Direct Sampling of Orujo Oil for Determining Residual Hexane by Using a ChemSensor

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ABSTRACT: Hexane is used to extract edible oils from oleaginous seeds. The detection of hexane in orujo oil is mandatory, as its presence in the final product may negatively affect human health. Headspace-GC is the technique of choice for determining residual solvent in foods. In the present work, a new instrument based on the headspace principle and mass spectrometric detection without chromatographic separation, ChemSensor, is proposed for the direct screening of orujo oil to determine residual hexane. This instrument provided an overall response, corresponding to the volatiles profile, including that of hexane, which could not be directly discriminated. By selecting the *m/z* values corresponding to *n*-hexane (major component of commercial hexane), the selectivity of the method was good enough to determine residual hexane in the range of 2.0–65 μg mL^{-1} (corresponding to 2.3–75.6 mg of hexane per kg of oil) with high precision. The detection limit achieved (0.7 mg per kg of oil) was lower than the maximum residual limit established by the European Union (5 mg per kg of oil). Two multivariate techniques, partial least squares and principal components regression (PCR), were compared with univariate regression; PCR provided the best results.

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KEY WORDS: ChemSensor, hexane residues, mass spectrometry, orujo oil, partial least squares, principal components regression.

Hexane is used as solvent to extract edible oils from seed and vegetable crops (e.g., soybeans, peanuts, corn, and olives) (1). In Spain, hexane is used to extract orujo oil from olive residues remaining after obtaining virgin olive oil by pressing the olives (2). Commercial hexane is a mixture of different linear, branched, and cyclic saturated alkanes of low M.W., *n*-hexane being the most abundant. In Europe, the maximum residual limit (MRL) has been established at 5 mg/kg of fat or oil (3). Various residual levels are acceptable for certain products in the United States as approved by U.S. Food and Drug Administration (4). Acute (short-term) inhalation exposure of humans to high levels of hexane causes mild central nervous system effects, including dizziness, giddiness, slight nausea, and headache; chronic (long-term) exposure to hexane in air is associated with polyneuropathy in humans, with numbness in the extremities, muscular weakness, blurred vision, headache, and fatigue. Neurotoxic effects have also been exhibited in rats. Studies do not indicate *n*-hexane is a carcinogen (5).

A large number of methods for detecting and quantifying residual solvent in several matrices have been developed. The majority of them use chromatographic techniques, mainly GC. Owing to the high volatility of the solvent residues, all of the methods employ headspace (HS) techniques (6–8) to introduce the analytes into the GC column; that is, samples are sealed into vials and heated to a specified temperature for a prescribed period of time, depending on the characteristics of the sample. Recent developments have implemented solid-phase microextraction techniques (9) in which different types of fibers are used to preconcentrate residual traces of volatile solvents. Compounds are usually detected by an FID, although MS (9,10) and FTIR (10) have been proposed recently. Solvent residues have been determined in different matrices such as pharmaceutical products (6), synthetic drugs (10), water, food, and animal feeding-stuffs (11), and industrial sludges (12). Among the volatile solvents used, hexane has been usually determined in oil samples such as seed oils (8,13–15) and together with other organic compounds, namely, trichloroethylene (14), tetrachloroethylene (15), or styrene (17). DuPuy *et al.* (18,19) introduced a direct sampling GC method to determine volatiles (hexane) in oils.

Recently, Agilent Technologies (Palo Alto, CA) commercialized a new instrument, the ChemSensor 4440, which is based on direct sampling and MS by means of a headspace autosampler. Few applications for this instrument have been developed to date. We recently proposed its application to characterize olive oil classes by using pattern recognition techniques (20). The aim of the present work was to develop a new application of the ChemSensor for detecting and quantifying residual hexane in refined orujo oil, taking into account that no Official Methods for the determination of hexane residues in oil samples have been established yet, although there are Official Methods for halogenated solvents in oil samples (21). Because chromatographic separation is not necessary with the ChemSensor, the proposed method is simple and provides high sample throughput in comparison to conventional HS-GC. With the ChemSensor, oil samples contaminated with hexane residues are automatically transferred from the autosampler to the heating unit; 3 mL of the generated headspace is directly carried by a helium stream into the mass spectrometer. Volatile fractions of oil samples in the *m/z* range 41 to 100 are mainly obtained; however, to increase the selectivity of the method, only those *m/z* values characteristic

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of *n*-hexane were evaluated. Both univariate and multivariate [partial least squares (PLS) and principal components regression (PCR)] regression techniques were applied for quantifying hexane.

EXPERIMENTAL PROCEDURES

Chemicals and standards. All reagents were of analytical grade or better. *n*-Hexane (HPLC grade) was obtained from Merck (Darmstadt, Germany); commercial hexane (mixture of linear, branched, and saturated cyclic alkanes of low M.W., containing at least 50% *n*-hexane) was purchased from Albus (Madrid, Spain); and pure refined orujo oil samples (without virgin olive oil) were provided by a Spanish oil manufacturer. Standard solutions were prepared from a stock solution of *n*-hexane in pure refined orujo oil at 1.0 mg mL^{-1} and stored in glass-stoppered bottles in the dark at 4°C.

Apparatus. Experiments were carried out with a ChemSensor 4440 system (Agilent Technologies), which included a Hewlett-Packard HP7694 headspace autosampler and a Hewlett-Packard HP5973 mass spectrometer detector. The autosampler included a robotic arm, a 44-space autosampler carousel, and an HS generation unit, which combined an oven to heat the samples inside the vials and a six-port injection valve with a 3-mL loop filled with the HS fraction. Helium (5.0 grade; Air Liquide, Seville, Spain), regulated by a digital pressure and flow controller, was used for both pressurizing the vial (18.0 psi) and carrying the headspace to the detector (2.0 psi). All tubing and the transfer line connected to the heated interface of the detector were passivated with Silicosteel. The second module was a quadrupole mass spectrometer detector operated in full scan mode with a mass range from *m/z* 41 to 100; EI ionization was used with an ionization energy of 70 eV. The transfer line, source, and quadrupole temperatures were maintained at 120, 200, and 120°C, respectively. Total ion current chromatograms were acquired and processed using G1701BA Stand-alone data analysis software (Infometrix Inc., Woodinville, WA) on a Pentium II computer that also controlled the whole system.

Glass flat-bottomed vials (10 mL) for HS analysis (Agilent Technologies) with 20 mm polytetrafluoroethylene/silicone septa (Supelco, Madrid, Spain) were also employed.

Procedure. HS vials (10 mL) were filled with 5.0-mL aliquots of commercial orujo oil samples contaminated with hexane or with standard solutions containing variable amounts of *n*-hexane (prepared in a refined orujo oil blank) and placed into the autosampler. A robotic arm took each vial from the 44-space autosampler carousel and placed it into the oven; the sample was then heated at 90°C with mechanical agitation for 30 min in order to release the hexane residues from the oil matrix to the gaseous phase. Afterward, a needle connected to the injection valve (IV) entered the vial and a helium line pressurized the headspace for 12 s (Fig. 1A). As a result of the difference in pressure between the inside of the vial and the end of the tubing (atmospheric pressure), opening the vent valve caused the headspace fraction containing

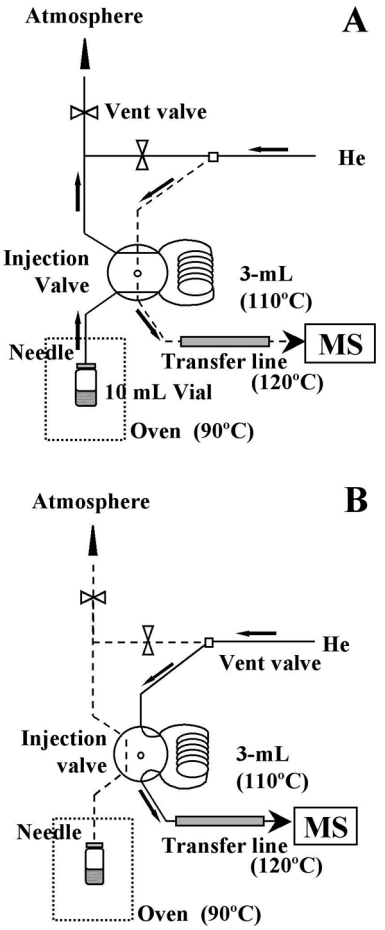

FIG. 1. Diagram of pressurizing/venting (A) and injection (B) positions of the headspace generation unit.

hexane residues to flow out of the vial *via* the needle and fill the 3-mL loop of the IV previously heated at 110°C and released to the atmosphere during 12 s. In a second step (Fig. 1B), the IV was switched and the helium stream carried the loop contents to the mass spectrometer *via* the transfer line, heated at 120°C.

In this method, as no chromatographic separation is used, all the volatile constituents of the oil sample reach the detector simultaneously providing a global signal; however, it is possible to discriminate hexane from other volatiles of the refined orujo oil sample because none of the *m/z* values characteristic of *n*-hexane was significant in any quantity detected in the refined orujo oil blank sample (without hexane).

Regression techniques. Both univariate and multivariate regression techniques were used for data treatment. Univariate calibration was performed for each of the *m/z* values characteristic of *n*-hexane. Multiple linear regression (MLR), PLS, and PCR were applied to the data set obtained from the detector. MLR uses independent variables (i.e., detector responses, x) to predict a dependent variable (i.e., hexane concentration, y): $y = b_1x_1 + b_2x_2 + \cdots + b_nx_n$, where b_i are the partial regression coefficients, whereas PLS and PCR algorithms are based on the combined properties of principal components analysis (PCA), in which new and independent variables are

obtained by linear combination of the original ones. PLS regression uses the same basic data structure as MLR, but the regression algorithm decomposes both the responses matrix (**X**) and concentration matrix (**Y**) into the sum of simpler matrices: $\mathbf{X} = \mathbf{tP}^T + \mathbf{E}_x$ and $\mathbf{Y} = \mathbf{uq}^T + \mathbf{E}_y$, where **t** and **P** are the scores and loading vectors associated with the detector response, **u** and **q** are the scores and loading vectors associated with the concentration matrix, and $\mathbf{E}_x$ and $\mathbf{E}_y$ are the respective residual matrices (22). PCR first applies a PCA algorithm to the original data, raw or preprocessed, and then generates a MLR calibration model from the scores obtained. PLS and PCR both are applied in preference to MLR because they accept collinear data and separate out sample noise (23).

The effects of different preprocessing techniques on the results were tested, namely, no preprocessing, mean-centering, and autoscale. In addition, each algorithm was applied using a different number of variables (*m/z* values): all the scanned variables from *m/z* 41 to 100; eight *m/z* values, characteristic of *n*-hexane; and the three most representative values obtained for the same solvent (*m/z*: 56, 57, and 86). All chemometric analyses were performed by means of the statistical software Pirouette: Multivariate Data Analysis, developed by Infometrix Inc.

RESULTS AND DISCUSSION

Orujo oil is obtained by hexane extraction of the olive residue (called orujo) remaining after pressing olives to obtain virgin olive oil. Its poor sensory characteristics hinder human consumption. Orujo oil must be refined and then mixed with varying amounts of virgin olive oil (*ca*. 5–10%) to improve its organoleptic properties. Residual hexane in the orujo oil is eliminated during deodorization.

Selection of m/z *values.* The ChemSensor used in the proposed method provides only a global response, called a volatiles profile, because all of the volatile fraction present in the oil sample is directly transferred to the MS detector (there is no chromatographic column). Figure 2A shows the volatiles profile (*m/z* 41–100) obtained for a pure refined orujo oil sample containing 100 µg/mL of *n*-hexane standard. In this broad, single signal, the contribution of the organic solvent cannot be distinguished from that of the other volatile compounds. *n*-Hexane can be discriminated/determined by selecting its characteristic *m/z* values. The mass spectrum of a pure refined orujo oil blank spiked with 100 µg/mL of *n*-hexane is depicted in Figure 2B; the characteristic *m/z* values assigned to *n*-hexane were 41 ($C_3H_5^+$, base peak), 43, 53, 55, 56, 57 ($C_4H_9^+$ ion fragment), 71, and 86 (molecular ion, M^+). Because none of these *m/z* values was observed in the mass spectrum of a pure refined orujo oil sample without *n*-hexane (Fig. 2C), they were selected to monitor *n*-hexane in further experiments. Considering that the commercial hexane used in obtaining orujo oil is a mixture of different low-M.W. linear, branched, and cyclic saturated alkanes, *n*-hexane being the most abundant, several experiments were carried out by spiking pure orujo oil with commercial hexane and *n*-hexane. No differences in the mass spectra were observed due to the higher percentage of *n*-hexane in the commercial mixture (at least 50% *n*-hexane) and the similar fragmentation profile obtained for all the isomers.

Optimization of ChemSensor variables. To obtain the best performance of the method for the identification/determination of *n*-hexane, a preliminary optimization step of the instrumental variables involved in the headspace generation was carried out. Samples of pure refined orujo oil containing 50 µg/mL *n*-hexane were analyzed. Initially, all *m/z* values were monitored, but finally *m/z* 57 was chosen to study different variables because of its high signal-to-noise ratio.

First, the sample volume was optimized, because of the marked effect of the HS volume on the analytical signal (9). The volume of sample was varied between 4 and 7 mL. As can be seen in Figure 3A, the *m/z* abundance increased as the sample volume increased, likely a result of the higher *n*-hexane concentration in the HS. Finally, a 5-mL sample volume was selected because it provided better repeatability, 6.6% [as relative SD (RSD), $n = 5$] compared to 8.0% obtained for 7 mL. Other variables related to the *n*-hexane concentration in the headspace were oven temperature and equilibration time; both variables were studied in the interval 70 to 100°C, and between 20 and 35 min, respectively. The signal increased as conditions favoring volatilization of *n*-hexane increased (Figs. 3B,C). An oven temperature and an equilibration time of 90°C and 30 min were chosen to decrease analysis time.

Once the HS has been generated and enriched with the residues of *n*-hexane, sample injection into the detector involves two steps: vial pressurization, and filling of the 3-mL loop of the injection valve by venting the vial. The length of time of both steps affects the amount of *n*-hexane detected by the mass spectrometer. Pressurization and venting times were finally assayed between 0.1 and 0.5 min. No significant changes in the signals of *n*-hexane (*m/z* = 57) were obtained for pressurization times greater than 0.2 min (Fig. 3D), and variation in the venting time (Fig. 3E) showed no effect within the assayed interval. To decrease the analysis time, a value of 0.2 min (12 s) was selected for both variables.

Analytical performance of the method. Several calibration graphs were run for refined orujo oil samples, spiked with different amounts (2.0–65 µg/mL) of an *n*-hexane standard. The abundance for each *m/z* value selected (41, 43, 53, 55, 56, 57, 71, and 86) was plotted against the analyte concentration. The best analytical features were obtained for the three most representative fragments of *n*-hexane, i.e., *m/z* values 56, 57, and 86 (Table 1). Detection limits were calculated as the minimum concentration providing an analytical signal three times higher than background noise. The precision of the method was checked on 11 replicates of a refined orujo oil sample spiked with 10 µg/mL of *n*-hexane. The sensitivity, detection limit, and precision were more favorable at the *m/z* value of 57, which therefore can be used for quantification purposes (Table 1).

Two multivariate regression techniques were studied, namely, PLS and PCR. Both were also applied three times

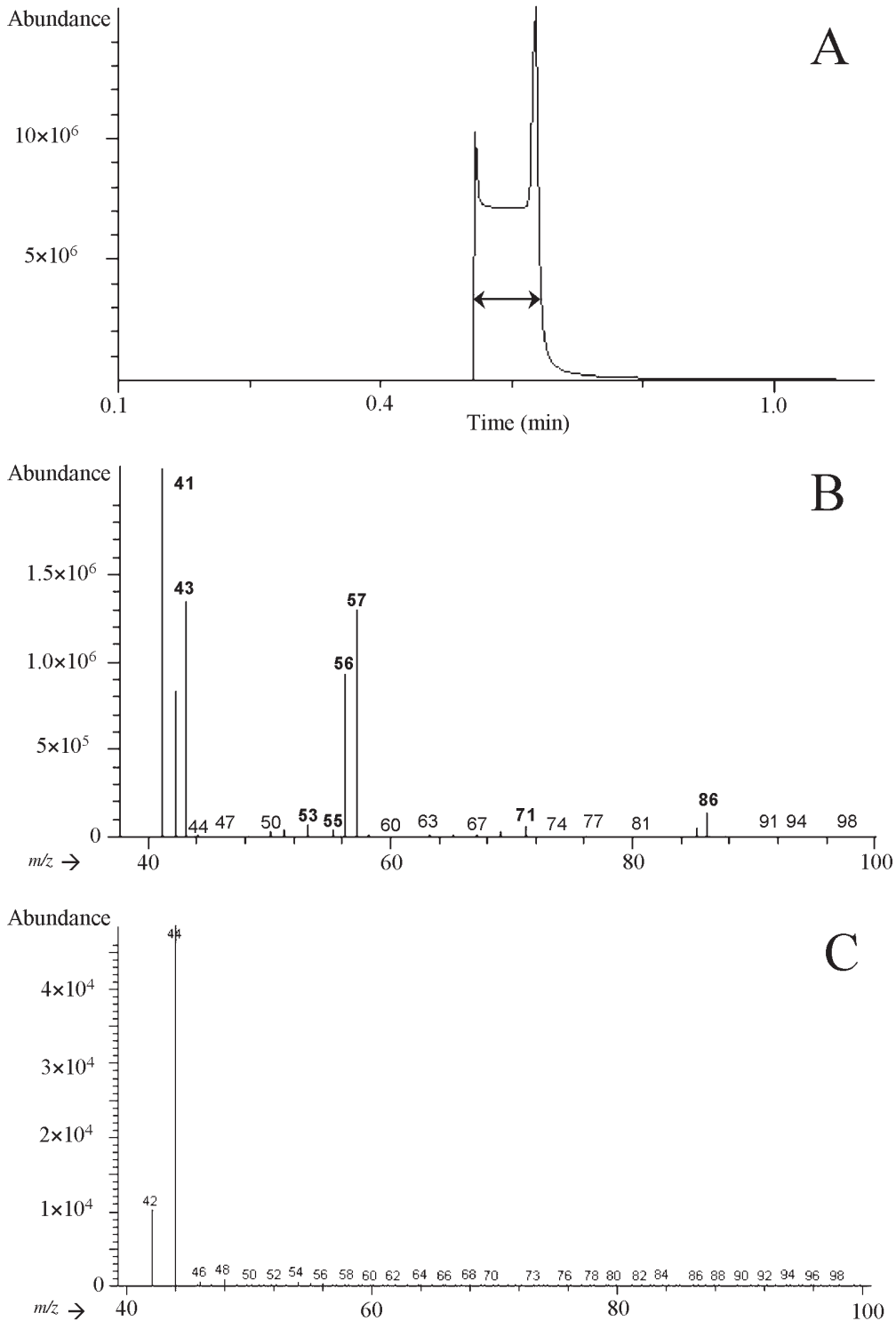

FIG. 2. Volatiles profile (A) and mass spectrum (B) of a refined orujo oil spiked with 100 µg/mL of *n*-hexane, and mass spectrum (C) of a pure refined orujo oil sample without *n*-hexane.

each time using a different number of variables: 3 (using the three most representative *m/z* values, 56, 57, and 86), 8 (the eight *m/z* values selected as characteristic of *n*-hexane, 41, 43, 53, 55, 56, 57, 71, and 86), and 60 (the studied interval of the volatiles profile, from *m/z* 41 to 100). No apparent difference exists between PLS and PCR results. Both yield calibration curves with similar characteristics; however, the correlation coefficient, *r*, of the curves was improved as the number of

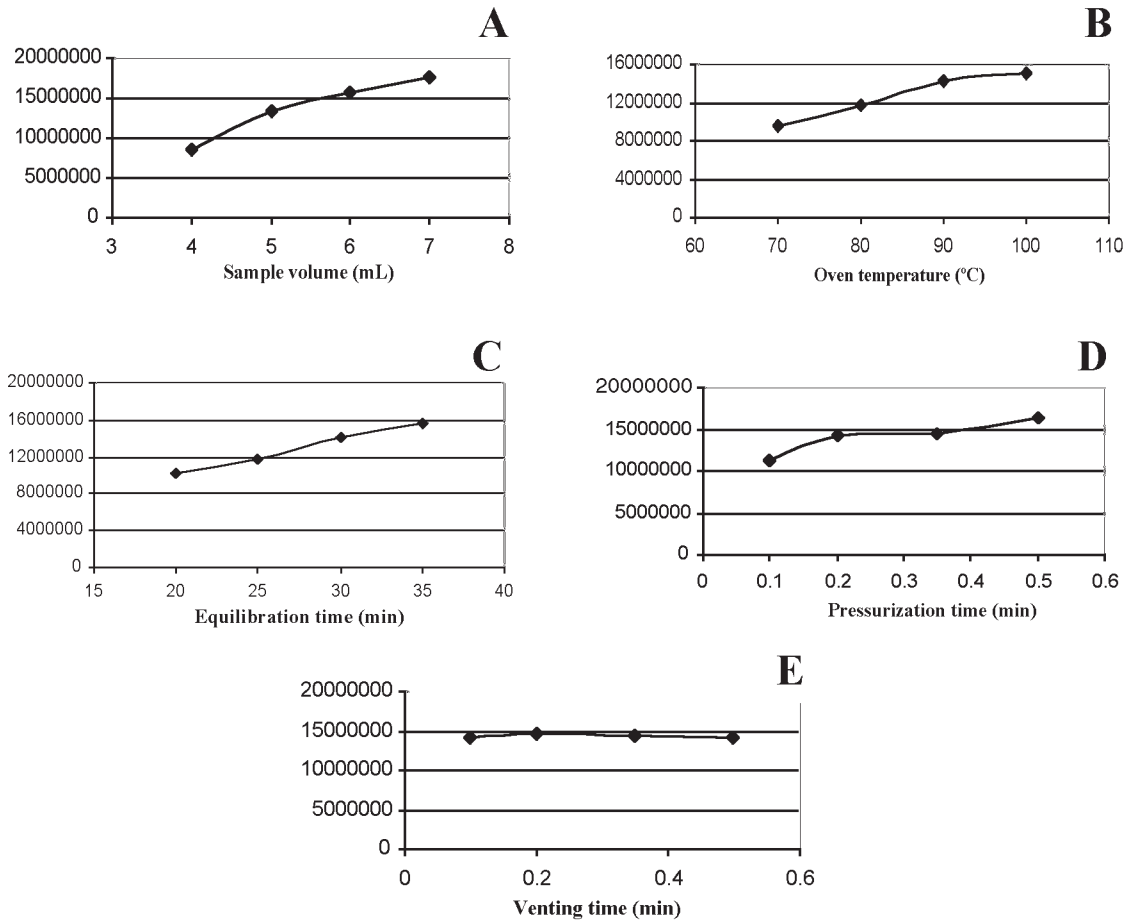

FIG. 3. Influence of five variables related to the headspace generation of a pure refined orujo oil spiked with 50 µg/mL of *n*-hexane. Units for *y* axes: abundance.

variables used increased (the values of *r* were 0.996, 0.998, and 0.999, for 3, 8 and 60 variables, respectively). In addition, various pretreatment techniques (no preprocessing, mean-centering, and autoscale) were also applied to the original data before regressing, with negligible effect on the results.

Application of the method to commercial orujo oil samples. To validate the proposed method, various refined orujo oil samples containing 7.5% of virgin olive oil (commercial orujo oil) were prepared and then spiked with different amounts of hexane (commercial product consisting essentially of a mixture of linear and saturated alkanes of low M.W.), which is used in the industrial extraction of orujo oil. Quality control standards of commercial hexane at six concentration levels (2.5, 5.0, 10.0, 15.0, 25.0, and 50.0 µg/mL) were spiked to commercial orujo oil. The samples were all run in quintuplicate, and the recovery study was done by applying the univariate and multivariate (PLS and PCR) calibration curves obtained in the previous section. The values found by each regression technique are listed in Table 2. The relative errors obtained by the difference between the amount

TABLE 1
Figures of Merit of Calibration Graphs for the Determination of Residual Hexane in Refined Orujo Oil Samples

m/z	Sensitivity[a]	Linear range (µg/mL)	Detn. limit (µg/mL)	RSD (%)	*r*
56	3151	2.5–65	0.8	6.6	0.996
57	4916	2.0–65	0.7	6.2	0.998
86	423	5.0–65	2.0	7.1	0.996

[a]Slope of the calibration curve: abundance/(µg/mL). RSD, relative standard deviation.

TABLE 2
Analysis of Commercial Refined Orujo Oil Containing 7.5% of Virgin Olive Oil, Spiked with Different Amounts of Hexane, and Using the ChemSensor and Several Regression Techniques

Conc. spiked (µg/mL)	Concentration found ± SD (µg/mL)		
	Univariate[a]	PLS[b]	PCR[c]
2.5	2.6 ± 0.2	2.5 ± 0.2	2.6 ± 0.2
5.0	5.2 ± 0.3	5.3 ± 0.3	5.1 ± 0.2
10.0	9.6 ± 0.6	10.3 ± 0.6	10.4 ± 0.5
15.0	14.7 ± 0.8	14.0 ± 0.7	14.8 ± 0.7
25.0	25.4 ± 1.2	24.9 ± 0.9	25.3 ± 0.8
50.0	50.6 ± 2.0	50.3 ± 1.6	49.6 ± 1.2

[a]Univariate regression by using *m/z* 57 calibration graph.
[b]Partial least squares (PLS) regression by employing 8 *m/z* values (41, 43, 53, 55, 56, 57, 71, and 86).
[c]Principal components regression (PCR) by employing 8 *m/z* values (41, 43, 53, 55, 56, 57, 71, and 86).

found and spiked, divided by the concentration added, were low for the three regression techniques (relative errors ranged between −7 and +6%), showing the absence of systematic errors in the proposed method. On the other hand, the precision of the results ($n = 5$), expressed as RSD, was better for multivariate regressions (average RSD values were 5.2 and 4.4 for PLS and PCR, respectively) than for univariate regression by employing *m/z* 57 (RSD = 5.6).

Finally, the proposed method was applied to commercial orujo oil samples, containing different percentages of virgin olive oil (5–10%), which were purchased at various local markets, and about 50 samples were analyzed in quintuplicate. Negative results were obtained for the majority of the samples (concentrations found were lower than the detection limit), and only two samples provided positive results. The concentration of residues of hexane in both positive orujo oil samples (2 and 3 mg/kg oil) were lower than the European Union maximum residue limit (MRL: 5 mg/kg oil), however.

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