Journal of Chromatography A, 1546 (2018) 36–45

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Dispersive liquid–liquid microextraction and gas chromatography accurate mass spectrometry for extraction and non-targeted profiling of volatile and semi-volatile compounds in grape marc distillates

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ARTICLE INFO

Article history:
Received 12 January 2018
Received in revised form 27 February 2018
Accepted 2 March 2018
Available online 3 March 2018

Keywords:
Volatile compounds profiling
Grape marc distillates
Dispersive liquid–liquid microextraction
Gas chromatography time-of-flight mass spectrometry

ABSTRACT

The suitability of dispersive liquid–liquid microextraction (DLLME) and gas chromatography accurate mass spectrometry (GC–MS), based on a time-of-flight (TOF) MS analyzer and using electron ionization (EI), for the characterization of volatile and semi-volatile profiles of grape marc distillates (grappa) are evaluated. DLLME conditions are optimized with a selection of compounds, from different chemical families, present in the distillate spirit. Under final working conditions, 2.5 mL of sample and 0.5 mL of organic solvents are consumed in the sample preparation process. The absolute extraction efficiencies ranged from 30 to 100%, depending on the compound. For the same sample volume, DLLME provided higher responses than solid-phase microextraction (SPME) for most of the model compounds. The GC-EI-TOF-MS records of grappa samples were processed using a data mining non-targeted search algorithm. In this way, chromatographic peaks and accurate EI-MS spectra of sample components were linked. The identities of more than 140 of these components are proposed from comparison of their accurate spectra with those in a low resolution EI-MS database, accurate masses of most intense fragment ions of known structure, and available chromatographic retention index. The use of chromatographic and spectral data, associated to the set of components mined from different grappa samples, for multivariate analysis purposes is also illustrated in the study.

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1. Introduction

Grape marc distillates (usually named as *grappa*) are alcoholic beverages elaborated in several European wine producing countries [1]. Production of quality distillates has a significant economic interest, as complementary activity to wine elaboration [1]. Grappa contains hundreds of volatile and semi-volatile species, either coming from seeds and peels of grape marc or generated during the fermentation of the pomace [2]. These compounds are responsible for the organoleptic properties, and thus the quality, of the spirits. In addition, they serve as markers of grape variety and maturation state at harvest, geographic origin, storage conditions of grape pomace and/or distillation techniques [3,4]. Most of grappa volatile components are amenable to gas chromatography (GC) analysis. In fact, the combination of GC with mass spectrometry (MS) is the preferred technique for their characterization [5,6].

Solid-phase microextraction (SPME) is the most utilized sample preparation technique for GC determination of volatile and semi-volatile compounds in distillate samples [7–9]. SPME conditions frequently involve the use of mixed-sorbents fibers and headspace (HS) extractions, at temperatures in the range between 25 and 55 °C, during a period of 15–60 min [5,7–9]. The applicability to a large number of compounds, the null consumption of organic solvents and automation easiness, if a dedicated SPME autosampler is available, are the principal advantages of the SPME technique [10]. However, less volatile and high water soluble compounds, still amenable to GC analysis, are difficult to extract in the HS SPME mode. Consequently, there is a demand of alternative sample preparation approaches enable to couple with the extraction of a wide set of volatile, semi-volatile, polar and non-polar

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https://doi.org/10.1016/j.chroma.2018.03.003
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organic compounds from distillates samples. Simplified methods combining low cost, reduced solvent consumption, high enrichment factors and sample throughput, without the need of dedicated instrumentation, have gained interest. Dispersive liquid–liquid microextraction (DLLME) fits most of the above premises being a technique in constant evolution, with an increasing number of application fields, from its introduction by Rezaee et al. [11]. DLLME has been used for the extraction of insecticides in honey liqueur [12] and, more recently, for the extraction of phthalate esters in distillates [13]. However, to the best of our knowledge, its suitability for profiling the volatile and semi-volatile compounds of distillate spirits has not been explored yet.

Chemical profiling of minor volatile and semi-volatile compounds in distillates, without a foregoing selection of the compounds of interest, is a characteristic example of non-targeted analysis. Responses for chromatographic entities can be used for geographic and botanic origin discrimination purposes [14,15]. The accurate mass and full scan high sensitivity features of time-of-flight (TOF) MS systems, following GC separation, have been demonstrated as exceptionally useful for target and non-targeted determination of a very broad range of minor compounds (natural products and contaminants) in complex matrices [10,16–20], with relevant advantages in terms of sensitivity and selectivity versus unit mass resolution techniques, such as GC quadrupole MS. In combination with electron ionization (EI), GC-TOF-MS provides characteristic fingerprints for any compound recovered from the spirits during sample preparation. The larger the number of compounds, the higher the latent information existing in the full scan GC-EI-TOF-MS records. On the other hand, managing such high number of chromatographic peaks (quite often partially overlapped) and ions requires the use of semi-automated software tools in order to drawn information of usefulness for characterization and/or discrimination of distillate samples.

The aim of this work was to investigate the suitability of the DLLME sample preparation technique, followed by GC-TOF-MS analysis, for the profiling of volatile and semi-volatile organic compounds in grappa distillate samples. Following optimization of DLLME conditions, the responses measured for a selection of compounds were compared to those provided by SPME, as an indirect indicative of the efficiency of the proposed extraction process. Thereafter, identification of major and minor compounds in the DLLME GC-TOF-MS records was carried out. Finally, a preliminary evaluation of chromatographic profiles to discriminate different distillates by using the *molecular features* extracted from samples is presented.

2. Experimental

2.1. Solvents and sorbents

Methanol and acetonitrile (HPLC grade) were obtained from Merck (Darmstadt, Germany). Pesticide grade acetone, chloroform ($CHCl_3$) and carbon tetrachloride (CCl_4) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium chloride (NaCl) was acquired from Merck. A standard of 4-decanol (97%) was purchased from Alfa Aesar (Kandel, Germany). This compound was employed as internal surrogate (IS), added to grappa distillates samples before starting the sample preparation process, in some of the performed extractions. A mixture of *n*-alkanes (C_8-C_{40}) in dichloromethane, provided by Supelco (Bellefonte, PA, USA), was used to calculate the linear retention index (LRI) of compounds in GC-TOF-MS records. Ultrapure water was obtained from a Milli-Q Gradient A-10 system (Millipore, Bedford, MA, USA).

A manual SPME holder and fibers coated with divinylbenzene-carboxen-poly (dimethylsiloxane) (DVB/CAR/PDMS, 50/30 μm film thickness, 1 cm length) and PDMS/DVB (65 μm film thickness, 1 cm length) were obtained from Supelco (Bellefone, PA, USA).

2.2. Samples and sample preparation

Grappa samples were either provided by wine makers from Galicia (North West Spain) or purchased in retail markets. Information regarding the grape variety and distillation technique (direct or steam distillation) was obtained from producers and/or labels on commercial samples. After reception, grappas were stored at room temperature, in capped glass bottles, protected from light with aluminum foil. Table S1 shows the features of the distillates used in the current research.

Under optimized conditions, DLLME extractions were performed in 10 mL volume, conical-shaped bottom glass tubes containing a 2.5 mL aliquot of grappa spiked with the IS (0.1 mL of a 10 μg mL^{-1} acetone solution), 6.5 mL of ultrapure water and 1 g of NaCl. Then, 0.5 mL of the extraction solution, consisting in 0.4 mL of acetonitrile and 0.1 mL of $CHCl_3$, was added using a gas tight syringe. Tubes were capped, shaken for 1 min and centrifuged for 5 min (room temperature) at 3500 rpm. After removing most of the upper aqueous phase, the $CHCl_3$ settled extract (0.047 ± 0.002 mL) was recovered and transferred to an insert for injection in the GC-QTOF-MS system.

SPME extractions were carried out in 20 mL volume glass vessels containing the same sample solution (2.5 mL of distillate, 6.5 mL of water and 1 g of NaCl) as DLLME tubes, plus a stir bar. Tubes were closed with a Teflon-lined septum and an aluminum cap. Extractions were carried at under different conditions (fiber coating, sampling mode and temperature) for 30 min. The SPME fibers were thermally desorbed in the hot splitless injector of the GC–MS system.

2.3. Determination conditions

Compounds were determined using a GC-QTOF-MS instrument, comprised of a 7890A gas chromatograph and a 7200 QTOF mass spectrometer, both acquired from Agilent (Wilmington, DE, USA). The QTOF system was equipped with an electron ionization (EI) source and operated in the single MS mode, at 2 GHz acquisition frequency. Accurate EI-MS scan spectra were recorded every 0.2 s (2715 transients per spectrum), in the centroid mode, between 40 and 650 *m*/*z* units. The mass axis of the TOF mass analyzer was re-calibrated every three injections using a commercial solution of perfluorotributylamine (PFTBA). Under the above conditions, mass resolution varied from 4600, at *m*/*z* 69, to 9100, at *m*/*z* 414. The transfer line and the EI source were set at 250 and 230 °C, respectively. The *Mass Hunter* software (B.08 version, Agilent) was employed to control all instrumental parameters in the GC-TOF-MS. The *Find by Molecular Feature (FMF)* function, integrated in the above software package, was used to mine the different *molecular features* (species) from raw GC-TOF-MS records.

Compounds were separated in a DB-WAXETR column (30m × 0.25 mm i.d., 0.5 μm Carbowax-type film thickness) acquired from Agilent. Helium was used as carrier gas at a constant flow of 1.2 mL min^{-1}. The column temperature was programed as follows: 60 °C (1 min), rated at 4 °C min^{-1} to 220 °C, and finally at 20 °C min^{-1} to 240 °C with a hold time of 5 min. The total analysis time was 47 min. Injections (2 μL) were made in the splitless mode with the injection chamber set at 260 °C. The splitless time and the split flow were 1 min and 60 mL min^{-1}, respectively. The solvent delay was fixed at 5.5 min. One injection of organic solvent ($CHCl_3$) was made between replicates of samples to prevent column contamination with low volatile species. SPME fibers were desorbed for 2 min, with the injection chamber maintained at 260 °C.

Table 1
Summary of model compounds considered during optimization of DLLME conditions.

Compound.	Molecular formula	CAS number	Chemical class	Retention time (min)	Quantification ion (*m*/*z*)	Log K_{ow}
Isoamyl acetate	$C_7H_{14}O_2$	123-92-2	Ester	6.84	55.0560	1.53
Isoamyl alcohol	$C_5H_{12}O$	123-51-3	Alcohol	9.01	70.0782	1.09
Ethyl hexanoate	$C_8H_{16}O_2$	123-66-0	Ester	9.62	88.0534	2.31
2-Furaldehyde	$C_5H_4O_2$	98-01-1	Aldehyde	16.77	95.0130	0.83
Benzaldehyde	C_7H_6O	100-52-7	Benzene derivative	18.59	105.0339	1.69
Isoamyl octanoate	$C_{13}H_{26}O_2$	2035-99-6	Ester	22.21	70.0794	4.46
Alpha-terpineol	$C_{10}H_{18}O$	98-55-5	Monoterpene	23.49	93.0704	2.20
1,1,5-Trimethyl-1,2-dihydronaphthalene (TDN)	$C_{13}H_{16}$	30364-38-6	C13-Norisoprenoid	24.87	157.1016	4.25
Methyl salicylate	$C_8H_8O_3$	119-36-8	Benzene derivative	25.85	120.0222	2.32
Ethyl phenyl acetate	$C_{10}H_{12}O_2$	101-97-3	Benzene derivative	25.98	91.0555	2.11
Benzyl alcohol	C_7H_8O	100-51-6	Benzene derivative	28.55	108.0575	1.21
Butanedioic acid, ethyl isoamyl	$C_8H_{14}O_4$	28024-16-0	Ester	28.87	101.0231	1.86
Phenyl ethyl alcohol	$C_8H_{10}O$	60-12-8	Alcohol	29.48	91.0553	1.49
Beta-ionone	$C_{13}H_{20}O$	14901-07-6	C13-Norisoprenoid	29.95	177.1279	3.28
4-Ethylguaiacol	$C_9H_{12}O_2$	2785-89-9	Phenol	32.46	137.0593	2.47
Eugenol	$C_{10}H_{12}O_2$	97-53-0	Phenol	35.76	164.0829	2.61
4-Ethylphenol	$C_8H_{10}O$	123-07-9	Phenol	36.04	107.0489	2.63

2.4. Compounds identification

A non-targeted search of compounds existing in the GC-EI-TOF-MS chromatograms of distillates was carried out using the FMF function. This algorithm groups those ions appearing at the same retention time, considering also that mass differences among them are compatible with a given empirical formula. The software reports a list of molecular entities for which retention time, deconvoluted spectrum and chromatographic signal intensity (area or height) are available. Spectra from molecular entities can be compared with any available EI-MS database. In our case, the low resolution NIST 2017 library was used.

The *Mass Profiler Professional* (version 14.8) software, provided by Agilent, was employed to manage the information (spectra, retention times and response intensities) corresponding to compounds mined from GC-TOF-MS records. This software integrates different chemometric tools for the treatment of data associated to molecular entities extracted from raw GC-EI-TOF-MS records, either as non-identified species (the option used in this study) or after confirming the identities of these compounds.

3. Results and discussion

3.1. Optimization of DLLME conditions

Grappa is a complex matrix with hundreds of compounds amenable to GC separation, but displaying different behaviors during sample extraction. In order to obtain a combination of experimental variables which provide the highest possible extraction yields for most sample components, a group of 17 compounds with different chemical functionalities (aldehydes, ketones, esters, alcohols and alkanes), structures (saturated, unsaturated, aromatic and polycyclic species), volatilities and polarities, as well as displaying different response ranges in distillates, was selected. Most of them have been employed in previous studies dealing with characterization of grappa distillates [9].The list of compounds, together with the employed quantification ions, their octanol-water (Log K_{ow}) partition coefficients and retention times (RT) are compiled in Table 1.Retention times ranged from 7 to 36 min and Log K_{ow} from 0.8 to 4.3. Selective responses for this set of compounds was obtained considering a *m*/*z* window of 0.010 Da centered in the *m*/*z* values of their quantification ions. Then, the influence of different DLLME parameters on their extraction efficiency was evaluated. Optimization experiments were performed using aliquots from a pooled sample of different grappas. Unless otherwise stated, extraction conditions were optimized using a univariate approach. The preliminary evaluation of the repeatability of the analytical procedure (sample extraction plus GC-QTOF-MS determination, n = 6 replicates) showed RSDs in the range from 2% to 16%, without using IS correction. The standard deviations of *m*/*z* values for their quantification ions (Table 1) remained below 0.005 Da.

3.1.1. Selection of extractant and disperser solvents

$CHCl_3$ and CCl_4 were evaluated as extractant solvents due to their short retention times in capillary columns considering also previous DLLME applications, and the easiness of phases separation after compounds extraction [10,11,21]. Less volatile solvents, such as toluene 1-octanol or 1-undecanol, also used in previous DLLME applications [22], were not investigated because of their potential co-elution with distillate components. Fig. 1 shows the responses of target compounds obtained with the above extractants (0.1 mL of $CHCl_3$ or CCl_4 combined with 0.9 mL of acetone as dispersant). Depicted data are normalized to those measured for $CHCl_3$, without any correction for differences between the volumes of the final extract. As observed, $CHCl_3$ rendered the highest responses for all the model compounds, with statistically significant differences (95% confidence level) except for eugenol. Thus, this solvent was selected as extractant.

Methanol, acetone and acetonitrile were evaluated as disperser solvents by the addition of 1 mL of the binary mixture, consisted in 0.90 mL of each disperser and 0.1 mL of $CHCl_3$, to diluted spirit samples. The results showed significant differences depending on the experimental conditions, for most of the compounds (Fig. S1). Globally, acetonitrile achieved equivalent (isoamyl alcohol, alpha-terpinol, beta-ionone and 4-ethylphenol) of higher responses (rest of model compounds) than acetone. On the other hand, five of the compounds presented much lower signals when methanol was employed as dispersant. So, acetonitrile was used as dispersant solvent in further experiments.

3.1.2. Selection of sample volume

DLLME experiments were performed (n = 3) introducing different volumes of sample (from 0.5 to 2.5 mL) in the glass extraction tubes. Then, the required volume of ultrapure water was added to made up the aqueous phase to 9 mL. The higher the sample intake, the larger the concentration of aroma compounds in the extraction solution. On the other hand, the percentage of ethanol (42% in the undiluted pooled distillate) also increased reducing the affinity of the compounds by the chlorinated solvent. Fig. S2 summarizes the obtained results as relative responses normalized to those measured for the 2.5 mL sample. Linear increase in the responses measured for selected compounds with the sample intake were observed. The only exception was the isoamyl alcohol, which showed better results for 2 mL of sample. Thus, taking into

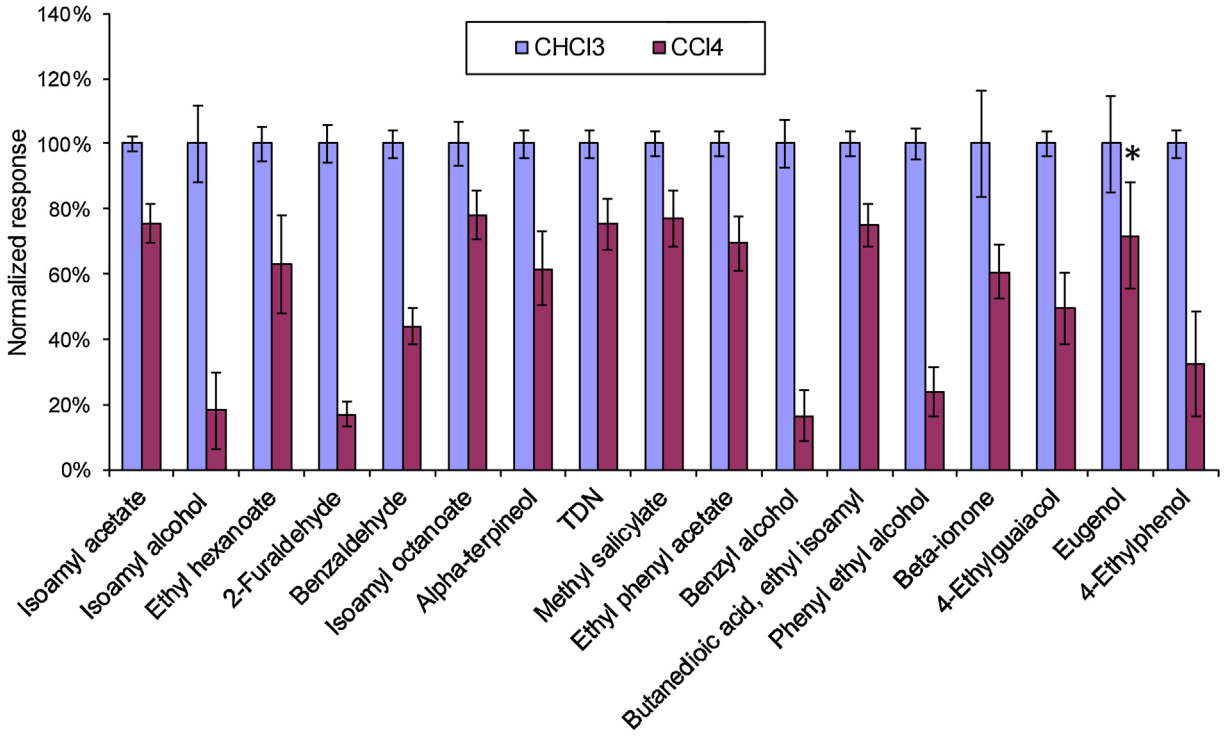


Fig. 1. Effect of the extractant solvent in the performance of DLLME. Normalized responses to $CHCl_3$, n = 3 replicates. Compounds marked with asterisk present non-significant changes in their extraction efficiency (95% confidence level).

account the achieved results and with the aim to attain a higher sensitivity for the majority of compounds, 2.5 mL sample volume was selected for further studies.

3.1.3. Ionic strength effect

The addition of NaCl is a common practice to enhance the extractability of high and medium polarity, non-ionic, compounds from aqueous solutions in organic solvents. On the other hand, less polar analytes might suffer a significant reduction in their extraction efficiencies due to slower mass transfer kinetics, related to the increased viscosity of the sample. In the specific case of DLLME, the increase in the ionic strength of the aqueous sample also reduces the solubility of the extractant solvent, slightly increasing the volume of the recovered phase.

The effect of NaCl in the responses of compounds compiled in Table 1 was investigated at three different levels (0, 0.50 and 1.0 g). Fig. 2 shows the obtained responses, as normalized values to those measured for 0 g NaCl. Six of the most polar selected compounds, particularly those with an alcohol functionality, undergo considerable increases (i.e. more than 100% for isoamyl alcohol) in analytical responses when NaCl amount was increased up to 1.0 g. For a second group of compounds, with a more lipophilic behavior (i.e. ethyl hexanoate, TDN, etc.) a steady decrease of their responses with the amount of NaCl was observed. Finally, responses of alpha-terpineol and 4-ethylguaiacol remained practically unaffected.

In general, polar compounds (which were positively affected by the addition of sodium chloride) displayed relative low chromatographic responses in comparison to more lipophilic species

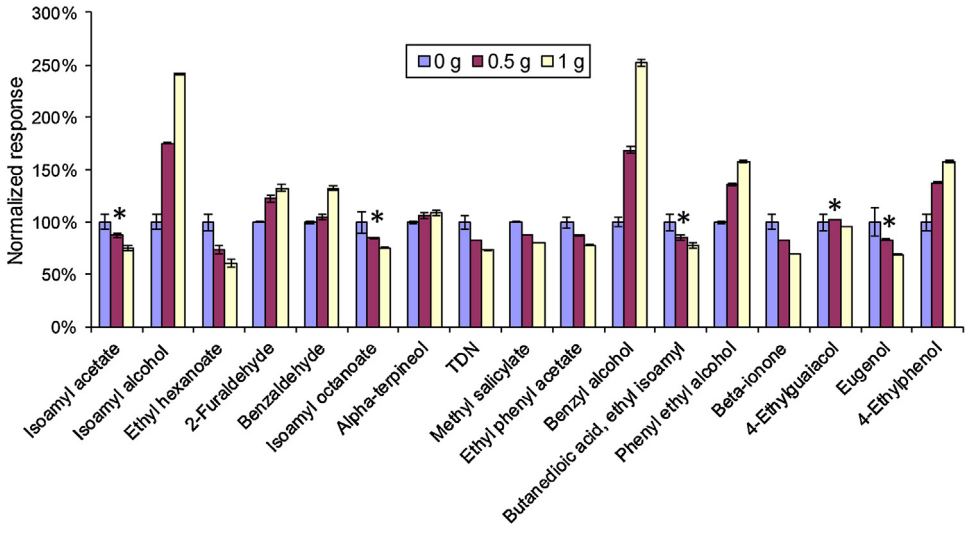


Fig. 2. Influence of NaCl addition in the efficiency of DLLME extraction, n = 3 replicates. An asterisk is employed to indicate when extraction yields are similar (95% confidence level) to those attained without NaCl addition.

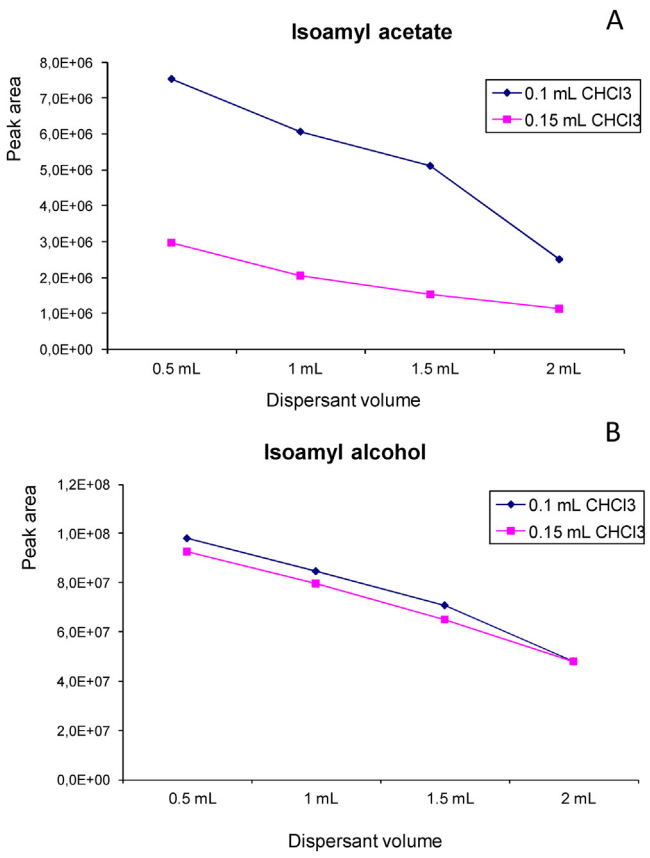


Fig. 3. Comparison of responses (peak areas) as function of dispersant (acetonitrile) and extractant ($CHCl_3$) solvent volumes. Average values for duplicate extractions.

present in the distillates, such as esters. Thus, further extractions were performed adding 1 g of NaCl to diluted grappa samples.

3.1.4. Selection of dispersant and extractant volumes

The effects of both parameters in the yield of the extraction were evaluated simultaneously. To this end, four different volumes of acetonitrile (from 0.5, 1.0, 1.5 and 2 mL) were combined with two different volumes of $CHCl_3$ (0.1 and 0.15 mL). For the lowest dispersant level, the volume of the settled chloroform phase accounted for 47 and 101 microliters, respectively. These volumes decreased in a 10% extend when the dispersant volume rise up to 2 mL.

Whatever the extractant amount, increasing the dispersant volume turned in lower responses since the solubility of the compounds in the hydro-alcoholic phase increases. Regarding the amount of extractant, two different trends were noticed. For most compounds, increasing the extractant volume led to a reduction in their responses (as a consequence of a more diluted extract) as illustrated in Fig. 3A for isoamyl acetate. On the other hand, a few compounds (i.e. isoamyl alcohol) presented very similar responses for the two volumes of $CHCl_3$, Fig. 3B. In this case, the extra dilution of the CCl_3H extract is balanced with the increase in the yield of the DLLME extraction Taking into account the above comments, 0.5 mL of the extraction solution, consisted of 0.4 mL of acetonitrile and 0.1 mL of $CHCl_3$, was selected.

3.1.5. Extraction and centrifugation time

The first variable was evaluated at different levels from 1 to 5 min, and the second one between 3 and 15 min. In all the experiments, the closed extraction tubes were centrifuged at 3500 rpm. None of the above parameters affected the DLLME efficiency (data not shown), thus extraction and centrifugation times were maintained at the lowest evaluated levels: 1 and 3 min, respectively

3.2. Efficiency of DLLME sample preparation

The extraction efficiency (EE, %) of the DLLME process was evaluated following an indirect method, using liquid–liquid extraction (LLE) of aqueous solutions of grappa distillate (2.5 mL of distillate, 6.5 mL of water and 1 g of NaCl) with 2 mL $CHCl_3$. EEs were calculated as: EE(%) = (Ans – Aes)/Ans)*100, being Aes and Ans the responses for each compound in the LLE extracts from hydro-alcoholic solutions previously submitted (Aes), and not submitted (Ans) to DLLME concentration, respectively. EEs (%) varied between 31 and 100%, with 11 out of 17 species presenting EEs above 86%, Table 2. Considering a volume of distillate of 2.5 mL and 0.047 mL as the average volume of the settled $CHCl_3$ phase, enrichment factors up to 52 times were obtained, Table 2.

In order to obtain a further evidence of the concentration capabilities of the developed DLLME methodology, responses for the set of model compounds were compared with those obtained using SPME extraction. Following previous literature methods [9,23,24], SPME extractions were performed under three different conditions: direct SPME at room temperature (20 °C), HS SPME at room temperature and HS SPME with samples thermostated at 50 °C. Obtained results (peak area for each compound, n = 3 replicates) were normalized to those achieved by DLLME under optimized conditions. Relative efficiencies provided by the SPME technique varied depending on the considered compound and the experimental SPME conditions, Table 2. TDN was systematically better extracted by SPME. On the other hand, whatever the tested SPME conditions, 12 out of 17 compounds rendered relative SPME responses below 100%. So, they are better extracted with the DLLME technique. The extraction repeatability (see SD values in Table 2) was similar for both techniques.

The obvious limitations of the DLLME technique are (1) the use of chlorinated solvents in the extraction process and (2) the automation difficulties. On the other hand, DLLME offered a much higher sample throughput since many samples can be simultaneously extracted; moreover, extracts can be handled with a conventional GC autosampler for liquid samples.

3.3. Profiling of volatile compounds in grappa distillates

GC-EI-TOF-MS records of grappa distillates contain thousands of ions (mostly fragment ions) belonging to hundreds of compounds, displaying a broad range of signal intensities and, quite often, overlapped peaks. Thus, the use of automated data mining strategies is compulsory for the characterization of compounds appearing in different samples, and for the further comparison of these samples, following a metabolomics-like approach. The workflow proposed to reach both targets is depicted in Fig. 4.

3.3.1. Mining and identification of distillate components

The FMF algoritm was used to assign a chromatographic signal (retention time and intensity) and an accurate MS spectrum to each species (component) in the GC-EI-QTOF-MS chromatograms. Threshold values of 0.05% and 5% (as relative intensities to the highest peak in each chromatogram and to the base ion in the spectrum of each compound, respectively) were set. To prevent bias of m/z ratios of fragment ions due to too weak or too intense (saturating) peaks, it is important to define the regions where the spectral information is obtained. For non-saturating peaks, the average spectrum in the region above 20% of the apex was used. For saturating species, the spectrum was averaged in the regions below 20% of saturation.

Around 200–250 components were found depending on the grappa sample. Fig. 5A and B show the raw chromatographic data

Table 2
Calculated extraction efficiencies (EEs, %) and enrichment factors (EFs) of the optimized DLLME method. SPME relative efficiencies versus DLLME extraction. Values for n = 3 replicates.

Compound	DLLME performance		[a]SPME relative extraction efficiencies (%) ± SD					
	EEs (%) ± SD	[b]EFs	Direct SPME PDMS/DVB	Direct SPME DVB/CAR/PDMS	HS SPME PDMS/DVB	HS SPME DVB/CAR/PDMS	HS SPME,50 °C, PDMS/DVB	HS SPME, 50 °C, DVB/CAR/PDMS
Isoamyl acetate	95 ± 8	49	12 ± 1	8.5 ± 0.2	27 ± 3	29 ± 3	15 ± 4	7.3 ± 0.2
Isoamyl alcohol	38 ± 1	20	1.0 ± 0.1	1.4 ± 0.1	1.2 ± 0.1	1.3 ± 0.2	1.3 ± 0.2	2.6 ± 0.2
Ethyl hexanoate	100 ± 14	52	18 ± 1	24 ± 2	21 ± 2	28 ± 5	8 ± 1	10 ± 3
2-Furaldehyde	32 ± 1	17	4.3 ± 0.1	74 ± 3	4.6 ± 0.3	53 ± 2	2.7 ± 0.1	45 ± 2
Benzaldehyde	72 ± 2	37	71 ± 6	171 ± 6	74 ± 1	157 ± 11	49 ± 1	93 ± 1
Isoamyl octanoate	100 ± 3	52	46 ± 2	57 ± 3	65 ± 3	85 ± 2	116 ± 1	131 ± 5
Alpha-terpineol	91 ± 2	47	11 ± 1	14 ± 1	5.4 ± 0.6	6.4 ± 0.4	70 ± 6	80 ± 1
1,1,5-Trimethyl-1,2-dihydronaphthalene (TDN)	100 ± 5	52	112 ± 3	140 ± 21	201 ± 10	332 ± 15	300 ± 22	355 ± 23
Methyl salicylate	95 ± 2	49	58 ± 3	142 ± 3	3 5± 2	46 ± 2	21 ± 1	65 ± 5
Ethyl phenyl acetate	97 ± 3	50	35 ± 1	55 ± 5	14 ± 1	19 ± 2	12 ± 1	17 ± 1
Benzyl alcohol	31 ± 3	16	8 ± 1	21 ± 2	5.3 ± 0.4	8.2 ± 0.2	2.6 ± 0.3	9.7 ± 0.5
Butanedioic acid, ethyl isoamyl	100 ± 2	52	29 ± 1	37 ± 4	3.3 ± 0.1	3.3 ± 0.2	11 ± 1	12 ± 1
Phenyl ethyl alcohol	42 ± 3	22	8.3 ± 0.3	17 ± 1	4.1 ± 0.1	6.1 ± 0.3	4.5 ± 0.3	6 ± 1
Beta-ionone	100 ± 5	52	80 ± 2	127 ± 5	14.3 ± 0.5	127 ± 7	48 ± 1	53 ± 2
4-Ethylguaiacol	86 ± 5	45	16 ± 1	31 ± 2	3.3 ± 0.2	4.3 ± 0.5	7.2 ± 0.3	12 ± 2
Eugenol	100 ± 8	52	13 ± 1	15 ± 2	2.1 ± 0.1	3.6 ± 0.2	37 ± 6	31 ± 5
4-Ethyl phenol	74 ± 9	38	19 ± 1	37 ± 5	4.3 ± 0.4	4.3 ± 0.2	7.1 ± 0.1	10 ± 1

[a] Relative responses provided by SPME, under investigated conditions, versus those attained by DLLME.
[b] Average EFs considering 2.5 mL of sample intake and 0.047 mL as the volume of the settle chloroform extract.

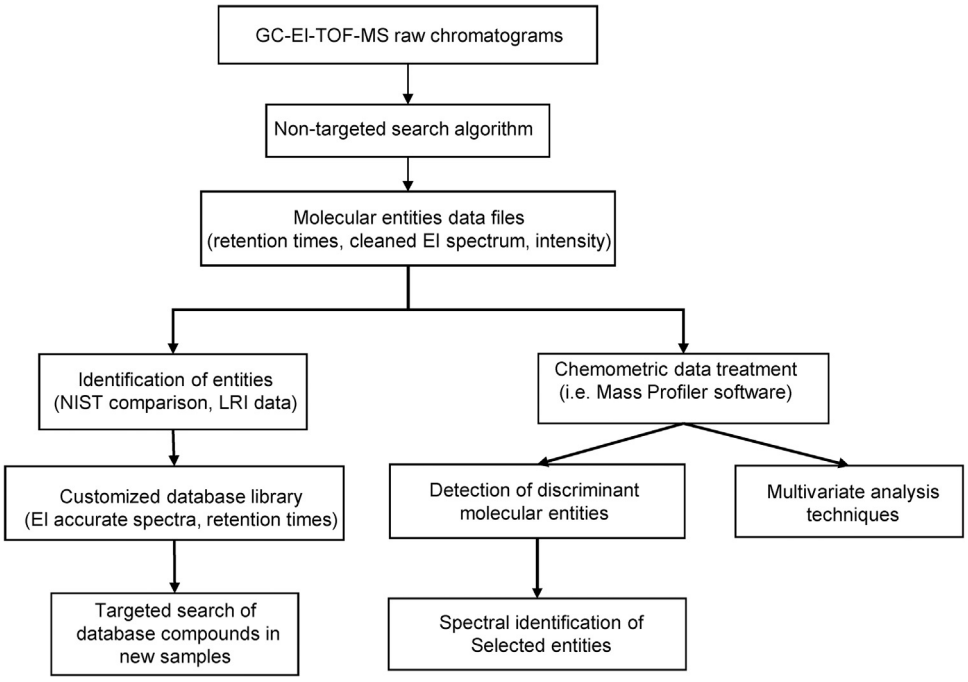


Fig. 4. Workflow scheme followed for data mining, and samples characterization, from GC-EI-TOF-MS raw chromatograms.

and the signals of the *molecular features* (11 components) mined in a given region of the chromatogram (c.a. 0.5 min) The spectrum assigned to one of these entities is shown in Fig. 5C. As appreciated in Fig. 5D, the raw spectrum in the region of this compound contains ions from different species; thus, the latter is of little interest for sample characterization/classification purposes.

The identities of compounds isolated in the previous step were proposed from comparison with the NIST low −resolution database, Fig. 4. Several requirements were established for a tentative identification [16]. First, the match between the low resolution spectrum of the candidate species, in the NIST library, and the accurate experimental one must stay above 80%. Second, *m*/*z* values for, at least, three intense ions in the experimental spectrum fit the calculated ones for fragments, with a known empirical formula, in the low resolution spectrum of the candidate, within a maximum error of 10 mDa. Third, when available, the LRI values of *molecular features* and candidate compounds, using the same or an equivalent coating GC column, must be coherent. Fig. 5E shows the low resolution NIST spectrum of benzyl alcohol, with calculated *m*/*z* values of several fragments. The difference with values in experimental spectrum assigned to this species (Fig. 4C) remained below 2 mDa.

Taking into account the above criteria, the identities of more than 140 compounds were tentatively assigned. Table S2 summarizes some relevant chromatographic and spectral data for these

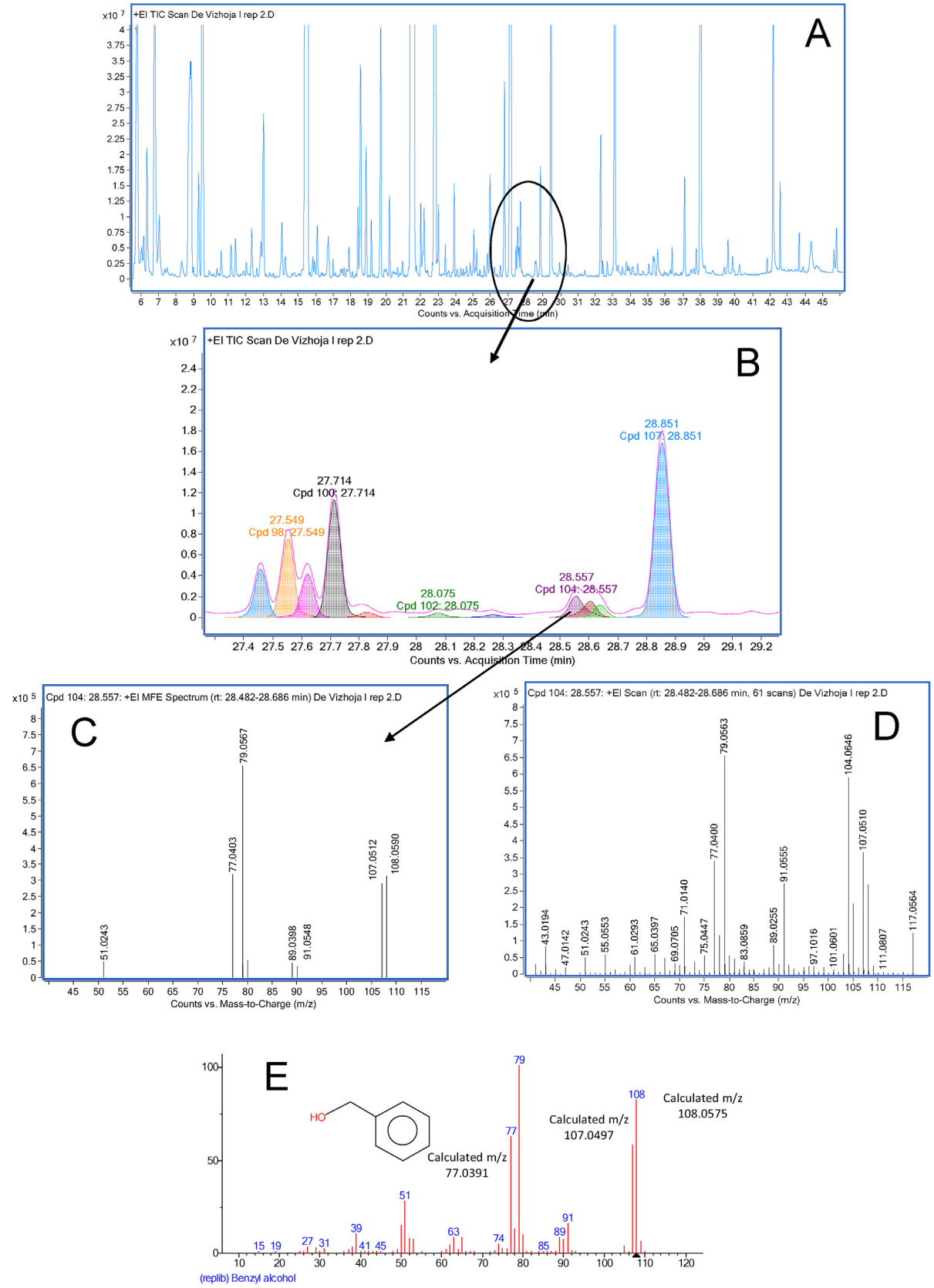
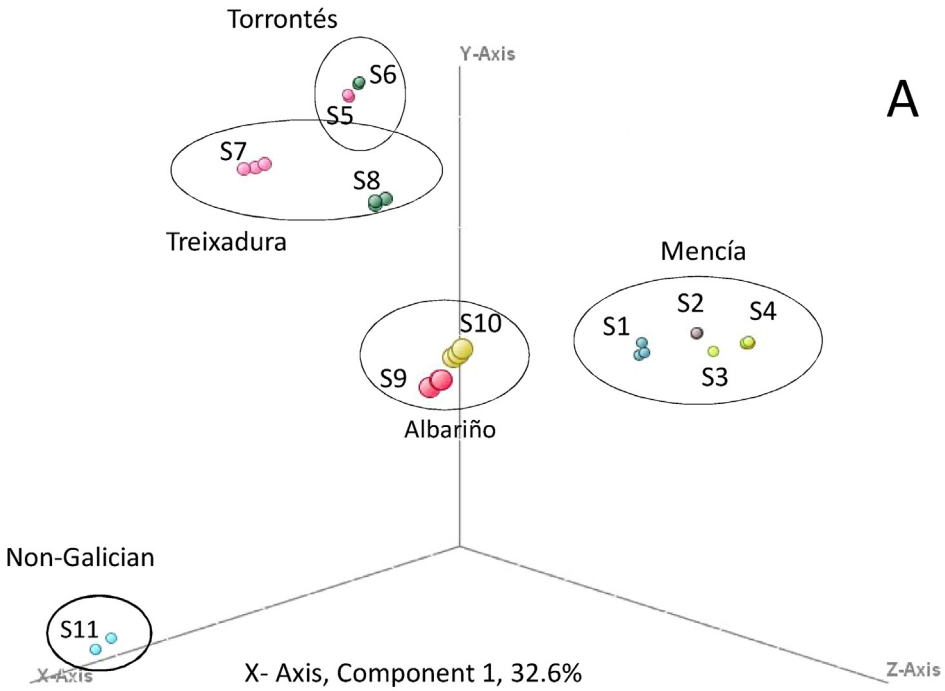


Fig. 5. A, raw GC-EI-TOF-MS spectra of a grappa sample. B, detail of *molecular features* (components) mined from the raw chromatogram. C, cleaned spectrum of the compound at retention time 28.55 min. D, raw spectra at the same retention time. E, NIST spectrum of benzyl alcohol with calculated *m*/*z* values of known fragment ions.



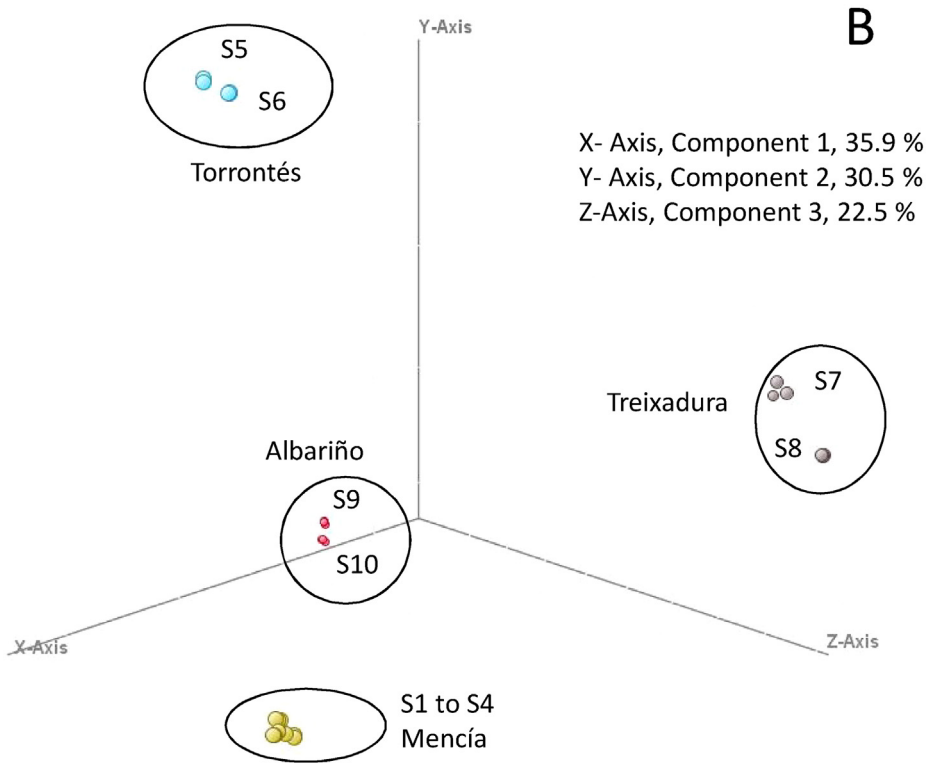


Fig. 6. PCA plots of grappa samples obtained from non-targeted data mined compounds. Each sample processed in triplicate. A, plot for the set of 11 grappa samples. B, PCA plot obtained after removing the grappa sample not produced using Galician grape marc varieties.

compounds. The largest group (c.a. 60 species) corresponds to free carboxylic acids and their esters with the major alcohols formed during fermentation of grape pomace. The list of acetals, alcohols, aldehydes and ketones, which are directly correlated with the aroma of distillates is also relevant. Likewise, more than 30 terpenes (from monoterpenes to sesquiterpenes) and 18 aromatic (benzene derivatives), which have been correlated with grape variety, are also identified [2,9,25], Table S2. Obviously, final identification of these compounds requires confirmation against authentic standards (in this research just the identities of around 20 compounds, highlighted in bold in Table S2, was confirmed). After this step, a customized database library, with retention times and accurate EI-MS spectra, can be created with the *Mass Hunter* software. Thereafter, this database will allow their target search in new samples [26].

3.3.2. Characterization of grappa samples from deconvoluted components

The workflow in Fig. 4 also shows the use of deconvoluted component data for the characterization of grappa samples. To this end, the *Mass Profiler Professional (MPP)* software was used to align compounds from different chromatographic injections, and to compare the fragment ions in their deconvoluted spectra. The maximum variations for retention time and m/z values were set at 0.1 and 10 mDa, respectively. After peak alignment, the same software permits to see changes in the responses of each compound among the different considered samples, which might be useful to detect discriminating species and/or to detect undesirable components, from the point of view of sensorial properties, in a particular sample, figure not shown.

Data of aligned components can be processed using multivariate chemometric tools (integrated in the above software package) useful for exploration and/or classification purposes. As example, the principal component analysis (PCA) plot corresponding to the processed grappas (11 different samples, extracted and analysed in triplicate) is shown in Fig. 6. For PCA analysis, responses for components mined in every triplicate of each sample were first divided by that of the IS and then, normalized to the average component response in each chromatogram. A few components, with large, saturating chromatographic peaks (i.e. the ethyl esters of C8, C10 and C12 carboxylic acids) were not employed for the PCA analysis, which was based on around 170 species.

When considering the 11 different distillates, the sample from Cantabria (code S11, Table S1) was well separated from those elaborated with different Galician grape varieties (codes S1 to S10), Fig. 6A. On the other hand, samples from Torrontés (S5 and S6) and Treixadura (S7 and S8) varieties are poorly discriminated, Fig. 6A. After repeating the PCA analysis with the 10 samples from Galicia, a clear separation among the four different grape varieties (Torrontés, Albariño, Treixadura and Albariño) was observed, Fig. 6B. Obviously, the number of distillates, from each of the four grape marc Galician varieties, used in the current study is too low for a reliable PCA study; however, the workflow and the projections depicted in Figs. 4 and 6, respectively, serve to illustrate the way to handle the accurate spectral data together with retention times of deconvoluted components for chemometric studies.

4. Conclusions

DLLME offers interesting features for the extraction of volatile and semi-volatile compounds from grape marc distillates such as low solvent consumption, fast extraction times, compatibility with GC analysis and acceptable yields for a large range of species from different chemical families. The combination of data mining strategies with the unique fingerprint information contained in the GC accurate EI-MS records of distillates permitted the extraction of a set of valuable data (retention time, intensity and fragment ions) to feed metabolomics software, aiming to classify and/or to discriminate distillate samples. Independently of the sample preparation technique, such methodology is of inherent interest in the spirit industry, and in any other field where GC amenable compounds can be useful for characterization purposes (i.e. alcoholic and non-alcoholic beverages, food and cosmetic industry). Given the universal acceptance of EI for ionization of volatile and semi-volatile species in GC–MS, the development of accurate EI-MS spectral libraries is required to fully exploit the potential offered by GC hyphenated to EI accurate MS.

Declarations of interest

None.

Acknowledgments

Financial support from Xunta de Galicia, Spanish Government and FEDER funds (projects GRC-ED431C 2017/36 and CTQ2015-68660-P) is acknowledged. A.R.F. thanks the external postdoctoral fellowship to CONICET, Argentina

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.chroma.2018.03.003.

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