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Comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry of monoterpenoids as a powerful tool for grape origin traceability

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Abstract

The establishment of the monoterpenoid profile of *Vitis vinifera* L. cv. 'Fernão-Pires' white grape was achieved by headspace solid-phase microextraction coupled with comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry (GC × GC–ToF-MS). The plot of the first dimension *versus* the second dimension retention times using the *m/z* 93, 121, and 136 was used. The grapes were found to contain 56 monoterpenoids identified by GC × GC–ToF-MS. From these, 20 were reported for the first time in grapes. According to their chemical structure, the compounds were organized in different groups: monoterpene hydrocarbons and monoterpene oxygen-containing compounds, this later divided in oxides, alcohols (monoterpenols and monoterpendiols), aldehydes, esters, and ketones. A database composed by the retention indices of monoterpenoids calculated in the bi-dimensional column set was created, representing a developmental step in monoterpenoid analysis using a GC × GC system. Remarkable results were also obtained in terms of compound classification based on the organized structure of the peaks of structurally related compounds in the GC × GC contour plot. This information represents a valuable approach for future studies, as the ordered-structure principle can considerably help the establishment of the composition of samples. This study proposes a methodology and provides data that can be applied to determine the monoterpenoid profile of grapes, and its extension to the analysis of musts, and wines. As monoterpenoids are secondary metabolites whose synthesis is encoded by variety-related genes, the terpenoid profile may be used as a way to trace its varietal origin.

Keywords: *Vitis vinifera* L.; Fernão-Pires; White grapes; Monoterpenoids; HS-SPME; GC × GC–ToF-MS

1. Introduction

Several studies carried out on grapes characterization recognized a relationship between the wine varietal character and the grape and musts volatile and semi-volatile compounds, namely monoterpenoids [1–6]. Thus, these compounds play an important role in the differentiation of wine varieties [3]. Different types of monoterpene compounds have been reported to be present in grapes, which include monoterpene hydrocarbons and monoterpene oxygen-containing compounds, particularly monoterpenols, monoterpendiols, and monoterpene possessing cyclic structures [6]. The monoterpenols appear as the dominating group, especially in white varieties, represented by linalool, hotrienol, α-terpineol, geraniol, and nerol [3,6]. These compounds, which contribute to the varietal characteristics, have specific aroma descriptors: linalool has characteristic citrus-like, sweet and flowery notes, hotrienol, α-terpineol, and geraniol exhibit flowery and sweet aromas [1,3,7], and nerol has a rose scent [8]. The monoterpendiols are the polyhydroxylated forms of the monoterpenes, being 3,7-dimethyl-1,5-octadien-3,7-diol (terpendiol I) and 3,7-dimethyl-1,7-octadien-3,6-diol (terpendiol II) the most widespread in grapes. These compounds make no direct contribution to the aroma, although some of them are reactive and can breakdown to give pleasant volatiles. For example, terpendiol I is odourless but represent a major potential source of hotrienol by dehydration at wine pH [6,9]. As con-

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cerns the monoterpene cyclic structures, the pyran and furan forms of the linalool oxides were the main compounds detected in *Vitis vinifera* L., which may contribute to the floral and citrus aromas [6,10]. The different monoterpenoids may appear in the free and odourant form, and/or in a glycosidically-linked and odourless form. They are located essentially in the skin of the grape [6].

The monoterpenoids of grapes are generally present in trace amounts ($\mu g\,kg^{-1}$) and their analyses require a previous step of isolation and/or concentration. In the last years, fast, simple and solvent-free methodologies have been used, such as solid-phase microextraction (SPME) [11,12] and stir bar sorptive extraction [13]. This step is normally followed by a capillary one-dimensional gas chromatography coupled with quadrupole mass spectrometry detection (GC–qMS). In spite of the great separation power of the conventional one-dimensional modern chromatographic techniques, the complex nature of the samples, including different kinds of chemical classes beyond monoterpenoids, such as aromatic and aliphatic alcohols, sesquiterpenoids, and C_{13} norisoprenoids [11–13], requires extended GC runs. Furthermore, deep analyses of the chromatograms frequently indicate that some peaks are the result of two or more co-eluting compounds. As a consequence of chromatographic co-elution, reliable MS identification is not possible.

One-dimensional chromatographic processes are widely applied in the analysis of food products. Although such methods often provide rewarding analytical results, the complexity of many naturally occurring matrices exceeds the capacity of any single separation system. As a consequence, in the past years considerable research has been dedicated to the combination of independent techniques with the aim of strengthening resolving power [14]. Comprehensive two-dimensional gas chromatography ($GC \times GC$) employs two orthogonal mechanisms to separate the constituents of the sample within a single analysis. The technique is based on the application of two GC columns coated with different stationary phases, such as one apolar and one polar, connected in series through a special interface (modulator). The interface cuts small (several seconds) portions of the first dimension eluate by cryofocusing, and re-injects it onto the second column. Each first dimension peak is modulated several times, which allows the preservation of the first dimension separation. The second column is very short and narrow and consequently each modulated portion is "*flash*" separated before the next modulation starts. Using this instrumental approach, compounds co-eluting from the first column undergo additional separation on the second one [15]. Therefore, the separation potential is greatly enhanced when compared to the one-dimensional GC. Besides chromatographic separation, sensitivity and limits of detection are also improved due to the focusing of the peak in the modulator and the separation of analytes from chemical background [16]. $GC \times GC$ also offers new opportunities to develop relationships between molecular structure and retentions in the two-dimensional separation space defined by the $GC \times GC$ retention in the combined dimensions [17].

Since the second column produces peaks as narrow as 0.1 s, a detection technique must be fast enough to describe the peaks properly. This represents a problem for classical scanning mass spectrometers, which are capable of scanning rates up to 50 spectra s^{-1}. On the other hand, the high-speed time-of-flight mass spectrometry (ToF-MS), with the maximum acquisition rates of 500 spectra s^{-1}, provides sufficient data density to address the requirements of $GC \times GC$ separations [15]. Besides that, ToF-MS brings other advantages such as full mass spectra acquisition at trace level sensitivity and mass spectral continuity, which allows for deconvolution of spectra of co-eluted peaks. The $GC \times GC$ has recently been used for food analysis [14,16,18,19] and, more recently, for wines [20], although it is not yet applied on grapes characterization.

The aim of this study is to develop a methodology based on the headspace SPME (HS-SPME) coupled with comprehensive $GC \times GC$–ToF-MS in order to obtain a deep qualitative characterisation (profile) of the monoterpenoids of grape. Although this methodology allows to study the whole volatile and semi-volatile composition of the grapes, considering the complexity of the data obtained, this manuscript was focused only on the monoterpenoid's fraction. Thus, to reduce the complexity and the time of analysis, specific *m/z* and a $GC \times GC$ space characteristic of monoterpenoids were established. The one-dimensional GC–qMS detection mode was also applied as a comparative approach.

2. Experimental

2.1. Samples

Healthy mature-state *Vitis vinifera* L. cv 'Fernão-Pires' (FP) grapes from the 2002 harvest were collected in Bairrada Appellation, from Talhão da Avenida vineyard, in Portugal. Samples were transported immediately to the laboratory and were stored in a freezer at −80 °C until analysis.

2.2. HS-SPME methodology

The SPME coating fibre and the experimental parameters were established according to a methodology previously developed in our laboratory for the grape analysis [11]. The SPME holder for manual sampling and the fibre used were purchased from Supelco (Aldrich, Bellefonte, PA, USA). The SPME device included a fused silica fibre coating partially cross-linked with 65 μm Carbowax-divinylbenzene (CW-DVB). The SPME fibre was conditioned at 250 °C for 30 min in the GC injector, according to the manufacturer's recommendations. For headspace sampling, 50 g of grapes was crushed manually in a plastic bag and introduced into a 120 ml glass vial, which corresponds to a ratio of the volume of the liquid phase to the headspace volume ($1/\beta$) of 0.5. The vial was capped with a PTFE septum and an aluminium cap (Chromacol, Welwyn Garden City, UK). After the addition of 8 g of NaCl and stirring (25 × 5 mm bar) at 1000 rpm, it was placed in a thermostatted bath adjusted to 40.0 ± 0.1 °C for 60 min to promote the transference of the compounds from the sample to the headspace. After this step, the SPME fibre was manually inserted into the sample vial headspace for 60 min.

2.3. GC–qMS analysis

The SPME coating fibre containing the headspace volatile compounds was manually introduced into the GC injection port at 250 °C and kept for 15 min for desorption. The injection port was lined with a 0.75 mm I.D. splitless glass liner. The desorbed volatile compounds were separated in an Agilent Technologies 6890N Network gas chromatograph, equipped with a 30 m × 0.32 mm I.D., 0.25 μm film thickness DB-FFAP fused silica capillary column (J&W Scientific, Folsom, CA, USA), connected to an Agilent 5973 quadrupole mass selective detector. Splitless injections were used (5 min). The oven temperature was programmed from 35 to 220 °C at 2 °C min^{-1}, and the transfer line was heated at 250 °C. Helium carrier gas had a flow rate of 1.7 ml min^{-1} and the column head pressure was 12 psi. The mass spectrometer was operated in the electron impact mode (EI) at 70 eV scanning the range 33–300 *m/z* in a 1-s cycle, in a full scan acquisition mode. Identification of volatile compounds was achieved comparing the GC retention times and mass spectra with those of the pure standard compounds, when available. All mass spectra were also compared with the library data system of the GC–MS equipment (Wiley 275), other published spectra [21], and according to the compounds previously described for musts and wines of this variety [2,22,23].

2.4. GC × GC–ToF-MS analysis

The SPME coating fibre containing the headspace volatile compounds was manually inserted into the GC × GC–ToF-MS injection port at 250 °C and kept for 15 min for desorption. The injection port was lined with a 0.75 mm I.D. splitless glass liner. Splitless injections were used (5 min). LECO Pegasus 4D (LECO, St. Joseph, MI, USA) GC × GC–ToF-MS system consisted of an Agilent GC 6890N gas chromatograph with a dual stage jet cryogenic modulator (licensed from Zoex) and a secondary oven. The detector was a high-speed ToF mass spectrometer. An Equity-5 60 m × 0.25 mm I.D., 1 μm film thickness (Supelco) was used as the first dimension column and a Supelcowax-10 2.5 m × 0.1 mm I.D., 0.1 μm film thickness (Supelco) was used as a second-dimension column. The carrier gas was helium at a constant flow rate of 1.0 ml min^{-1}. The primary oven temperature was programmed from 40 (1 min) to 260 °C (15 min) at 5 °C min^{-1}. The secondary oven temperature was programmed from 45 (1 min) to 265 °C (15 min) at 5 °C min^{-1}. The MS transfer line temperature was 250 °C and the MS source temperature was 220 °C. The modulation time was 4 s; the modulator temperature was kept at 30 °C offset (above primary oven). The ToF-MS system was operated at a spectrum storage rate of 125 spectra s^{-1}. The mass spectrometer was operated in the EI mode at 70 eV using a range of *m/z* 33–350 and the voltage was −1650 V. Total ion chromatograms (TIC) were processed using the automated data processing software ChromaTOF (LECO) at S/N threshold 500 (see detailed description of data processing procedure in Section 3.2). Contour plots were used to evaluate the general quality of the separation and for manual peak identification. A signal-to-noise threshold of 500 was used. The methods for identification described in Section 2.3 were also used for GC × GC–ToF-MS analysis. Two commercial databases (Wiley 275 and US National Institute of Science and Technology (NIST) V. 2.0- Mainlib and Replib) and a laboratory-made database of terpenoids comprising 40 elements were used. Mass spectral match factor, similarity>850, was used to decide whether a peak was correctly identified or not. Furthermore, more careful attention was provided by the manual inspection of the mass spectra and/or by the use of additional data, such as the experimentally determined retention index (RI) values and the values reported in the bibliography for chromatographic columns similar to that used as the first dimension column in the present work (Table 1). For the determination of the RI a C_6–C_{24} *n*-alkanes series was used.

3. Results and discussion

The volatile composition of the musts and monovarietal wines showed that monoterpenoids, aromatic alcohols, and C_{13} norisoprenoids were the chemical groups of compounds that contribute to the peculiarity varietal volatile composition of FP variety [2,22,23]. The work presented in this manuscript was focused exclusively on the monoterpenoids due to their contribution to the varietal character of *Vitis vinifera* L., as extensively reported in the bibliography. The HS-SPME–GC–qMS methodology used comprises a preliminary step, in which the grapes were crushed and macerated before HS-SPME analyses. This procedure allows to obtain in the grapes headspace a fraction of the free volatile components from the skin and pulp plus the compounds arising from the reactions that may take place by the acidic conditions used (pH of the grapes *ca.* 3.8) and by the endogenous enzymatic activity [11]. The compounds detected under these conditions were named "variety- and pre-fermentation-related volatile compounds", which include the monoterpenoids.

3.1. HS-SPME–GC–qMS analysis

The monoterpenoid profile of FP grapes obtained by HS-SPME–GC–qMS is presented in Table 1, showing that 26 compounds were detected. From these, seven were perceived only after being detected by GC × GC–ToF-MS, which allowed a deep re-analysis of the one-dimensional chromatograms using the ion extraction analysis mode. The specific *m/z* fragments of the mass spectra of the different compounds were used to more selectively search the monoterpenoid compounds. According to their chemical structure, these 26 compounds were organized in different chemical groups: monoterpene hydrocarbons and monoterpene oxygen-containing compounds, such as oxides, alcohols (monoterpenols and monoterpendiols), aldehydes, esters, and acids. The monoterpenols were the predominant group, representing seven compounds, namely, linalool, hotrienol, (+)-α-terpineol, γ-isogeraniol, citronellol, nerol, and geraniol. This group was followed, in number of compounds, by the monoterpene hydrocarbons and the monoterpene oxides, representing six compounds each. The monoterpene hydrocarbons comprised of 1*S*-α-pinene, β-myrcene, limonene, β-ocimene, 2,6-dimethyl-2,6-octadiene, and α-terpinolene. The

Table 1
Monoterpenoids identified by GC–qMS and GC × GC–ToF-MS in *Vitis vinifera* L. cv. 'Fernão-Pires' white variety

^{1}D*tr* (s), ^{2}D*tr* (s)	Peak no.	RI_{calc}[a]	RI_{lit}[b]	Compound	Identification[c]	GC–qMS	GC × GC–ToF-MS	Lit.[d]
Monoterpene hydrocarbons								
2020, 2.312	1	959	939	1*S*-α-Pinene	B, C	×[e]	×	×
2104, 2.376	2	995	992	β-Myrcene	B, C	×[e]	×	×
2164, 2.408	5	1022	1004	2-Carene	B, C	–	×	–
2176, 2.408	6	1028	1006	α-Phellandrene	B, C	–	×	–
2180, 2.408	7	1030	1022	4-Carene	B, C	–	×	–
2200, 2.424	8	1039	–	1*R*-α-Pinene	B, C	–	×	×
2228, 2.440	9	1052	1033	Limonene	A, B, C	×	×	×
2240, 2.456	10	1058	1030	β-Phellandrene	B, C	–	×	–
2240, 2.560	11	1058	1056	β-Ocimene	B, C	×[e]	×	×
2256, 2.616	13	1064	–	2,6-Dimethyl-2,6-octadiene	B, C	×[e]	×	–
2288, 2.472	15	1080	1074	γ-Terpinene	B, C	–	×	×
2360, 2.520	19	1113	1088	α-Terpinolene	B, C	×	×	×
2412, 2.576	22	1138	1132	*neo*-allo-ocimene	B, C	–	×	×
Sub-total (number of compounds)						6	13	8
Oxygen-containing compounds								
Monoterpene oxides								
2128, 2.432	3	1006	973	*Z*-Herboxide (dehydroxylinalool oxide)	B, C	×	×	×
2160, 2.456	4	1021	988	*E*-Herboxide (dehydroxylinalool oxide)	B, C	×	×	×
2248, 2.464	12	1061	1030	1,8-Cineole	B, C	–	×	×
2320, 2.656	16	1095	1087	Linalool *Z*-furanic oxide	B, C	×	×	×
2388, 2.576	21	1128	1113	*Z*-Rose oxide	B, C	–	×	×
2436, 2.608	23	1149	1130	*E*-Rose oxide	B, C	–	×	×
2456, 2.768	25	1159	–	α-Pinene oxide	B, C	–	×	–
2484, 2.704	26	1172	1131	Nerol oxide	B, C	×[e]	×	×
2524, 2.816	30	1191	–	*E*-2,3-Epoxycarane	B	–	×	–
2540, 3.064	31	1199	–	Linalool *E*-pyranic oxide	B, C	×	×	×
–	–	–	–	Linalool *Z*-pyranic oxide	B, C	×	–	×
Sub-total (number of compounds)						6	10	9
Monoterpenols								
2280, 2.616	14	1076	–	Dihydromyrcenol	B, C	–	×	×
2320, 2.712	17	1095	–	2,6-Dimethyl-1,7-octadien-3-ol	B, C	–	×	–
2352, 2.816	18	1109	1100	Linalool	A, B, C	×	×	×
2360, 2,816	20	1114	1101	Hotrienol	B, C	×	×	×
2448, 2.816	24	1155	–	Plinol C	B, C	–	×	–
2500, 3.048	28	1179	–	Ocimenol	B, C	–	×	×
2556, 2.920	33	1206	–	*p*-Menthan-1-ol	B, C	–	×	–
2572, 3.064	36	1213	1162	Borneol	A, B, C	–	×	–
2572, 3.272	37	1213	1190	*p*-Cymen-8-ol	B, C	–	×	×
2576, 2.904	38	1215	1179	4-Terpinenol	B, C	–	×	×
2596, 3.024	39	1224	1195	(+)-α-Terpineol	A, B, C	×	×	×
2612, 3.096	40	1232	–	γ-Isogeraniol	B, C	×	×	–
2616, 3.032	41	1234	1230	Citronellol	A, B, C	×	×	×
2624, 3.168	42	1237	1229	Lilac alcohol D	B, C	–	×	–
2624, 3.208	43	1237	1197	Myrtenol (α-pinene-10-ol)	B, C	–	×	–
2640, 3.136	46	1245	1233	Nerol	A, B, C	×	×	×
2684, 3.224	49	1265	1260	Geraniol	A, B, C	×	×	×
Sub-total (number of compounds)						7	17	10
Monoterpendiols								
2540, 3.376	32	1199	–	3,7-Dimethyl-1,5-octadien-3,7-diol	B, C	×	×	×

Table 1 (*Continued*)

^{1}D*tr* (s), ^{2}D*tr* (s)	Peak no.	RI_{calc}[a]	RI_{lit}[b]	Compound	Identification[c]	GC–qMS	GC × GC–ToF-MS	Lit.[d]
2556, 3.400	34	1206	–	3,7-Dimethyl-1,7-octadien-3,6-diol	B, C	×[e]	×	×
2624, 3.408	44	1237	–	3,7-Dimethyl-1-octen-3,7-diol	B, C	–	×	×
				Sub-total (number of compounds)		2	3	3
Monoterpene aldehydes								
2488, 2.832	27	1174	1136–1146	Lilac aldehyde B	B, C	–	×	–
2568, 3.000	35	1211	1244	*Z*-Citral (neral)	A, B, C	×	×	×
2632, 3.056	45	1241	1197	Safranal	B, C	–	×	–
2660, 3.024	47	1254	–	*p*-Menth-1-en-9-al	B, C	–	×	×
2676, 3.064	48	1261	1250	β-Ciclocitral	B, C	×	×	×
2732, 3.176	51	1287	1277	*E*-Citral (geranial)	A, B, C	×	×	×
Sub-total (number of compounds)						3	6	4
Monoterpene esters								
2788, 3.160	52	1312	1302	Geranyl formate	B, C	×[e]	×	–
2832, 3.184	53	1332	1304	Isobornyl acetate	B, C	–	×	–
2832, 3.216	54	1332	–	*E*-Ethyl geranate	B, C	–	×	–
2960, 3.512	56	1386	1376	Neryl acetate	B, C	–	×	×
Sub-total (number of compounds)						1	4	1
Monoterpene ketones								
2508, 2.976	29	1183	1152–1155	1*R*-(+)-Norinone	B, C	–	×	–
2720, 3.256	50	1282	1250	Carvone	B, C	–	×	×
Sub-total (number of compounds)						0	2	1
Monoterpene acids								
2888, 1.568	55	1353	–	Geranic acid	A, B, C	×	×	×
Sub-total (number of compounds)						1	1	1
Total						26	56	36

[a] RI: retention index obtained through the modulated chromatogram.

[b] RI: retention index reported in the literature for 5% phenyl polysilphenylene-siloxane GC column or equivalents [13,31,32,36–44].

[c] The reliability of the identification or structural proposal is indicated by the following: (A) mass spectrum and retention time consistent with those of an authentic standard; (B) structural proposals given on the basis of mass spectral data (Wiley 275); (C) mass spectrum consistent with spectra found in literature.

[d] Compounds previously detected in grapes [5,6,9,25–28].

[e] Perceived only after their detection by GC × GC–ToF-MS, which allowed a deep re-analysis of the one-dimensional chromatograms using the ion extraction analysis mode.

monoterpene oxides included two herboxide isomers, linalool *Z*-furanic oxide, nerol oxide, and linalool *E*- and *Z*-pyranic oxide. The presence of two monoterpendiols (terpendiol I and II), three aldehydes (*Z*- and *E*-citral, and β-ciclocitral), one ester (geranil formate), and one acid (geranic acid) was also noticed.

From these 26 monoterpenoids, 11 compounds were previously found as components of FP musts obtained by liquid–liquid dichloromethane continuous extraction followed by GC–qMS analysis [2,23], namely, linalool, hotrienol, nerol, geraniol, α-terpineol, linalool *Z*-furanic oxide, linalool *E*- and *Z*-pyranic oxide, terpendiol I and II, and geranic acid. The differences observed might be due to the different volatile extraction methodologies used, the different vintage, and/or the higher extent of pre-fermentative phenomena that occur in musts.

3.2. HS-SPME–GC × GC–ToF-MS analysis

In the first step of HS-SPME–GC × GC–ToF-MS analysis, automated data processing was used to find all peaks in the GC × GC chromatograms with a signal-to-noise at a minimum of 500. Within the automated data processing, the software finds peaks at individual single ion traces over the whole mass range measured. Therefore, not only major sample components but also trace level compounds, hidden under TIC baseline, can be detected. After the peak detection, modulated peaks are automatically combined by mass spectral deconvolution, i.e. mathematical separation of spectra of co-eluted peaks is performed. In this work, the peak table generated automatically by ChromaTOF software has been further examined and the identification has been confirmed or changed based on the crite-

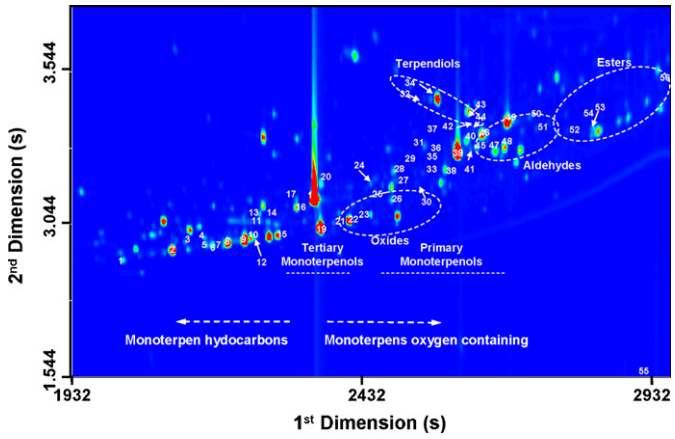

Fig. 1. GC × GC extracted ion chromatogram contour plot of *m/z* 93, 121 and 136. Bands or clusters formed by structurally related compounds are indicated (attribution of peak numbers shown in Table 1).

ria described in Section 2.4. The obtained GC × GC total ion chromatogram contour plot (data not shown) exhibited several hundreds of peaks. As the present study was focused on the establishment of the grape monoterpenoids profile, in a second approach, extracted ion chromatogram contour plot of *m/z* 93, 121 and 136 ions, characteristic of these compounds, was obtained. This approach was very helpful to eliminate the majority of the non-monoterpenic compounds, allowing the definition of a two-dimensional chromatographic space containing the monoterpenoids. This approach simplified the data analysis and, consequently, reduced its time of analysis. Fig. 1 shows the GC × GC extracted ion chromatogram contour plot of *m/z* 93, 121, and 136 ions. The monoterpenoids were detected in the first dimension range of 2016–2906 s and in a second dimension range of 2.360–3.512 s. Compound numbers correspond to those presented in Table 1. This contour plot (cf. Fig. 1) was used to locate the peaks and find the corresponding section of the linear chromatogram from where a deep manual inspection analysis of the mass spectra needed to be done.

Table 1 summarizes the information obtained about the 56 monoterpenoids identified by GC × GC–ToF-MS in *Vitis vinifera* L. cv. 'Fernão-Pires' white variety and the respective RIs calculated according to the van den Dool and Kratz equation [24]. An exhaustive search was done in the literature in order to obtain the RI values for the compounds detected in this study (see Table 1, RI_{lit}). The most intensive peaks, called base peaks, were used for the calculation of the RIs. These values, when compared with the RIs reported in the literature for 5% phenyl polysilphenylene-siloxane GC column or equivalents (Table 1), indicated that a maximum difference of 30–40 was observed for the absolute RI ($|RI_{cal} - RI_{lit}|$). Although this is a slightly great difference than that usually used, this variation can be considered reasonable because (i) the values reported in the literature were obtained in a one-dimensional system, and the modulation causes some inaccuracy in first dimension retention time and (ii) the literature data is obtained from a large range of GC stationary phases (several commercial GC columns are composed of 5% phenyl polysilphenylene-siloxane or equivalent stationary phases), which had a slight different separation selectivity than Equity-5. These data allowed to prepare a database composed of the retention indices of monoterpenoids, which may represent an improvement step in the development of monoterpenoid analysis by GC × GC systems.

According to their chemical structure, the compounds were organized in the already established groups (Table 1). The analysis by GC × GC–ToF-MS allowed to detect twice more compounds than detected by GC–qMS (Table 1). With the exception of ketones, not detected by GC–qMS, and acid, this tendency was observed for all chemical groups. Comparing with the data obtained by GC–qMS, the analysis by GC × GC–ToF-MS allowed an additional detection of: seven monoterpene hydrocarbons (2- and 4-carene, α-and β-phellandrene, 1*R*-α-pinene, γ-terpinene, and *neo*-allo-ocimene), four monoterpene oxides (1,8-cineole, *Z*- and *E*-rose oxide, and *E*-2,3-epoxycarane), 10 monoterpenols (dihydromyrcenol, 2,6-dimethyl-1,7-octadien-3-ol, plinol C, ocimenol, *p*-menthan-1-ol, borneol, *p*-cymen-8-ol, 4-terpinenol, lilac alcohol D, and myrtenol), one monoterpendiol (3,7-dimethyl-1-octen-3,7-diol), three monoterpene aldehydes (lilac aldehyde B, safranal, and *p*-menth-1-en-9-al), three monoterpene esters (isobornyl acetate, *E*-ethyl geranate, and neryl acetate), and two monoterpene ketones (1*R*-(+)-norinone and carvone).

The above results show that GC × GC–ToF-MS provides much higher potential for the detection and identification of compounds than GC–qMS detection mode. The reasons are, mainly, the better chromatographic separation of analytes, and also the better sensitivity of the ToF-MS detector in full mass range acquisition. The automated peak finding and deconvolution algorithm represent powerful tools to detect peaks that are co-eluted. It should also be noted that in this work a relatively high signal-to-noise threshold (500) was used, which limited the number of detected compounds.

According to the data available in the literature [5,6,9,25–28], from the 56 monoterpenoids detected by GC × GC–ToF-MS, 20 of these compounds are reported for the first time in grapes: 2- and 4-carene, α- and β-phellandrene, 2,6-dimethyl-2,6-octadiene, α-pinene oxide, *E*-2,3-epoxycarane, 2,6-dimethyl-1,7-octadien-3-ol, plinol C, *p*-menthan-1-ol, borneol, γ-isogeraniol, lilac alcohol D, myrtenol, lilac aldehyde B, safranal, geranyl formate, isobornyl acetate, *E*-ethyl geranate, and 1*R*-(+)-norinone. These compounds have already been detected in other natural products and some of them have been reported to have sensory properties and biological activity. Several studies reported the significant contribution of lilac aldehydes and lilac alcohols to the characteristic aroma of widespread plants, such as *Syringa oblata* [29] and *Origanum vulgare* L., and flowers of many plant families (Violaceae, Orchidaceae, Rosaceae) [30]. These compounds exhibit very low sensory odour thresholds (few ng L^{-1}) and aroma descriptors related to flowery, fresh, and sweety notes [31]. Other compounds, such as borneol [32] and safranal [33] have been reported to have antifungal, pesticide, antibacterial, and antioxidant activities. Borneol has a camphoraceous odour [8] and safranal is the main compound responsible for the aroma of saffron spice [34].

The GC × GC analysis was performed on a system with a non-polar thick-film column in the first dimension and a second dimension column containing a thin-film Supelcowax-10 polar stationary phase. The column combination used in this study provided two almost independent separations (orthogonal). On the first (non-polar) column, analytes were separated according to their vapour pressure/volatility, and on the second dimension column, analytes were separated according to their polarity. Consequently, compounds with similar vapour pressures had similar retention times in the first dimension and compounds with similar polarities had similar retention times in the second dimension. Thus, structurally related compounds exhibited similar elution order, i.e. they eluted within a cluster in a GC × GC plane [35]. Therefore, it was possible to relate their chemical structures with their chromatographic position [17].

According to the first dimension, the GC × GC extracted ion chromatogram contour plot in Fig. 1 shows that the compounds were organized into two groups: the monoterpene hydrocarbon compounds placed in the lower retention times (higher volatility) and the monoterpene oxygen-containing compounds placed in the higher retention times (lower volatility). The exceptions were the monoterpene oxides *Z*- and *E*-herboxide, 1,8-cineole, and linalool *Z*-furanic oxide. This profile was expectable according to the RI data reported in literature for the different types of monoterpenoids identifiable in grapes and other analogous, when using chromatographic columns similar to those used as the first dimension columns in the present work [13,31,32,36–44]. The monoterpene oxygen-containing compounds showed similar volatility ranges, however, clusters were observed in the GC × GC plane. The clusters corresponded to oxides, aldehydes, terpendiols, and esters. The monoterpenols were also separated into two bands corresponding to tertiary monoterpenols (aliphatic and aromatic), such as 2,6-dimethyl-1,7-octadien-3-ol, linalool, hotrienol, plinol C, ocimenol, and *p*-menthan-1-ol and primary monoterpenols (aliphatic and aromatic), namely, γ-isogeraniol, citronellol, lilac alcohol D, myrtenol, nerol, and geraniol. Geranic acid, which was the only one acid detected, occurred separated from all other compounds. Being very polar, this compound was more retained on the second dimension column. Therefore, it was not eluted in its own modulation cycle, moving into the next one and appearing in the lower part of the contour plot. This effect is called "wrap around".

The application of a second dimension that separated the compounds according to their polarity increased the GC chromatographic space and enhanced the separation potential. An example regarding the practical usefulness of the second dimension is shown in Fig. 2: β-phellandrene (peak 10) ($^1Dtr = 2240$ s; $^2Dtr = 2.456$ s) and β-ocimene (peak 11) ($^1Dtr = 2240$ s; $^2Dtr = 2.560$ s) were placed on the same vertical line, i.e. co-elute on the Equity-5 column. They were also lined with another not yet identified compound. However, these compounds exhibited different polarities and, therefore, were separated on the Supelcowax-10 column in the second dimension. Other pairs of compounds that were vertically lined were borneol/*p*-cymen-8-ol ($^1Dtr = 2572$ s), lilac alcohol/myrtenol ($^1Dtr = 2624$ s), and isobornyl acetate/*E*-ethyl geranate ($^1Dtr = 2832$ s).

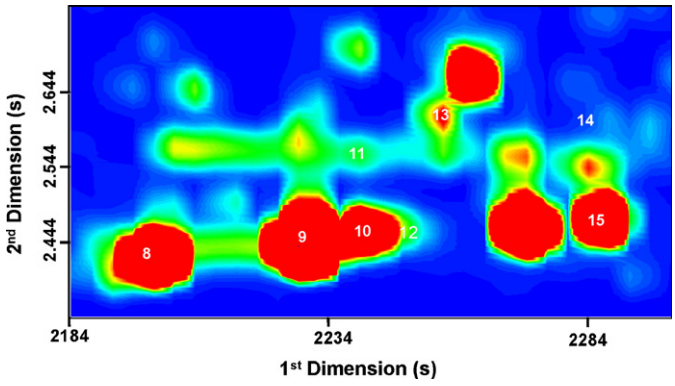

Fig. 2. Blow-up of part of the GC × GC extracted ion chromatogram contour plot of *m/z* 93, 121 and 136 in Fig. 1 (attribution of peak numbers shown in Table 1).

4. Concluding remarks

The grape samples (*Vitis vinifera* L. var. 'Fernão-Pires') were found to contain 56 monoterpenoids identified by GC × GC–ToF-MS. According to the data available in the literature, from these 56 monoterpenoids, 20 compounds were identified for the first time in grapes; some of them have been reported as having interesting aroma properties and biological activities. As this manuscript not comprises any quantitative approach, it is not possible to evaluate the real contribution of these novel-grape components, however this data open several research possibilities.

The GC × GC–ToF-MS analysis allowed the detection of higher number of compounds in comparison with those detected by GC–qMS mode, which was due to the: (i) enhanced separation by GC × GC technique (ii), better sensitivity of ToF-MS in full mass range acquisition, and (iii) utilization of automated peak finding and deconvolution algorithm. Furthermore, the use of specific *m/z* ions and the establishment of GC × GC chromatographic space that comprises the monoterpenoids reduced the complexity and the time of analysis. This represents a helpful approach for the establishment of the monoterpenic profile of the grapes. The combination of the first with the second dimension allowed the formation of clusters and sub-clusters within the monoterpenoids: the monoterpene hydrocarbons and the monoterpene oxygen-containing compounds represented the two main clusters. However, sub-clusters were also observed within the monoterpene oxygen-containing compounds; oxides, alcohols (monoterpenols and monoterpendiols), aldehydes, esters, and ketones. This classification, based on the presence of ordered structures in the GC × GC chromatogram of structurally related compounds, represents a valuable approach for future studies, as the ordered-structure principle can be a helpful tool in the identification of compounds and establishment of the composition of samples. Mass spectral match factors can be used to evaluate the library search results. The additional database composed of the retention indices for monoterpenoids,

calculated in the bi-dimensional column set, can substantially improve the identification of the monoterpenoids.

In conclusion, this study proposes a methodology and provides data that can be applied to determine the monoterpenoid profile of grapes, and its extension to the analysis of musts and wines. As monoterpenoids are secondary metabolites whose synthesis is encoded by variety-related genes, the terpenoid profile may be used as a way to trace its varietal origin. Thus, a potential application of the proposed methodology is the classification of wines, according to their varietal or even geographical origin.

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References

[1] C.R. Strauss, B. Wilson, P.R. Gooley, P.J. Williams, in: T.H. Parliment, R. Croteua (Eds.), Role of monoterpenes in grape and wine flavor (ACS Symposium Series, No. 317), American Chemical Society, Washington, DC, 1986, p. 222.
[2] S. Rocha, P. Coutinho, A. Barros, M.A. Coimbra, I. Delgadillo, A.D. Cardoso, J. Agric. Food Chem. 48 (2000) 4802.
[3] A. Rapp, Nahrung 42 (1998) 351.
[4] L.C. Vázquez, M.S. Pérez-Coello, M.D. Cabezudo, Anal. Chim. Acta 458 (2002) 39.
[5] M. Fernández-González, R. Di Stefano, Lebensm.-Wiss. Technol. 37 (2004) 467.
[6] J.J. Mateo, M. Jiménez, J. Chromatogr. A 881 (2000) 557.
[7] I. Marais, S. Afr. J. Enol. Vitic. 4 (1983) 49.
[8] H.-D. Belitz, W. Grosch, P. Schieberle, Food Chemistry, 3rd ed., Springer, Berlin, Heidelberg, 2004.
[9] P.J. Williams, C.R. Strauss, B. Wilson, J. Agric. Food Chem. 28 (1980) 766.
[10] F. Luan, D. Hampel, A. Mosandl, M. Wüst, J. Agric. Food Chem. 52 (2004) 2036.
[11] E. Coelho, S.M. Rocha, I. Delgadillo, M.A. Coimbra, Anal. Chim. Acta 563 (2005) 204.
[12] E. Sánchez-Palomo, M.C. Díaz-Maroto, M.S. Pérez-Coello, Talanta 66 (2005) 1152.
[13] M.R. Salinas, A. Zalacain, F. Pardo, G.L. Alonso, J. Agric. Food Chem. 52 (2004) 4821.
[14] P.Q. Tranchida, P. Dugo, G. Dugo, L. Mondello, J. Chromatogr. A 1054 (2004) 3.
[15] T. Górecki, O. Panić, N. Oldridge, J. Liq. Chromatogr. Rel. Technol. 29 (2006) 1077.
[16] J. Zrostlíková, J. Hajšlová, T. Čajka, J. Chromatogr. A 1019 (2003) 173.
[17] P.J. Marriott, T. Massil, H. Hűgel, J. Sep. Sci. 27 (2004) 1273.
[18] M. Adahchour, L.L.P. van Stee, J. Beens, R.J.J. Vreuls, M.A. Batenburg, U.A.Th. Brinkman, J. Chromatogr. A 1019 (2003) 157.
[19] A. Williams, D. Ryan, A.O. Guasca, P. Marriott, E. Pang, J. Chromatogr. B 817 (2005) 97.
[20] D. Ryan, P. Watkins, J. Smith, M. Allen, P. Marriott, J. Sep. Sci. 28 (2005) 1075.
[21] Eight Peak Index of Mass Spectra, The Mass Spectra Data Centre, Nottingham, UK, 2nd ed., 1974.
[22] S.M. Rocha, P. Coutinho, I. Delgadillo, A. Dias Cardoso, M.A. Coimbra, J. Sci. Food Agric. 85 (2005) 199.
[23] S.M. Rocha, P. Coutinho, A. Barros, I. Delgadillo, M.A. Coimbra, J. Sci. Food Agric. 87 (2007) 1667.
[24] H. van den Dool, P.D. Kratz, J. Chromatogr. 11 (1963) 463.
[25] D.J. Caven-Quantrill, A.J. Buglass, J. Chromatogr. A 1117 (2006) 121.
[26] G.K. Skouroumounis, M.A. Sefton, J. Agric. Food Chem. 48 (2000) 2033.
[27] S.C. Diéguez, L.C. Lois, E.F. Gómez, M.L.G. de la Peña, Lebensm.-Wiss. u.- Technol. 36 (2003) 585.
[28] M.J. Ibarz, V. Ferreira, P. Hernández-Orte, N. Loscos, J. Cacho, J. Chromatogr. A 1116 (2006) 217.
[29] Z.-G. Li, M.-R. Lee, De-L. Shen, Anal. Chim. Acta 576 (2006) 43.
[30] S. Dötterl, D. Burkhardt, B. Weißbecker, A. Jürgens, S. Schütz, A. Mosandl, J. Chromatogr. A 1113 (2006) 231.
[31] M. Kreck, A. Mosandl, Agric. Food Chem. 51 (2003) 2722.
[32] M.J. Jordán, R.M. Martínez, K.L. Goodner, E.A. Baldwin, J.A. Sotomayor, Ind. Crops Prod. 24 (2006) 253.
[33] C.D. Kanakis, P.A. Tarantilis, H.A. Tajmir-Riahi, M.G. Polissiou, J. Agric. Food Chem. 55 (2007) 970.
[34] M. Carmona, A. Zalacain, M.R. Salinas, G.L. Alonso, Crit. Rev. Food Sci. Nutr. 47 (2007) 145.
[35] J. Dallüge, L.L. van Stee, X. Xu, J. Williams, J. Beens, R.J.J. Vreuls, U.A.Th. Brinkman, J. Chromatogr. A 974 (2002) 169.
[36] M.Z. Özel, F. Göğüş, A.C. Lewis, Anal. Chim. Acta 566 (2006) 172.
[37] T.Y. Chung, J.P. Eiserich, T. Shibamoto, J. Agric. Food Chem. 41 (1993) 1693.
[38] Á. Högnadóttir, R.L. Rouseft, J. Chromatogr. A 998 (2003) 201.
[39] T.E. Acree, http://www.flavornet.org/d_kovats_db5.html, Cornell University, NYSAES, Geneva, NY, 2007.
[40] J. Liu, P. Nan, Q. Tsering, T. Tsering, Z. Bai, L. Wang, Z. Liu, Y. Zhong, Flavour Fragr. J. 21 (2006) 435.
[41] A. Gauvin, H. Lecomte, J. Smadja, Flavour Fragr. J. 19 (2004) 455.
[42] L. Jirovetz, G. Buchbauer, M.B. Ngassoum, M. Geissler, J. Chromatogr. A 976 (2002) 265.
[43] F. Juteau, V. Masotti, J.M. Bessiere, M. Dherbomez, J. Viano, Fitoterapia 73 (2002) 532.
[44] X. Xu, L.L.P. van Stee, J. Williams, J. Beens, M. Adahchour, R.J.J. Vreuls, U.A.Th. Brinkman, J. Lelieveld, Atmos. Chem. Phys. 3 (2003) 665.