		Ungrouped cases	25.0	25.0	25.0	25.0	100.0
Cross-validated[a]	Count	VB	7	0	0	0	7
		VM	0	7	0	0	7
		VS	0	0	7	0	7
		VV	0	0	0	7	7
	%	VB	100.0	0.0	0.0	0.0	100.0
		VM	0.0	100.0	0.0	0.0	100.0
		VS	0.0	0.0	100.0	0.0	100.0
		VV	0.0	0.0	0.0	100.0	100.0

VB, Boal wine; VM, Malvazia wine; VS, Sercial wine; VV, Verdelho wine.
[a] Cross-validation is done only for those cases in the analysis. In cross-validation, each case is classified by the functions derived from all cases other than that case.
[b] 100.0% of original grouped cases correctly classified.
[c] 100.0% of cross-validated grouped cases correctly classified.

The HS-SPME/GC–MS was successfully applied to the differentiation and classification of Madeira wine samples according to their origin. The results show that Boal, Malvazia, Sercial and Verdelho varieties have different profiles of terpenoid compounds. Malvazia has a higher total amount of these compounds than the other varieties. β-Damascenone, the most abundant C_{13} norisoprenoid in young Madeira wines, is present at higher levels than its perception threshold (45 ng/l) and for this reason it can contribute to the "fruity" and "exotic" character to the young wines studied. The most discriminating terpenoids are shown in Table 8.

The content of monoterpenols and C_{13} norisoprenoids shown by these wines remains relatively constant throughout the three vintages studied (1998–2000), allowing the definition of varietal profiles that are typical of each variety.

Table 8
Most discriminating variables found in studied wines

Wines	Variable
Boal	Vitispirane
Malvazia	α-Terpineol
Sercial	Farnesol
Verdelho	Linalol

Boal, Malvazia, Sercial and Verdelho wines were independently grouped according to variety when terpenoid compounds were submitted to the multivariate analysis.

Boal wines samples are characterized, primarily by the C_{13} norisoprenoids vitispirane and 1,1,6-trimethyl-1,2-dihydronaphtalene, whereas the Malvazia wines by β-damascenone, (*E*,*E*)-farnesal and α-terpineol. Sercial wines are mostly associated with α-terpineol and trans-furan linalool oxide. Geraniol, linalool and nerolidol are the variables that most characterize Verdelho wines.

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