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Development and validation of an ultra high performance liquid chromatography-tandem mass spectrometry method for the determination of phthalate esters in Greek grape marc spirits

Dimitra Diamantidou[a,b,c], Olga Begou[a,b,c], Georgios Theodoridis[a,b,c], Helen Gika[b,c,d], Emmanouil Tsochatzis[e], Stavros Kalogiannis[c,f], Natalia Kataiftsi[g], Evangelos Soufleros[g], Anastasia Zotou[a,c,*]

[a] Laboratory of Analytical Chemistry, Department of Chemistry, Aristotle University of Thessaloniki, 54124, Thessaloniki, Greece
[b] BIOMIC Auth, Center for Interdisciplinary Research and Innovation (CIRI-AUTH), Balkan Center, Buldings A&B, Thessaloniki, 10thkm Thessaloniki-Thermi Rd, P.O. Box 8318, GR, 57001, Greece
[c] FoodOmicsGR, Center for Interdisciplinary Research & Innovation, Aristotle University of Thessaloniki, 57001, Thessaloniki, Greece
[d] Laboratory of Forensic Medicine and Toxicology, School of Medicine, Aristotle University of Thessaloniki, 54124, Thessaloniki, Greece
[e] Department of Chemical Engineering, Aristotle University of Thessaloniki, 54124, Thessaloniki, Greece
[f] Department of Nutrition and Dietetics, Alexander Technological Educational Institute of Thessaloniki, 57400, Thessaloniki, Greece
[g] Laboratory of Oenology and Alcoholic Beverages, Department of Food Science and Technology, School of Agriculture, Aristotle University of Thessaloniki, 54124, Thessaloniki, Greece

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ABSTRACT

An Ultra High Performance Liquid Chromatography – Tandem Mass Spectrometry method has been developed for the analysis of 12 phthalate esters in Greek grape marc spirits. The phthalates were separated on a U-VDSpher PUR 100 C18-E (100 mm x 2.0 mm, 1.8 μm) column by gradient elution. The analytes were ionized by positive electrospray ionization using the multiple reaction monitoring mode. The standard addition method was used for quantification and the Student's *t*-test was carried out to evaluate the matrix effect. The accuracy of the method was assessed by recovery experiments resulting in values from 81.6 to 109.6%. The detection limits ranged from 0.3 to 33.3 μg L^{-1}.The proposed method was validated and successfully applied to the analysis of 45 samples collected from Greece and Cyprus. All phthalate esters proved to be present at least once in the analysed grape marc spirits samples, except only in cases of diphenyl phthalate and diisodecyl phthalate, while for the regulated phthalates only bis (2-ethylhexyl) phthalate was quantified above the legislative concentration limits.

1. Introduction

Phthalate esters (PAEs) are widely used as plasticizers in the production of polymeric materials for improving plasticity, flexibility and elasticity [1]. They are also found in personal care products, such as cosmetics, lotions, perfumes and nail polish, even in medication or nutritional supplement coatings [2]. However, these additives are not chemically bound to the materials, thus they can migrate into anything in contact or surroundings, e.g. to packaged food and beverages and food is the major source of human exposure to them [3,4]. Reports showed that PAEs and their metabolites and degradation products may have toxic effects on human health and potential endocrine disrupting properties, since they can cause hormonal alterations and birth defects [5–7]. Endocrine disruptors mimic naturally occurring hormones (i.e. estrogens and androgens), affecting human reproduction and pre-natal development [8]. In Europe, restrictions on the use of phthalates for materials in contact with foodstuffs are defined by the Framework Regulation (EU) No 1935/2004 [9] and Commission Regulation (EU) No 10/2011 [10].

Grape marc is the solid part of the grape (seeds, skins and pulps) which remain after grape crushing, removing of stalks and juice separation in the winemaking process. Countries around the Mediterranean basin, like France, Spain, Italy and Greece, use grape marc for the production of spirits, reaching economic valorization of this by-product. In Greece (mainland) this typical distillate is called tsipouro (tsikoudia in the island of Crete), marc in France, grappa in Italy, orujo in Spain etc. Grapes, juice and its resulting alcoholic beverage could encounter phthalate contamination at all stages of the process, from the vineyard to bottling. Potential con-

* Corresponding author.
E-mail address: azotou@chem.auth.gr (A. Zotou).

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tamination sources could be plastic storage containers, packaging bottles and containers, harvesting and spirits making equipment, additives and agrochemicals [3]. Consequently, fast and sensitive analytical methods for the monitoring of PAEs in samples are necessary.

In the literature, several analytical methods have been described for phthalate determination in different matrices including liquid-liquid extraction (LLE) [11–14], solid phase extraction (SPE) [4,15–18] or solid phase microextraction (SPME) [7,19,20] for sample pretreatment. Dispersive liquid-liquid microextraction (DLLME) has also been reported [21–24], as it offers the advantages of simplicity, rapidity, high recovery and low cost. However, those methods require tedious operation steps and large amounts of organic solvents. After these treatments, analytical techniques such as high performance liquid chromatography (HPLC) coupled with UV detectors [11,20–22] or mass spectrometry (MS) [3–7,25–27], gas chromatography-mass spectrometry (GC–MS) [12–15,17,19,28,29] and capillary electrophoresis (CE) [30,31] have been used. However, the number of reports in the literature on the determination of PAEs in alcoholic beverages is limited [3–5,12,14,19,27–30] and only three of them report methods for the analysis of PAEs in spirit samples [14,28,30].

Table 1 presents an overview of separation methods for the determination of PAEs in alcoholic beverages and provides information on matrix, sample preparation, analytical technique and the limits of detection (LOD) and limits of quantification (LOQ) achieved.

The limited number of studies reporting methods for the determination of PAEs in alcoholic beverages and the lack of LC–MS methods for the analysis of PAEs in grape marc spirits make it meaningful and imperative to develop a suitable method for this purpose. This study presents the successful development of a new, simple, fast, high-throughput, accurate and sensitive ultra high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method for the efficient separation and quantification of 12 PAEs in 45 grape marc spirit samples, mainly of Greek origin. The advantage over existing methods is that the samples could be injected directly without any previous extraction and/or enrichment, thus eliminating the steps of the analytical procedure and reducing the risk of phthalate contamination from laboratory materials. The chromatographic and mass spectrometric parameters were studied and carefully selected so as to obtain an efficient separation/detection of all the targeted compounds. The multiple reaction monitoring (MRM) acquisition mode offered high sensitivity and selectivity and a full validation protocol in terms of linearity, LODs, LOQs, precision, accuracy and matrix effects was carried out to determine the parameters of interest. To the best of our knowledge, this is the first report on a validated UHPLC-MS/MS method capable of direct analysis of Greek grape marc spirits and the abscence of tedious sample preparation steps is at the same time its major advantage when compared to existing LC–MS/MS methods with similar sensitivity.

2. Materials and methods

2.1. Reagents and materials

Dimethyl phthalate (DMP) (≥99%), diethyl phthalate (DEP) (99.5%), dipropyl phthalate (DPP) (99%), diphenyl phthalate (DPhP) (99%), benzyl butyl phthalate (BBP) (98%), dibutyl phthalate (DBP) (99%) and bis(2-ethylhexyl) phthalate (DEHP) (≥99%) were obtained from Sigma-Aldrich (Darmstadt, Germany). Diisopropyl phthalate (DiPP) (>98%), dipentyl phthalate (DPeP) (>99%), di-*n*-octyl phthalate (DnOP) (≥98%), diisononyl phthalate (DiNP) (≥99%) and diisodecyl phthalate (DiDP) (>99%) were supplied from Fluka (Buchs, Switzerland). Water was purified in a Milli-Q Direct-Q®3 UV Millipore Purification System (Millipore Corporation, Burlington, MA, USA) to deliver ultrapure water with a resistivity of 18.2 MΩ-cm (at 25 °C). HPLC-grade hexane and acetone were obtained from Chem-Lab (Zedelgem, Belgium). Acetonitrile (ACN), methanol (MeOH) and formic acid (99%) were all LC–MS analytical grade and were also obtained from Chem-Lab (Zedelgem, Belgium).

In order to minimize the risk of contamination, all plastic materials were avoided in method development and all solvents were checked for the presence of PAEs before use. All laboratory glassware were washed with acetone, rinsed with hexane and dried at 120 °C for 4 h before use [23]. During storage they were wrapped in aluminum foil to prevent contamination from suspended particles of plasticizers.

2.2. Calibration

2.2.1. Solution preparation for calibration in standard mixtures

Individual stock solutions of each compound were prepared at a concentration of 1000 mg L^{-1} in methanol and stored in amber vials at −20 °C for 1 month. Two multi-component solutions containing equal concentrations (50 and 10 mg L^{-1}) of each compound were prepared on the day of calibration, by transferring the appropriate volumes of the 1000 mg L^{-1} stock solutions in 10-mL volumetric flasks and diluting with methanol. A serial dilution of these solutions with methanol in 10-mL volumetric flasks followed, to prepare eight multi-component standard working mixtures of equal concentrations with respect to each compound (1, 5, 10, 50, 100, 500, 1000, 2500 μg L^{-1}).Calibration curves were constructed by plotting the means of triplicate measurements of peak areas against concentrations of the compounds.

2.2.2. Solution preparation for standard addition calibration

The Standard Additions Method (SAM) was adopted for quantification of PAEs in the distillates, in order to take into account the potential matrix effect on the measured signal. A representative pooled sample was prepared by blending equal quantities of the 45 samples. Aliquots of the pooled sample were fortified with known standard mixtures of the 12 PAEs giving final added concentrations of 0.3, 1, 5, 10, 50, 100, 500, 1000 and 2500 μg L^{-1}. For the preparation of a blank sample, the same procedure was followed without addition of a standard. The blank and the fortified samples were measured in triplicate. Calibration curves were constructed by plotting the means of peak areas against added concentrations of the analytes.

2.3. Samples

Totally, 44 grape marc spirit samples produced in Greece and 1 in Cyprus were analysed directly without any pretreatment. Details about the origin of the samples are supplied in Fig. 1. The samples, once transferred to the laboratory for analysis, were kept at room temperature in glass vials until analysed. For the quantification of highly concentrated samples with respect to DEHP, a suitable dilution with methanol was carried out.

2.4. UHPLC-MS/MS analysis

The UHPLC-MS/MS analysis was carried out on a Accela UHPLC-Triple Quadrupole Accela TSQ Quantum™ Access MAX Mass Spectrometer system (Thermo Scientific, San Jose, CA, USA). The UHPLC unit was equipped with an Accela autosampler, an Accela1250 pump, an Accela column oven and the MS unit with an electrospray ionization (ESI) source. Chromatography was performed on a U-VDSpher PUR 100 C18-E (100 mm x 2.0 mm, 1.8 μm) column (VDS optilab, Chromatographie Technik GmbH, Germany), protected by a U-VDSpher PUR C18-E (5.0 mm x 2.0 mm, 1.8 μm) pre-column (VDS

Table 1
Overview of analytical methods for the determination of PAEs in alcoholic beverages.

Compounds analysed	Matrix	Sample preparation	Analytical technique	LOD	LOQ	Reference
Di-iso-butyl phthalate Di-n-butyl phthalate Butyl benzyl phthalate Bis (2-ethylhexyl) phthalate	Beer, red wine and 52 other foodstuffs	Mechanical agitation (1 h) with ethyl acetate, centrifugation, at −20 °C(>12 h), evaporation to dryness, reconstitution in acetonitrile, vortex, at −20 °C (>12 h), evaporation to dryness, reconstitution in toluene	GC-MS/MS	Limits of reporting 0.18-3.4 μg kg^{-1}	–	29
Dimethyl phthalate, diethyl phthalate, di-n-butyl phthalate, di-n-pentyl phthalate, bis (2-methoxyethyl) phthalate	Alcoholic beverages (brandy, wine, sangria and beer)	In-vial membrane liquid-liquid extraction	GC-MS	0.1–0.4 μg L^{-1}	0.3–1 μg L^{-1}	12
Dibutyl phthalate, butyl benzyl phthalate, diethyl phthalate, dimethyl phthalate	Water and wine	SPE	HPLC-ESI-MS	0.03–0.84 μg L^{-1}	0.09–2.81 μg L^{-1}	4
Dimethyl phthalate, diethyl phthalate, dibutyl phthalate, benzyl butyl phthalate, bis (2-ethylhexyl) phthalate, dioctyl phthalate, (bis (2-ethylhexyl), adipate	Beer	HS-SPME	GC-MS	0.006–0.588 μg L^{-1}	0.020–1.959 μg L^{-1}	19
9 phthalates	Wine	Addition of 1.5 mL methanol, vortex	HPLC-MS/MS	0.5–8.8 μg L^{-1}	1.6-26.6 μg L^{-1}	3
23 phthalates	Wine and 9 other food matrices	Liquid samples :Addition of NaCl, addition of acetonitrile, vortex, centrifugation Solid samples: QuEChERS or Glass-based SPE	HPLC-MS/MS	0.8–15 μg kg^{-1}	10–100 μg kg^{-1}	5
24 phthalates	Liquor	SPE	UPLC-MS/MS	0.003–0.05 mg kg^{-1}	0.03–0.12 mg kg^{-1}	27
13 phthalates	Wine and grape spirits	LLE	GC-MS	0.004–0.020 mg L^{-1}	0.01–0.05 mgL^{-1}	14
Benzylbutyl phthalate, dibutyl phthalate, diethyl phthalate, diisobutyl phthalate	Spirits	DLLME	β-CD-MEKC -DAD	0.4-0.8 ng mL^{-1}	1.4–2.7 ng mL^{-1}	30
16 phthalates	Spirits	Evaporation, addition of n-hexane, vortex, centrifugation	GC-MS/MS	0.1 – 10 ng g^{-1}	0.3 – 33 ng g^{-1}	28
12 phthalates	Grape marc spirits	–	UHPLC-MS/MS	0.3 – 33.3 μg L^{-1}	1–100 μg L^{-1}	This work

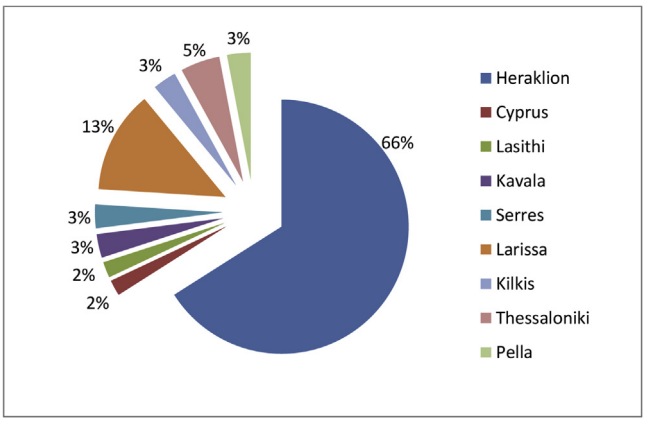

Fig. 1. Origin of the analysed samples (location of the vineyard).

optilab, Chromatographie Technik GmbH, Germany). XCalibur and TSQ Tune (Thermo Scientific, San Jose, CA, USA) software was used for data acquisition.

2.5. Chromatographic and mass spectrometric conditions

2.5.1. UHPLC conditions

The mobile phase consisted of solvent A: water-0.1% formic acid and solvent B: ACN/MeOH, 50:50 (*v/v*)-0.1% formic acid. The elution was performed in gradient mode during a time course of 14 min as follows: 0–3.5 min, 40–55% B; 3.5–7.5 min, 55–90% B; 7.5–8.2 min, 90–92% B; 8.2–9.0 min, 92–95% B; 9.0–10.0 min, 95–100% B; 10.0–14.0 min, held to 100% B. Finally, the composition was returned to the initial (40% B) in 0.01 min. The column was equilibrated for 4 min before the next injection. The flow rate started at 250 μL min^{-1} initially, increasing to 300 μL min^{-1} from 10.0 to 14.0 min and then decreasing to the initial during the final 4-min equilibration step. The injection volume was 5 μL and the column temperature was set at 40 °C.

2.5.2. MS/MS conditions

The detection was performed by multiple reaction monitoring (MRM). The ESI was operated in positive mode (ESI +) with the parameters in the source as follows: spray voltage, 3000 V; capillary and vaporizer temperature, 300 °C; sheath gas pressure, 40 arbitary units (Arb); aux gas pressure, 10 Arb; ion source discharge current, 4.0 μA; ion sweep gas pressure, 2.0 Arb and collision gas pressure, Argon at 1.5 mTorr. The collision energies and cone voltages were optimized for each target compound to give the best possible resolution and sensitivity. The conditions were optimized using direct injection of a 1000 μg L^{-1} standard solution of each compound.

2.6. Method validation

2.6.1. Linearity, limits of detection and quantification

The linearity and sensitivity of the method were evaluated by calibration using both standard solutions and spiked samples over the range of 1–2500 μg L^{-1} and 0.3–2500 μg L^{-1} (added concentrations) respectively. Linear least squares regression was used to calculate the slopes, intercepts and correlation coefficients, the latter expressing the linearity of the method. The LODs and LOQs were defined as the lowest concentrations of standard solutions or as the lowest spiked concentrations in fortified samples that produce a signal-to-noise ratio of 3 and 10, respectively.

2.6.2. Precision and measurement accuracy in standard solutions

The precision of the method in standard solutions was evaluated in terms of injection repeatability (intra-day precision) and intermediate precision (inter-day precision), both expressed as a relative standard deviation, and it was assessed at a low, medium and high concentration level of standard solutions. Repeatability was determined by performing eight replicate injections of each of three multi-component standards on the same day with the same instrument and the same operator while intermediate precision was calculated on the basis of the results from triplicate injections of three standard mixtures, as above, conducted during routine operation of the system over five consecutive days. Measurement accuracy was expressed as relative error.

2.6.3. Precision and accuracy in spiked samples

The within-day relative standard deviation and the recovery of the analytes, from spiked samples, were calculated by means of the SAM calibration, based on triplicate measurements, for the assessment of the within-day precision and accuracy of the assay in grape marc spirits samples, respectively. The accuracy was determined by the percentage recovery using the spiked samples, at three spiked levels of the analytes, as [(mean measured – initial)/added] concentrations.

3. Results and discussion

3.1. Method development

3.1.1. Optimization of chromatographic parameters

Chromatographic conditions were carefully chosen to obtain the highest sensitivity and efficient separation of all the targeted compounds. The composition of the mobile phase is closely related to the chromatographic retention and ionization efficiency of the target compounds. Solvents are typically chosen based on the solubility and compatibility of an analyte of interest with various ionization techniques used in LC/MS. Volatility and the solvent's ability to donate a proton are important in ESI. The mobile phase solvents used in the studies of available literature [3–5] are methanol, acetonitrile and water.

In view of the above, a mobile phase consisting of solvent A: water and solvent B: ACN, both containing 0.1% formic acid was initially tested in gradient mode and although it resulted in shorter retention times, gradient elution with a mobile phase consisting of solvent A: water-0.1% formic acid and solvent B: ACN/MeOH, 50:50 (*v/v*)-0.1% formic acid was finally chosen as it gave better ion intensities with ESI^+ and better peak resolution. Variations of gradient elution programs were tested in order to achieve the most efficient separation of the targeted compounds. The studies conducted for the selection of optimal separation conditions are presented in Tables S1-S3 in the Supplementary material. Separation of the 12 PAEs using the final gradient program is shown in Figs. 2–4 which illustrate the chromatograms for a standard multi-component mixture of PAEs, the non-spiked and the representative spiked pooled sample, respectively.

3.1.2. Optimization of MS/MS parameters

In the MRM mode, two parameters – cone voltage and collision energy – were optimized based on the target compounds' precursor and product ions, in order to maximize the signal of the selected fragment ion and the selectivity of its detection. Full-scan data acquisition from 50 to 500 m/z was performed for all PAEs, after direct injection of a 1000 μg L^{-1} standard solution of each compound. The responses for all of the target compounds were best in positive ESI mode. The $[M+H]^+$ ions of the compounds were therefore used as the precursor ions. The two most abundant product ions for each compound were used as the quantification and identification ions and the collision energies were optimized to maximize the sensitivity for each product ion. Each analyte was identified

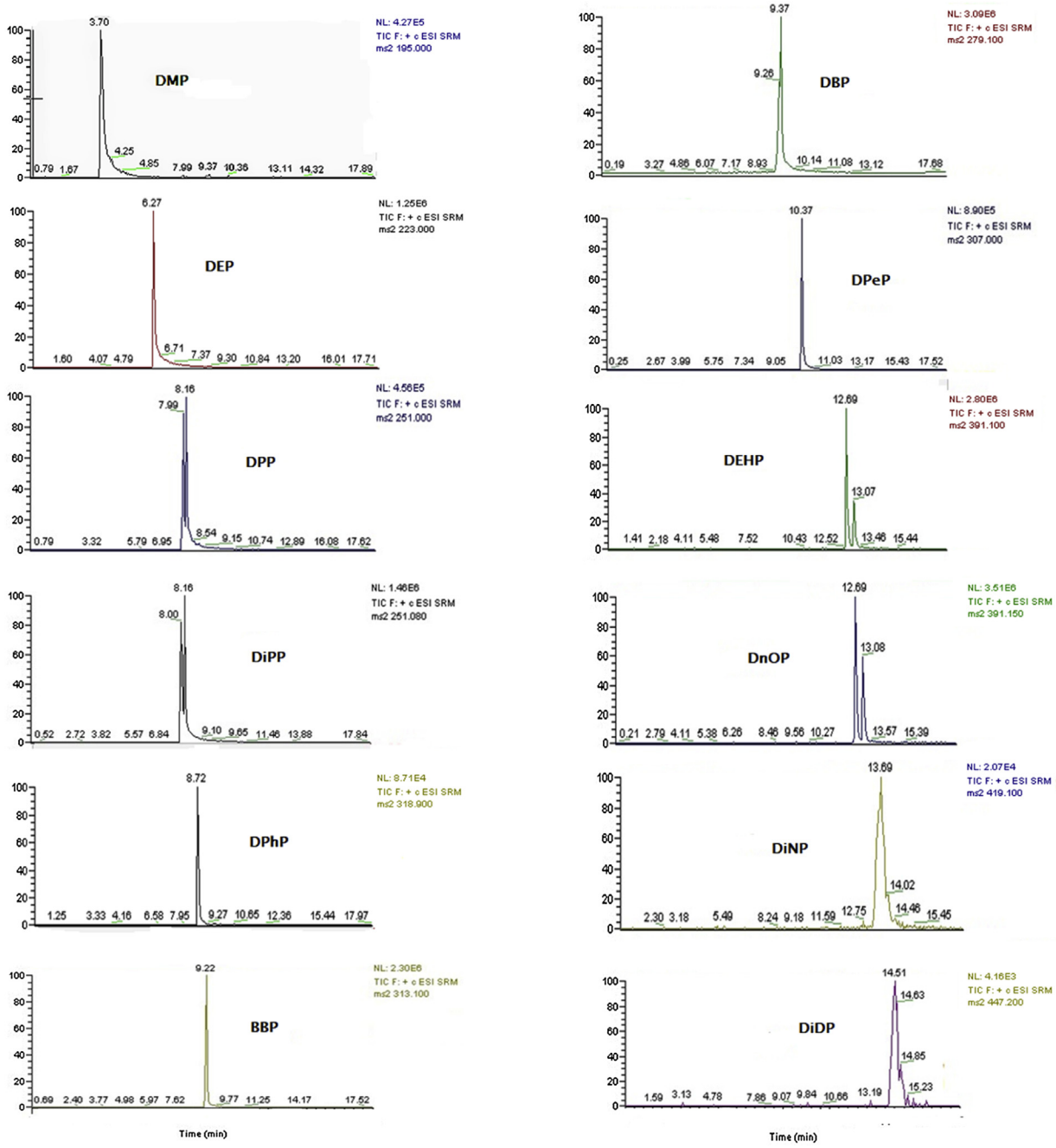

Fig. 2. UHPLC-MS/MS of a standard multi-component mixture of PAEs (1000 μg L^{-1}), at the optimal chromatographic and MS/MS conditions as described under Experimental.

from its retention time and two ion transitions and the more abundant ion transition was used to quantify the analyte. The relative molecular mass, precursor and product ions, cone voltage, collision energy and retention times of each compound are shown in Table 2.

3.2. Method validation

The validation of the method was carried out in terms of linearity, LODs, LOQs, precision accuracy and matrix effects.

3.2.1. Linearity and sensitivity

Based on the solvent calibration curves the linearity of the method for the target analytes was found to extend between 5–2500 μg L^{-1}. The standard addition curves exhibited linearity between 1–2500 μg L^{-1}. In all cases, the correlation coefficients (r) were greater than 0.99, demonstrating an excellent linearity. For solvent calibration the LODs ranged between 1.7–33.3 μg L^{-1} and the LOQs between 5.0–100 μg L^{-1}. For the SAM calibration the LODs were in the range of 0.3–33.3 μg L^{-1} and the LOQs in the range of 1.0–100 μg L^{-1}. Table 3 presents detailed data for each analyte.

3.2.2. Precision and measurement accuracy in standard solutions

The intra- and inter-day precision, expressed as relative standard deviation, ranged for all the analytes from 0.4 to 9.9% and from 1.8 to 10.2% respectively, in standard solutions. The results are summarized in Table 4.

3.2.3. Matrix effect

The effect of the matrix can be variable and unpredictable in the occurrence of measurable effects. The matrix-induced signal sup-

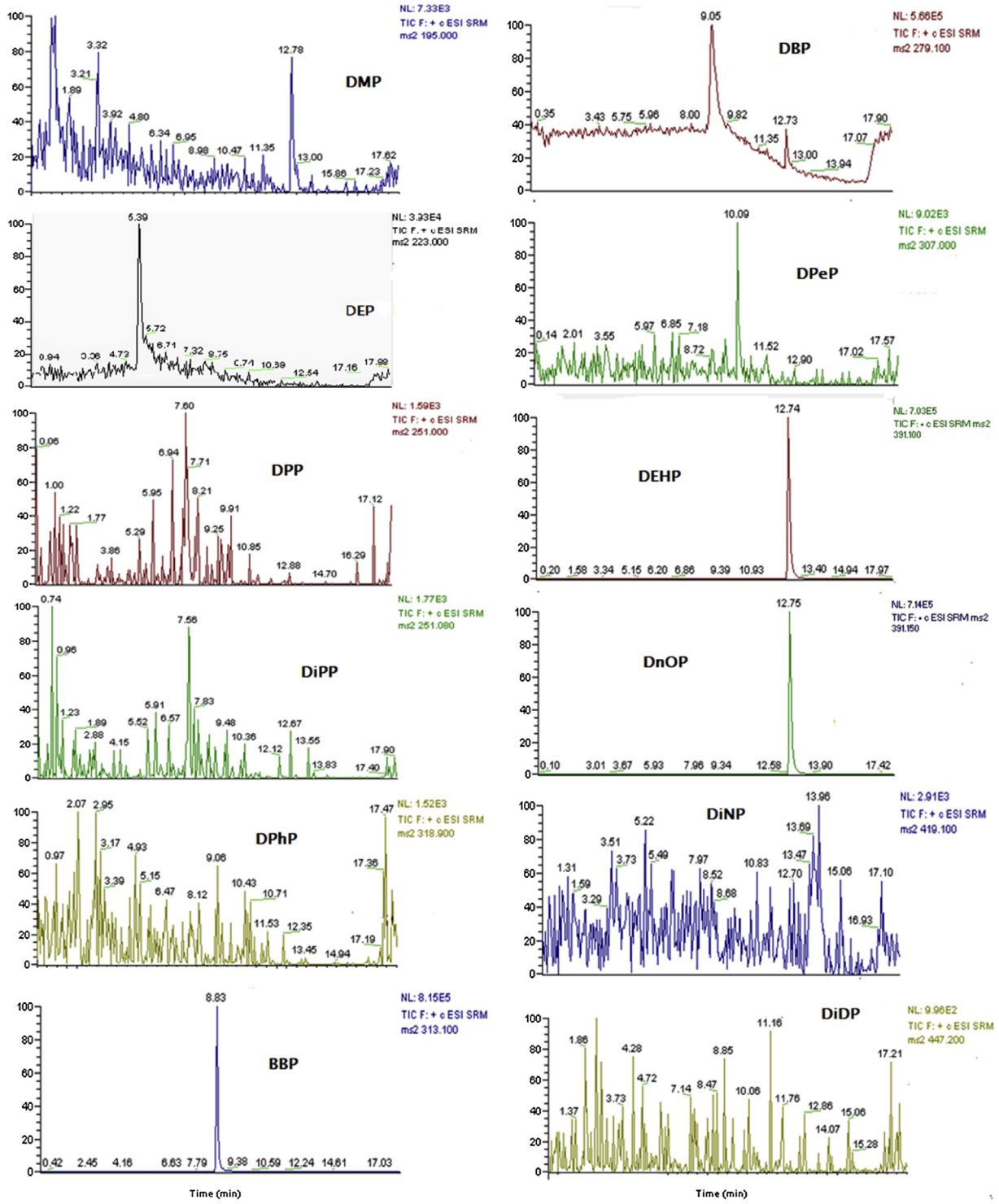

Fig. 3. UHPLC-MS/MS of the non-spiked representative pooled sample, at the optimal chromatographic and MS/MS conditions as described under Experimental.

pression/enhancement was determined by comparing the slopes of the SAM calibration curves with those of the pure solvent calibration curves, using the Student's *t*-test [32], according to the following equation,

$$t = \frac{b_1 - b_2}{s_{b_1 - b_2}}$$

Where b_1 and b_2 are the slopes of the regression lines and $s_{b_1-b_2}$ the standard error of the difference between the regression slopes calculated as,

$$s_{b_1-b_2} = \left[\frac{\left(s^2_{Y \cdot X}\right)_p}{\left(\sum x^2\right)_1} + \frac{\left(s^2_{Y \cdot X}\right)_p}{\left(\sum x^2\right)_2} \right]^{1/2}$$

where $\left(s^2_{Y \cdot X}\right)_p$ is the pooled residual mean square and the subscripts 1 and 2 refer to the two regression lines (SAM and solvent) being compared. The calculation of the critical values of *t*-test was performed taking into account $(n_1\text{-}2) + (n_2\text{-}2)$ degrees of freedom.

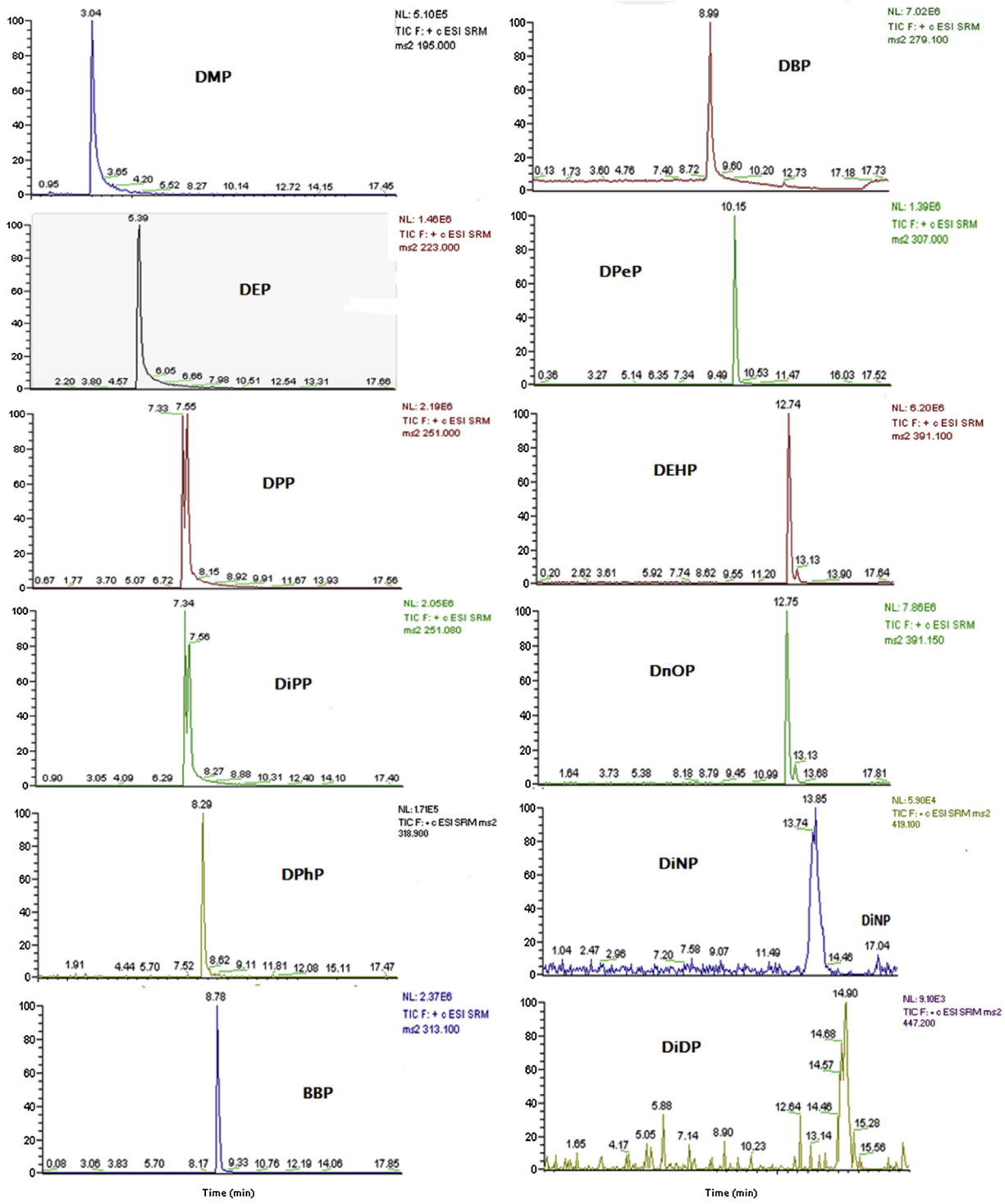

Fig. 4. UHPLC-MS/MS of the representative pooled sample spiked with a standard multi-component mixture of PAEs (1000 $\mu g\,L^{-1}$), at the optimal chromatographic and MS/MS conditions as described under Experimental.

The results, shown in Table 5, indicated that a significant difference (at 95% confidence level) exists between the SAM and the solvent calibration curves for almost all PAEs ($t_{experimental}$ = 2.890–21.624 > $t_{critical}$), except for DiPP ($t_{experimental}$ = 1.547 < $t_{critical}$ = 2.228), therefore the standard additions method was used for quantification.

3.2.4. Precision and accuracy in spiked samples

For the assessment of the method accuracy, the recovery of the analytes from spiked samples was calculated by the standard additions method, using the spiked pooled samples. The mean recoveries ranged between 81.6%–109.6% for all the compounds. The relative standard deviation of triplicate measurements of the

Table 2
List of the monitored PAEs with relative molecular mass, MS detection parameters and retention times.

Compound	Relative molecular mass ($gmol^{-1}$)	Precursor ion	Product ions	Tube lens (kV)	Collision energy (kV)	Detection window (min)	Retention time (standard solution) (min)	Retention time (pooled sample) (min)
DMP	194.186	195	164 78	48	5 32	2–5	3.70	3.04
DEP	222.24	223	149 65	26	20 39	4–7	6.27	5.39
DPP	250.294	251	149 65	29	16 45	6–9	8.16	7.55
DiPP	250.294	251	149 65	31	17 46	6–9	8.00	7.34
DPhP	318.328	319	77 226	72	36 13	7–10	8.72	8.29
BBP	312.365	313	149 91	25	14 31	8–11	9.22	8.78
DBP	278.348	279	149 65	28	15 50	8–11	9.37	8.99
DPeP	306.402	307	149 121	49	15 36	9–12	10.37	10.15
DEHP	390.654	391	149 121	35	30 48	11–14	12.69	12.74
DnOP	390.654	391	149 121	37	15 43	12–14	13.08	13.13
DiNP	418,609	419	149 71	78	29 20	11–15	13.69	13.95
DiDP	446,670	447	149 85	86	17 30	12–16	14.51	14.90

Table 3
Analytical features of the proposed method.

Compound	Linear range ($\mu g\ L^{-1}$)	$b \pm s_b$	α	r	LOD ($\mu g\ L^{-1}$)	LOQ ($\mu g\ L^{-1}$)
DMP	10–2500[c] 50–2500[d]	$(2.7 \pm 0.05)\ 10^{3c}$ $(2.5 \pm 0.02)\ 10^{3\ d}$	$-1.0 \cdot 10^{3c}$ $-34 \cdot 10^{3d}$	0.9996 [c] 0.9999 [d]	3.3 [c] 16.7 [d]	10.0 [c] 50.0 [d]
DEP	5–2500[c] 1–2500 [d]	$(26. \pm 0.3)\ 10^{3c}$ $(21 \pm 0.09)\ 10^{3\ d}$	$35 \cdot 10^{3c}$ $34 \cdot 10^{3d}$	0.9997[c] 0.9999 [d]	1.7[c] 0.3 [d]	5.0[c] 1.0 [d]
DPP	5–2500[c] 1–2500 [d]	$(19 \pm 0.2) 10^{3c}$ $(10 \pm 0.04)\ 10^{3\ d}$	$-84 \cdot 10^{3c}$ $21 \cdot 10^{3d}$	0.9997[c] 0.9999 [d]	1.7[c] 0.3 [d]	5.0[c] 1.0 [d]
DiPP	5–2500[c] 1–2500 [d]	$(18 \pm 0.2) 10^{3\ c}$ $(6.9 \pm 0.06) 10^{3\ d}$	$34 \cdot 10^{3c}$ $-9.9 \cdot 10^{3d}$	0.9998[c] 0.9998 [d]	1.7[c] 0.3 [d]	5.0[c] 1.0 [d]
DPhP	5–2500[c] 5–2500 [d]	$(1.9 \pm 0.03)\ 10^{3\ c}$ $(1.7 \pm 0.006)\ 10^{3\ d}$	$6.2 \cdot 10^{3c}$ $9.3 \cdot 10^{3d}$	0.9995[c] 0.9999 [d]	1.7[c] 1.7 [d]	5.0[c] 5.0 [d]
BBP	5–2500[c] 1–2500[d]	$(1.5 \pm 0.03)\ 10^{3\ c}$ $(4.5 \pm 0.03)\ 10^{3\ d}$	$-8.7 \cdot 10^{3\ c}$ $753 \cdot 10^{3d}$	0.9995[c] 0.9999[d]	1.7[c] 0.3[d]	5.0[c] 1.0[d]
DBP	10–2500[c] 1–2500[d]	$(23 \pm 0.8)\ 10^{3c}$ $(12 \pm 0.1)\ 10^{3d}$	$89 \cdot 10^{3c}$ $234 \cdot 10^{3d}$	0.9981[c] 0.9998[d]	3.3[c] 0.3[d]	10.0[c] 1.0[d]
DPeP	5–2500[c] 1–2500[d]	$(12 \pm 0.3)\ 10^{3\ c}$ $(6.1 \pm 0.03)\ 10^{3\ d}$	$-188 \cdot 10^{3c}$ $68 \cdot 10^{3d}$	0.9987[c] 0.9999[d]	1.7[c] 0.3[d]	5.0[c] 1.0[d]
DEHP	5–2500[c] 5–2500[d]	$(11 \pm 0.1)\ 10^{3c}$ $(18 \pm 0.1)\ 10^{3d}$	$369 \cdot 10^{3c}$ $17996 \cdot 10^{3d}$	0.9998[c] 0.9999[d]	1.7[c] 1.7[d]	5.0[c] 5.0[d]
DnOP	10–2500[c] 50–2500[d]	$(4.0 \pm 0.07)\ 10^{3c}$ $(0.59 \pm 0.008)\ 10^{3d}$	$235 \cdot 10^{3c}$ $239 \cdot 10^{3d}$	0.9996[c] 0.9996[d]	3.3[c] 16.7[d]	10.0[c] 50.0[d]
DiNP	50–2500[c] 100–2500[d]	$(1.1 \pm 0.06)\ 10^{3c}$ $(0.40 \pm 0.004)\ 10^{3\ d}$	$266 \cdot 10^{3c}$ $41 \cdot 10^{3d}$	0.9976[c] 0.9998[d]	16.7[c] 33.3[d]	50.0[c] 100.0[d]
DiDP	100–2500[c] 100–2500[d]	$(0.54 \pm 0.02)\ 10^{3\ c}$ $(0.14 \pm 0.002)\ 10^{3\ d}$	$-62 \cdot 10^{3c}$ $0.7 \cdot 10^{3d}$	0.9988[c] 0.9997[d]	33.3[c] 33.3[d]	100.0[c] 100.0[d]

a, slope; *b*, intercept; s_b, standard error of the intercept; [c]solvent calibration; [d]SAM calibration.

spiked samples at three concentration levels ranged from 1.6 to 10.2%. Table 6 summarizes the results.

3.3. Grape marc spirit samples analysis

Grape marc spirit samples - collected from different regions in Greece and Cyprus- were analysed in triplicate under the optimal conditions. The sensitivity and selectivity of the proposed method and the relatively simple nature of the samples permitted direct analysis with no sample pretreatment. The content of the analysed samples in the assayed compounds is presented in Table 7, and as it can be seen the PAEs were detected in the majority of the samples, except in samples nr. 5, 15, 28, 29, 36 and 40. DBP, BBP and DEHP seemed to be the main PAEs found in the samples. In three of the samples (nr. 27, 30 and 31) DEHP was found at concentrations hugely above its SML (1.5 $mg\ kg^{-1}$ food stimulant), as established by the European Union (Framework Regulation (EU) No 1935/2004 [9] and Commission Regulation (EU) No 10/2011 [10] and since it exceeded the upper limit of the linear range, a suitable dilution of these three samples took place to correctly calculate the amount of the compound. Also BBP and DBP have SMLs [10] of 30 $mg\ kg^{-1}$ and 0.3 $mg\ kg^{-1}$ [10] and BBP was found to be present in 25 samples with a concentration ranging from 1.37 up to 1526 $\mu g\ L^{-1}$, while DBP was found in 29 samples with a concentration range from 3.16 to 135.8 $\mu g\ L^{-1}$.

For the majority of the analysed samples (38 out of total 45 samples), there was available information on vine cultivation, alcoholic fermentation, the distillation process and finally the storage

Table 4
Intra-day and inter-day precision of assay in standard solutions.

	Intra-day				Inter-day			
Compound	Added (μg L^{-1})	Found [a] ± s (μg L^{-1})	s_r(%)	E_r (%)	Added (μg L^{-1})	Found [b] ± s (μg L^{-1})	s_r (%)	E_r (%)
DMP	10	10.16±0.08	0.8	+1.6	10	10.42±0.19	1.8	+4.2
	100	91.94±3.26	3.6	−8.1	100	102.4±9.8	9.6	+2.4
	1000	1006±4	0.4	+0.62	1000	991.0±38.0	3.8	−0.90
DEP	10	9.08±0.48	5.3	−9.2	10	9.51±0.93	9.8	−4.9
	100	109.7±3.1	2.8	+9.7	100	108.2±5.7	5.3	+8.2
	1000	986.3±42.3	4.3	−1.4	1000	978.6±50.0	5.1	−2.1
DPP	10	9.99±0.81	8.1	−0.10	10	9.22±0.38	4.1	−7.8
	100	96.43±2.05	2.1	−3.6	100	92.59±2.25	2.4	−7.4
	1000	972.0±30.2	3.1	−2.8	1000	1007±43	4.3	+0.70
DiPP	10	9.04±0.49	5.4	−9.6	10	9.09±0.19	2.1	−9.1
	100	97.40±1.72	1.8	−2.6	100	102.1±3.8	3.7	+2.1
	1000	1011±45	4.4	+1.1	1000	900.4±74.0	8.2	−9.9
DPhP	10	9.93±0.98	9.9	−0.70	10	10.60±0.40	3.8	+6.0
	100	101.4±6.3	6.2	+1.4	100	100.8±5.7	5.6	+0.79
	1000	1029±30	2.9	+2.9	1000	1031±43	4.2	+3.1
BBP	10	10.95±1.08	9.9	+9.5	10	10.79±1.02	9.4	+7.9
	100	105.3±6.9	6.5	+5.3	100	105.6±6.4	6.1	+5.6
	1000	951.5±38.0	4.0	−4.8	1000	1006±54	5.4	+0.58
DBP	10	10.14±0.66	6.5	+1.4	10	10.21±0.83	8.1	+2.1
	100	96.24±3.58	3.7	−3.8	100	96.47±6.21	6.4	−3.5
	1000	1028±40	3.9	+2.8	1000	1033±25	2.4	+3.3
DPeP	10	10.06±0.93	9.2	+0.60	10	9.82±1.00	10.2	−1.8
	100	109.0±4.0	3.7	+8.9	100	97.38±3.71	3.8	−2.6
	1000	1018±30	2.9	+1.8	1000	1049±36	3.4	+4.9
DEHP	10	10.03±0.45	4.5	+0.3	10	9.45±0.80	8.5	−5.5
	100	105.0±5.0	4.8	+5.0	100	109.4±10.2	9.3	+9.4
	1000	1109±13	1.2	+10.9	1000	1110±40	3.6	+11.0
DnOP	100	93.70±3.96	4.2	−6.3	100	110.2±3.6	3.3	+10.2
	1000	1101±23	2.1	+10.1	1000	1007±98	9.7	+0.70
	2500	2635±125	4.7	+5.4	2500	2623±241	9.2	+4.9
DiNP	100	106.1±9.7	9.1	+6.1	100	103.9±1.9	1.8	+3.9
	1000	903.7±32.4	3.6	−9.6	1000	1096±99	9.0	+9.6
	2500	2551±169	6.6	+2.0	2500	2677±141	5.3	+7.1
DiDP	100	102.8±9.7	9.4	+2.8	100	111.6±9.6	8.6	+11.7
	1000	1005±81	8.1	+0.46	1000	944.8±59.2	6.3	−5.5
	2500	2396±200	8.3	−4.2	2500	2387±177	7.4	−4.5

s, standard deviation; s_r, relative standard deviation; E_r, relative error; [a] Means of values calculated from the solvent calibration curves for eight determinations within a day; [b] Means of values calculated from the solvent calibration curves for three determinations per day over five days.

Table 5
Student's t-test.

Compound	$t_{experimental}$	$t^a_{critical}$
DMP	4.637	2.447
DEP	7.356	2.262
DiPP	1.547	2.228
DPP	17.267	2.228
DPhP	2.890	2.306
DBP	6.238	2.262
DPeP	9.631	2.228
DEHP	18.261	2.447
DnOP	21.624	2.365
DiNP	6.868	2.571
DiDP	10.562	2.776
BBP	19.027	2.306

[a] at the 95% confidence level and (n_1-2) + (n_2-2) degrees of freedom.

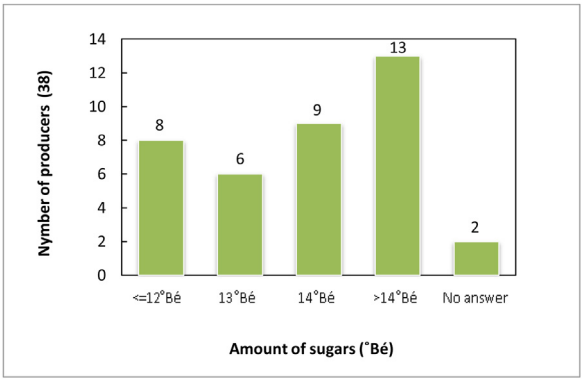

Fig. 5. Amount of sugars in the grapes when collected from the vineyard.

stage (Table S4 in the Supplementary material). These samples were provided by 38 producers and their selection was random. A questionnaire, related to the process and production conditions was given to the producers, however not all the questions were answered by the total number of producers. The questionnaire along with the related answers (Table S4) are presented in the Supplementary material.

The samples with high levels of DEHP migration originated from the wider region of Northern Greece (Larissa, Kilkis and Serres). PAEs can migrate from plastic materials to the environment, and they are often found around industrial areas, dispersing into the soil, water and air thereby creating potential contamination hazard [33]. All the samples (except one, in which only DEP, BBP and DBP were found at low concentrations) came from grapes harvested from vineyards which were not adjacent to some kind of production unit. Therefore, in our case, the external environment cannot be considered as a source of contamination of the samples.

Also, the amount of sugars in the grapes -when collected from the vineyard - which varied in the range of 12–15°Bé (Fig. 5), cannot be considered as a factor justifying the presence or absence of PAEs in the distillates.

Stainless steel (Inox) spare parts is the equipment mostly used for the alcoholic fermentation, while plastic transfer tubes are also

Table 6
Precision and accuracy data in the pooled sample spiked at three concentrations.

Compound	Initial concentration ($\mu g\ L^{-1}$)	Added concentration ($\mu g\ L^{-1}$)	Mean measured concentration[a] ± s ($\mu g\ L^{-1}$)	s_r[b] (%)	Recovery[c] (%)
	13.69	50	64.55±5.15	7.8	101.7
DMP	13.69	500	497.2±18.3	3.7	96.7
	13.69	2500	2509±192	7.6	99.8
	16.08	50	60.48±6.18	10.2	88.8
DEP	16.08	100	99.85±3.53	3.5	83.8
	16.08	1000	1007±58	5.8	99.1
	2.00	10	11.27±1.03	9.1	92.7
DPP	2.00	100	94.13±8.16	8.7	92.1
	2.00	1000	1002±101	10.1	100.0
	1.44	10	12.28±1.16	9.4	108.4
DiPP	1.44	100	103.0±10.0	9.7	101.6
	1.44	1000	957.5±75.4	7.9	95.6
	5.35	10	13.80±1.30	9.4	84.5
DPhP	5.35	100	101.6±7.2	7.1	96.3
	5.35	1000	991.2±89.7	9.0	98.6
	167.35	500	578.0±46.1	8.0	82.1
BBP	167.35	1000	983.3±66.6	6.8	81.6
	167.35	2500	2504±135.9	5.4	93.5
	18.08	100	108.0±10.4	9.6	89.9
DBP	18.08	500	584.7±40.9	7.0	100.6
	18.08	2500	2483±141	5.7	98.6
	11.27	50	55.30±5.02	9.1	88.1
DPeP	11.27	500	541.3±25.3	4.7	106.0
	11.27	2500	2492±112	4.5	99.2
	974.51	50	1024±104	10.2	98.2
DEHP	974.51	500	1502±113	7.5	105.5
	974.51	2500	3715±254	6.8	109.6
	403.62	500	903.0±42.4	4.7	99.9
DnOP	403.62	1000	1260±107	8.5	85.7
	403.62	2500	2499±212	8.5	83.8
	102.99	500	527.7±19.6	3.7	84.9
DiNP	102.99	1000	1053±61	5.8	95.0
	102.99	2500	2490±100	4.0	95.5
	4.78	100	101.0±10.1	10.0	96.2
DiDP	4.78	1000	966.0±15.9	1.6	96.1
	4.78	2500	2509±212	8.4	100.2

[a] Means of values calculated from the SAM calibration curves (n = 3) on the same day ± standard deviation.
[b] Relative standard deviation.
[c] Recovery (%) = [(Mean measured conc.-initial conc.)/added conc.]×100.

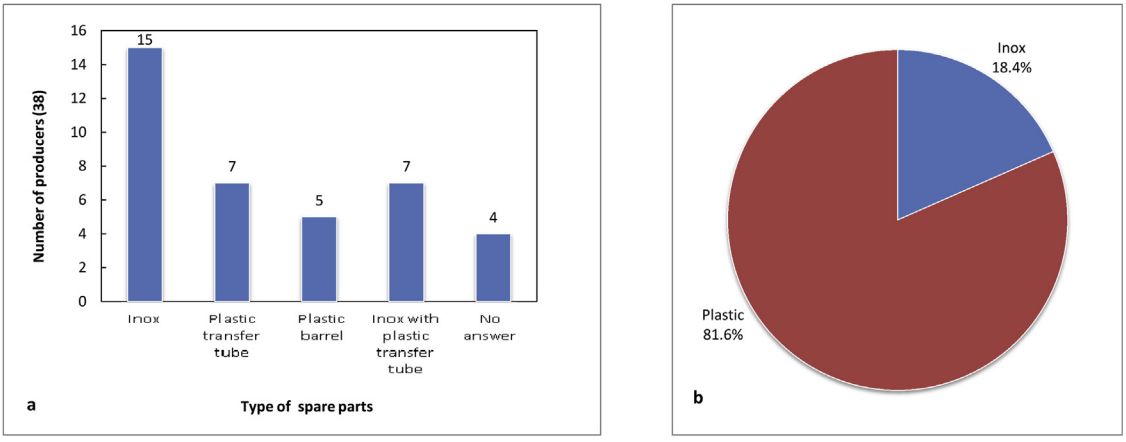

Fig. 6. Type of spare parts (a) and type of fermentation tank (b) used during alcoholic fermentation.

used to a considerable extent, combined or not with the former (Fig. 6a). Plastic tubes appear to be a potential contamination factor. The use of plastic fermentation tanks during alcoholic fermentation was predominant (Fig. 6b) for most of the test specimens, but this did not appear to be a contamination factor for all the samples. The relative molecular mass of the polymer, the thickness and amount of the plasticizer and the duration of plastification and stabilization of the plastic material used for the fermentation tank can also determine the migration levels of PAEs in the produced spirit.

A proportion of 81.6% of the producers (31 producers) used plastic tanks for alcoholic fermentation (Fig. 6b). Among them only a few kept the grape marc more than 2 months in plastic tanks (Fig. 7a). Regarding the temperature at which the marc remained in the plastic containers during alcoholic fermentation, 20 producers stated that the temperature exceeded 21 °C while the rest stated that the temperature was below 20 °C (Fig. 7b). However, the accuracy of the measurement and how closely was the temperature monitored cannot be estimated. Unfortunately, no conclusions can

Table 7
Concentration of PAEs in the analysed grape marc spirits' samples.

Samples	DMP (μg L^{-1})± s^a	DEP (μg L^{-1})± s^a	DiPP (μg L^{-1})± s^a	DPP (μg L^{-1})± s^a	DPhP (μg L^{-1})± s^a	BBP (μg L^{-1})± s^a	DBP (μg L^{-1})± s^a	DPeP (μg L^{-1})± s^a	DEHP (μg L^{-1})± s^a	DnOP (μg L^{-1})± s^a	DiNP (μg L^{-1})± s^a	DiDP (μgL^{-1})± s^a
1	–[b]	–	–	–	–	8.33±1.30	12.07±1.26	–	18.42±2.63	31.07±2.4	–	–
2	–	2.06±0.14	–	–	–	9.62±1.94	24.47±3.84	1.25±0.02	144.3±19.5	–	–	–
3	–	–	–	–	–	11.16±0.84	–	–	6.92±1.85	–	–	–
4	–	456.3±66.2	–	–	–	56.26±10.22	–	1.85±0.42	607.5±90.0	–	79.03±11.13	–
5	–	–	–	–	–	–	–	–	–	–	–	–
6	–	–	–	–	–	5.13±1.07	24.35±0.50	–	14.41±1.11	–	–	–
7	–	–	–	–	–	172.8±5.6	–	–	11.34±0.45	–	–	–
8	–	–	–	–	–	28.45±0.30	39.15±3.57	–	–	–	–	–
9	–	–	–	–	–	10.43±1.28	3.16±0.94	–	70.13±2.02	–	–	–
10	–	1.11±0.30	–	–	–	–	5.31±0.30	–	–	–	–	–
11	3.19±0.34	–	–	–	–	5.76±1.47	18.23±1.50	–	9.13±0.52	26.08±1.44	–	–
12	–	2.24±0.24	–	–	–	–	–	–	–	–	–	–
13	19.14±0.23	2.55±0.17	10.37±1.43	–	–	500.9±91.4	60.45±7.53	–	15.40±1.07	–	–	–
14	–	–	–	2.62±0.01	–	491.9±7.3	–	–	18.92±0.23	–	–	–
15	–	–	–	–	–	–	–	–	–	–	–	–
16	6.45±0.42	–	–	–	–	–	30.65±3.21	–	31.68±2.32	12.96±1.03	57.89±3.87	–
17	–	2.10±0.14	–	–	–	–	21.53±0.55	–	19.43±3.08	–	–	–
18	–	0.99±0.06	–	–	–	29.03±2.86	64.46±5.02	–	19.32±2.05	–	–	–
19	8.45±1.21	–	–	–	–	6.21±0.42	12.60±3.06	–	20.58±1.21	26.94±1.67	–	–
20	–	–	–	–	–	8.45±1.08	26.44±3.64	–	77.13±2.28	–	–	–
21	–	–	–	–	–	708.2±68.7	17.60±1.19	1.23±0.28	75.28±4.93	–	–	–
22	–	–	–	–	–	4.35±0.12	7.77±1.25	–	10.52±0.98	–	–	–
23	–	–	–	–	–	190.3±2.4	–	–	7.45±0.11	–	–	–
24	3.98±0.50	–	–	–	–	579.6±102.8	12.63±1.97	1.19±0.22	53.41±8.42	29.26±1.23	–	–
25	–	–	–	–	–	270.4±69.4	–	–	66.71±15.28	–	–	–
26	31.36±1.80	5.13±0.51	–	5.26±0.39	–	1526±194	31.90±0.57	–	194.2±21.6	–	883.0±12.0	–
27	–	–	–	1.48±0.12	–	–	135.8±1.1	103.8±3.1	93339±345	–	–	–
28	–	–	–	–	–	–	–	–	–	–	–	–
29	–	–	–	–	–	–	–	–	–	–	–	–
30	46.08±5.73	2.03±0.22	–	3.18±0.40	–	–	9.37±1.31	16.01±2.45	68829±2170	–	–	–
31	56.17±2.07	–	–	2.34±0.26	–	–	14.27±2.46	16.23±0.43	113220±897	–	–	–
32	66.61±4.25	–	–	–	–	134.3±30.8	29.41±5.44	1.11±0.13	783.2±98.3	–	172.9±2.8	–
33	–	–	–	–	–	–	–	–	446.4±10.2	–	174.1±2.3	–
34	–	1.25±0.23	–	–	–	2.49±0.42	10.32±2.54	–	–	–	–	–
35	–	–	–	–	–	–	6.93±1.56	–	–	–	–	–
36	–	–	–	–	–	–	–	–	–	–	–	–
37	–	–	–	–	–	–	26.60±2.02	–	36.85±3.45	27.40±3.89	–	–
38	–	–	–	–	–	–	9.93±0.70	–	68.13±3.21	–	–	–
39	–	–	–	–	–	–	30.43±0.79	–	20.33±2.68	–	–	–
40	–	–	–	–	–	–	–	–	–	–	–	–
41	–	1.02±0.27	–	–	–	79.85±11.54	23.67±2.83	3.33±0.52	228.7±27.4	–	–	–
42	–	–	–	–	–	–	26.98±0.96	–	–	–	–	–
43	–	–	–	–	–	1.37±0.10	–	–	–	–	–	–
44	17.87±1.27	1.56±0.47	–	–	–	–	35.05±1.99	–	–	30.65±2.21	–	–
45	24.34±3.11	–	–	–	–	2.40±0.67	–	–	–	–	–	–

[a] Means of values calculated from the SAM calibration curves (n = 3) ± standard deviation.
[b] "–" Not detected.

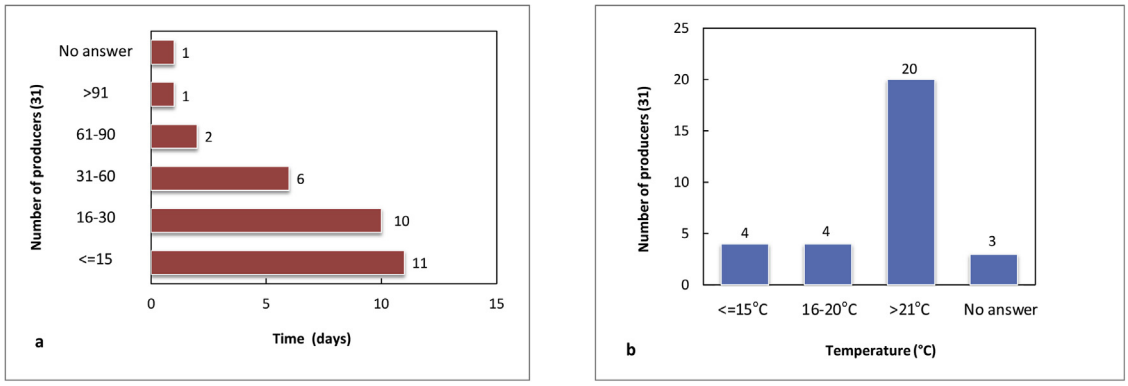

Fig. 7. Staying time (a) and temperature (b) of the grape marc in the plastic fermentation tanks.

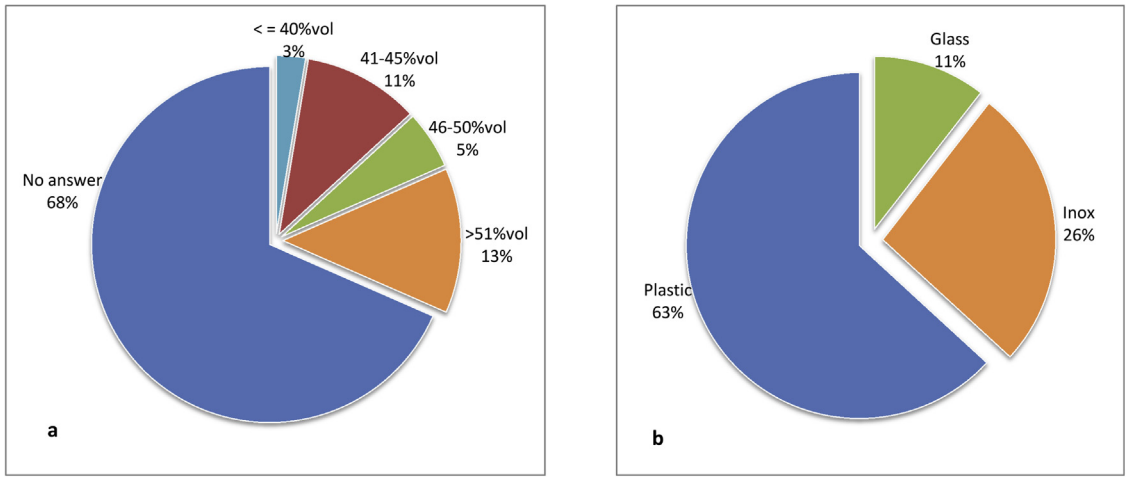

Fig. 8. Alcoholic strength of the produced distillate (a) and material of the container used for the storage of distillates (b).

be drawn regarding the effect of staying time and temperature of the marc in plastic fermentation tanks, as most producers of the DEHP contaminated samples did not answer the specific questions.

A proportion of 26% of the samples were flavored with anise (Fig. S1 in the Supplementary material) and our results showed that these samples exhibited higher concentrations of PAEs, especially DEHP. All three samples (nr. 27, 30 and 31) where DEHP was found at concentrations hugely above its SML were anise flavored (Table 7 and Table S4 in the Supplementary material). Since the processes of anise flavored and non-flavored tsipouro are almost identical, it may be concluded that spirits of poor quality are anise flavored with the intent to mask possible defects and off flavors possibly connected with production errors that also may lead to higher migration of PAEs to the spirits [34].

The majority of the producers (68%) did not provide information about the alcoholic strength of their distillate. Only 3% of them said that their distillate had less than 40% vol and 11% of them declared 41–45% vol. Some producers (13%) declared distillates with more than 51% vol (Fig. 8a).Two of the samples with a high concentration of DEHP also exhibited an alcoholic strength of 50% vol or above (Tables 7 and S4 in the Supplementary material) and this can be justified on the basis of Kampouris et al survey [35], who observed that the higher the concentration of alcohol in the sample, the greater the migration levels of PAEs. This finding supports that most DEHP contamination occurs during storage of the final product after distillation.

Elevated levels of BBP and DEHP migration were observed in cases where plastic transfer tubes were used during the alcoholic fermentation process and even higher when combined with plastic fermentation tanks and/or plastic containers used for the storage of distillates (Tables 7 and S4 in the Supplementary material). However, there were samples where PAEs were not detected, for which the producers declared the use of plastic materials. According to Kampouris et al. [35], the storage life of an alcoholic solution should be studied in parallel with the temperature, because, as they observed, 30 days of contact at 10 °C gave the same amount of migration with contact for 2 days at 30 °C, as well as 30 days of contact at 30 °C resulted in the same levels of migration with contact for 2 days at 60 °C. From the graph of Fig. 9a we observe that most producers stored their distillates for a period of between 2–6 months, at temperatures ranging for most distillates between 11–20 °C (Fig. 9b).

We performed principal component analysis (PCA) and partial least square analysis (PLS) using SIMCA P 13.0 (Umetrics, Sweden). No significant statistical model could fit in order to discriminate the samples in relation to the conditions of the treatment. This could be attributed to the large proportion of zero values as most of the analysed samples contained (if any) only a small number of phthalates. In addition, the questionnaire was not fully answered by all the producers (Table S4 in the Supplementary material).

Despite the detection of DEHP, at concentrations higher than its SML, in a limited number of samples, the largest number of Greek spirits is classified as safe for human consumption, which is confirmed by the reports of the State General Laboratory. To our knowledge the State General Laboratory, the competent authority for tsipouro control, did not report similar infringements in the last years, although the frequency of PAEs determination in tsipouro samples by the State General Laboratory is unknown. We could

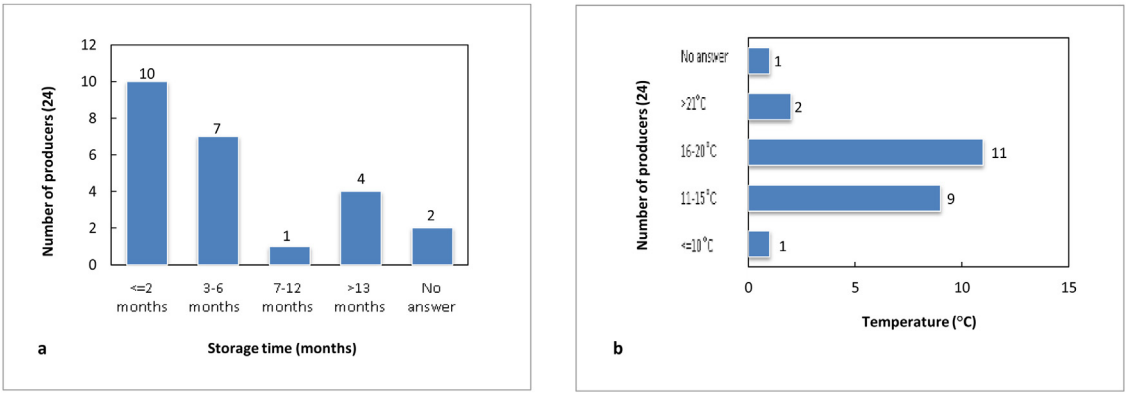

Fig. 9. Storage time (a) and storage temperature (b) of distillates in plastic containers.

therefore conclude that Greek distillates of small distilleries may be considered safe, provided that the suitable controls carried out by the competent authorities are continuous and strict. Moreover, grape marc spirit producers need to be thoroughly informed about the dangers posed by the use of plastic materials throughout the production process, especially during handling of the alcohol rich final product.

4. Conclusions

This paper describes the successful development of a fast, simple, high-throughput and sensitive UHPLC-MS/MS method for the determination of twelve PAEs in grape marc spirit samples originated from Greece and Cyprus. The number of reports in the literature on the determination of PAEs in alcoholic beverages is limited and to the best of our knowledge, this is the first report on a validated UHPLC-MS/MS method capable of direct analysis of grape marc spirits.

The samples could be injected directly without any pretreatment, thus eliminating the steps of the analytical process and reducing the phthalate contamination from laboratory materials. The MRM acquisition mode offered high sensitivity and selectivity. DBP, BBP and DEHP seemed to be the main PAEs found in the samples. Despite the detection of DEHP, at concentrations higher than its SML, in a limited number of samples, the largest number of Greek spirits is classified as safe for human consumption.

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Declaration of competing interest

None

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.chroma.2019.06.034.

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