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Analysis of Unsaponifiable Compounds of Edible Oils by Automated On-Line Coupling Reversed-Phase Liquid Chromatography−Gas Chromatography Using the Through Oven Transfer Adsorption Desorption Interface

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An automated method for analysis of unsaponifiable compounds in edible oils is presented. The method involves the on-line coupling of reversed-phase liquid chromatography and gas chromatography (LC−GC) using the through oven transfer adsorption desorption (TOTAD) interface. The oil is injected directly with no sample pretreatment step other than filtration. It may also be considered to dilute the oil sample. In the LC step, a short C4 column using a methanol/water eluent separates analytes from the other components of the oils, which are made up of mainly triglycerides. A LC fraction of up to 1.6 mL containing the analytes is transferred to GC at a flow rate of 0.1−2 mL/min. The TOTAD interface allows solvent venting and the introduction of the analytes into the GC column. The proposed fully automated method allows the analysis of different groups of compounds (free sterols, tocopherols, squalene, and erythrodiol and uvaol) in one chromatographic run or the analysis of these compounds in different groups. Sensitivity is more than necessary, and repeatability is good, the CV ranging from 3 to 12% for the full analysis.

KEYWORDS: Reversed-phase LC−GC; on-line coupling; TOTAD interface; unsaponifiable compounds; edible oils

1. INTRODUCTION

Vegetable oils are mainly composed of triacylglycerols (95−98%) and complex mixtures of minor compounds (2−5%) of a wide-ranging chemical nature. These minor constituents must be analyzed as they are used as a reference for edible oil regulation and for the analytical assessment of oil quality, origin, extraction method, refining procedure, and possible adulteration (*1*). The European Union (EU) has established maximum values for certain components to prevent adulteration (EEC 2568/91). For instance, analysis of the triterpene dialcohols, erythrodiol and uvaol, is used to distinguish between olive oils of different qualities such as cold-pressed (extra virgin) and solvent-extracted oils (*2*), in which the concentration of triterpene dialcohols is higher. An upper legal limit of the sum of erythrodiol and uvaol has been established for virgin olive oil. The sterol fraction is usually analyzed to identify a particular fat or an oil, for the detection of added nondeclared cheap oils to more expensive oils (*3*), to establish different qualities of the same oil (*4*), and to typify olive oils from certain geographic zones (*5*, *6*). Squalene is a terpenoid hydrocarbon found in high concentrations (60−75%) in the unsaponifiable fraction of olive oil. As this compound is present in olive oils in much higher quantities than in other vegetable oils, its assessment may be used to detect adulterations (*7*).

The methods mainly used for the analysis of different fractions of edible oils involve several steps, namely, the saponification of the oil to remove triglycerides, the fractionation of the unsaponifiable matter into several classes of compounds by thin layer chromatography (TLC) or preparative high-performance liquid chromatography (HPLC), and their subsequent analysis by gas chromatography as trimethylsilyl (TMS) derivatives (except for the hydrocarbons) in nonpolar capillary gas chromatography columns (*8*). These methods are described in different regulations (*9*, *10*).

It is widely recognized that these conventional methods are tedious and time-consuming and may also involve the loss of analytes. On-line coupling of reversed-phase liquid chromatography and gas chromatography (LC−GC) is a technique that combines the high separation efficiency of HPLC for sample preparation, avoiding saponification and replacing the subse-

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quent cleanup steps with the high performance of capillary gas chromatography separation (*11*−*14*). It eliminates almost all manual work, allowing one person to run a large number of samples in a short time.

Grob and co-workers proposed on-line LC−GC methods for the analysis of the minor components of edible oils, using the normal phase in the LC preseparation step and previous derivatization of the compounds to be analyzed (*1*, *2*, *15*, *16*). The use of the normal phase in the LC step requires back-flushing of the column after each analysis to eliminate any retained lipids and may cause several problems, which can affect the performance of the column, for example, deactivation of the silica gel by the triglycerides. The use of the reversed phase in the LC step instead of the normal phase prevents these problems. However, the fact that transfer of polar solvents can be difficult because of the very large volumes of vapor that are produced per unit of liquid must be taken into account (*3*, *17*).

Research on RPLC−GC has focused on the development of different interfaces suited to the transfer of aqueous eluents (*9*, *18*, *19*), and several problems have been prevented by extending the range of applicability of the technique. In this respect, previous work on RPLC-GC using a programmed temperature vaporizer (PTV) as an interface has already proven to be useful for the analysis of minor components, e.g., the free sterols of edible oils (*4*, *17*, *20*). The extremely large volume of vapor resulting from the aqueous eluent during LC−GC transfer was eliminated by removing the GC column end from the injector body. However, with this procedure, it is not possible to automate the system.

Our research work has developed an automated interface, named the TOTAD interface, which has been satisfactorily used for the on-line coupling RPLC−GC analysis of different type of pesticides in olive oil (*21*, *22*). The purpose of this work is to develop an automated and rapid method for the direct analysis of minor components in edible oils using the TOTAD interface, which allows analysis of the different groups of compounds in only one run or the analysis of only one group of compounds in determining quality, origin, extraction method, or possible adulteration of the oil.

2. EXPERIMENTAL PROCEDURES

2.1. Materials. Several edible oils were purchased from a local market: virgin olive oil, virgin sunflower oil, virgin soybean oil, virgin sesame oil, and virgin wheat-germ oil. Standards of some minor components (uvaol, erythrodiol, stigmasterol, β-sitosterol, α-tocopherol, δ-tocopherol, and squalene) were provided by Dr. Herraiz from the Instituto de Fermentaciones Industriales (Consejo Superior de Investigaciones Científicas, Madrid, Spain). The 2-propanol used to dilute the olive oil and methanol and water used as the mobile phase, all HPLC-grade, were purchased from LabScan (Dublin, Ireland).

Tenax TA 80−100 mesh (Chrompack, Middelburg, The Netherlands) was used as packing material in the liner of the PTV and placed between two plugs of glass wool to keep it in place. The packed liner was conditioned under a helium stream, which was heated from 50 to 350 °C at 50 °C/10 min and maintained for 60 min at this final temperature.

2.2. Sample Preparation. The oil samples were filtered through a 0.22 μm filter (Chromatography Research Supplies, Inc.). Nondiluted oils and the three different dilutions of the oils with 2-propanol [1:20, 1:50, and 1:200 (v/v)] were considered.

2.3. Instrumentation. The analyses were performed using on-line coupled LC−GC equipment fitted with an automated TOTAD interface, U.S. Patent 6,402,947 B1 (exclusive rights assigned to KONIK-Tech, Sant Cugat del Vallés, Barcelona, Spain). The TOTAD interface operation mode has been described previously (*23*−*25*).

The HPLC system comprised a manual injection valve (model 7125, Rheodyne) with a 20 μL loop, a quaternary pump (HP model 1100), a column oven (HP model 1100), and a diode array ultraviolet (UV) detector (Perkin-Elmer model LC 235). The gas chromatograph (Konik model HRGC 4000B) was equipped with a TOTAD interface and a FID detector. KoniKrom 32 (Konik, Sant Cugat Del Vallés) was used to obtain data from LC and GC runs and to automate the process.

2.4. LC Conditions. LC preseparation was carried out on a 50 mm × 4.6 mm (inside diameter) column packed with modified silica (C4, kromasil 100−10, Hichrom, Berks, U.K.) maintained at 45 °C. Methanol and water were used as the eluent.

Oil (20 μL) or the standard solutions (20 μL) were injected to ascertain the elution time of the fraction to be transferred to the gas chromatograph. The initial composition of the eluent [methanol/water, 70:30 or 95:5 (v/v)] at a flow rate of 2 mL/min was maintained for 5 min, before the gradient was varied to reach 100% methanol within 1 min and maintained for 20 min. UV detection was performed at 205 and 255 nm.

In the analysis of the minor components, the LC detector was not used and the LC column was directly connected to the six-port valve by a stainless steel tube (0.25 mm inside diameter). Oil (20 μL, diluted or not) was injected. The composition of the eluent [95:5 (v/v)] was kept constant. The flow rate was 2 mL/min until the beginning of the elution of the fraction of interest. During the transfer step, three different flow rates (0.1, 1, and 2 mL/min) were used. The flow rate was kept constant until the transfer step had reached completion. After the transfer, the flow rate was increased to 2 mL/min again, and the gradient was changed to 100% methanol within 1 min and maintained for 20 min to ensure complete elimination of the retained lipids.

2.5. LC−GC Transfer. The liner was packed with a 1 cm length of Tenax TA between two glass wool plugs. Initially, the TOTAD interface was stabilized at 80 °C. The helium flow rate used to push the eluent coming from the liquid chromatograph through the sorbent was 500 or 1600 mL/min, and a rate of 500 mL/min was used to prevent solvent condensation of the vented solvent. The GC oven temperature was maintained at 60 °C during the transfer.

Once the transfer step was completed, the temperature and helium flow were kept constant for 1 min to ensure that all the remaining solvent was eliminated from the glass liner and the transfer tubing. After this time, the helium flow was changed and the TOTAD interface was quickly heated to 325 °C for 5 min, leading to the thermal desorption of the analytes, which were pushed by the helium to the GC column.

2.6. GC Conditions. A fused-silica column [30 m × 0.32 mm (inside diameter)] coated with 5% phenyl methyl silicone (film thickness of 0.25 μm) from Quadrex (Weybridge, U.K.) was used for the gas chromatographic separations. During the transfer and the solvent elimination steps, the oven temperature was kept at 60 °C. During GC−FID analysis, the column temperature was maintained at 60 °C for 1 min. It was then increased to 240 °C at a rate of 40 °C/min for 5 min, to 290 °C at a rate of 4 °C/min for 5 min, and finally to 320 °C at a rate of 5 °C/min for 5 min. The FID temperature was kept at 320 °C. Helium was used as the carrier gas at a flow rate of 1.8 mL/min.

3. RESULTS AND DISCUSSION

3.1. LC Strategy. The experimental conditions that were established enabled the simultaneous analysis of four classes of compounds (sterols, tocopherols, squalene, and the triterpene dialcohols, erythrodiol and uvaol) either together or separately. In this respect, it is especially important to establish experimental conditions in such a way that the components of the different groups of compounds of interest do not overlap the major components of the oil, especially the triglycerides in the LC preseparation step. Previous results concerning the analysis of pesticide residues in olive oil (*21*, *22*) were taken into consideration, since in these previous studies the different fractions of the olive oil constituents were identified according to Señorans and co-workers (*20*). Other authors have used coupled RPLC−GC to analyze erythrodiol and uvaol in olive oils (*4*) and free sterols, tocopherols, and squalene in edible oil (*17*), but it was not possible to automate the analysis or to carry

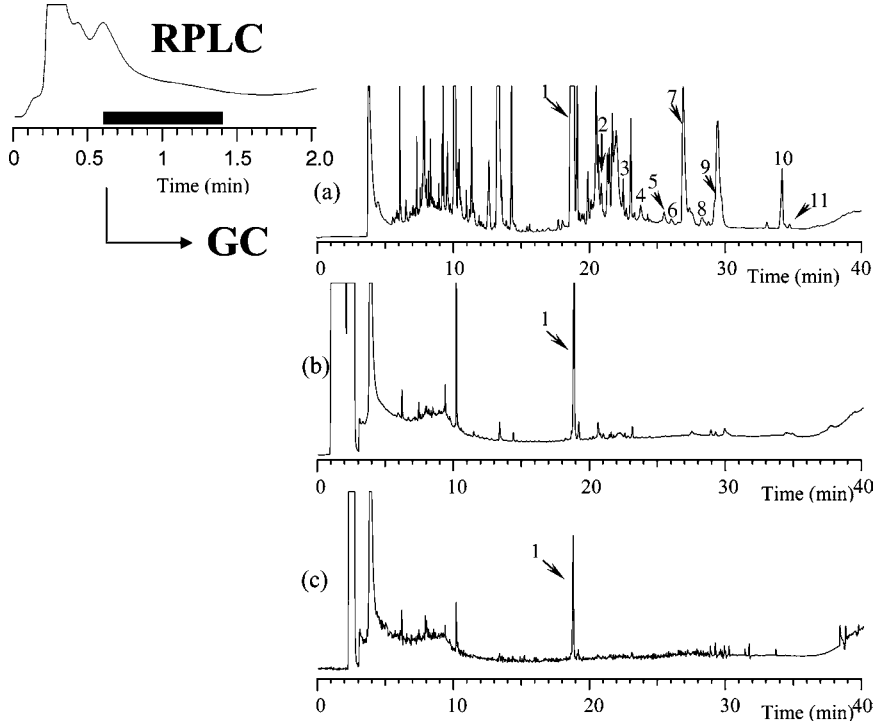

Figure 1. LC and GC chromatograms obtained from the direct RPLC–GC–FID analysis of a virgin olive oil sample. Twenty microliters of olive oil diluted 1:50 in 2-propanol was injected into the liquid chromatograph at different transfer rates: (**a**) 0.1, (**b**) 1, and (**c**) 2 mL/min. The thick line situated between the time axis and the chromatogram indicates the LC fraction that has been transferred from the liquid chromatograph to the gas chromatograph. Identified peaks: (1) squalene, (2) δ-tocopherol, (3) γ-tocopherol, (4) α-tocopherol, (5) campesterol, (6) stigmasterol, (7) β-sitosterol, (8) Δ^7-stigmasterol, (9) Δ^7-avenasterol, (10) erythrodiol, and (11) uvaol.

out the analysis of the four classes of compounds in only one run. In these previous papers (*4*, *17*, *20*−*22*), the characteristics of the LC column were exactly the same as those of the column employed in this work. According to these authors, for the simultaneous assessment of all the desired compounds, and using methanol and water (70:30) as the eluent in the LC preseparation step, the four classes of compounds must be transferred to the GC system within the same fraction. For this, the LC volume to be transferred would be at least 18 mL. It is worth emphasizing that this same volume can easily be transferred when using the TOTAD interface (*25*) because solvent elimination is almost complete as it takes place simultaneously in both the evaporative and nonevaporative mode. In this respect, obtaining a narrow LC fraction containing the components of interest was not a priority as it is in other LC−GC methods (*15*). Nevertheless, given that the flow transfer rate was 0.1 mL/min, it would take almost 3 h to carry out the transfer step. It would be possible to increase the transfer flow rate to shorten the transfer time, but the sensitivity decreases at higher speeds, as previously pointed out (*27*, *28*). Another option for decreasing the volume to be transferred is to change the eluent composition. Using a 95:5 (v/v) methanol/water eluent, the volume to be transferred is much lower. As seen in the LC chromatogram **(Figure 1**), the fraction corresponding to the total of minor compounds eluted between 0.6 and 1.4 min. The flow rate in the LC system was 2 mL/min; therefore, the resulting volume of the fraction to transfer was 1.6 mL, and the transfer step took 16 min at a flow rate of 0.1 mL/min.

3.2. Sample Dilution. When 20 μL of virgin olive oil was injected into the RPLC−GC system, the GC signal of some compounds became saturated, a limitation that was overcome by diluting the olive oil in 2-propanol. Two dilutions, 1:20 and 1:50, were tested. When the 1:20 dilution was used, some compounds remained saturated, so a 1:50 dilution was chosen to carry out the analysis. The GC chromatogram that was obtained is shown in **Figure 1a**. As we can see, free sterols, tocopherols, squalene, and the triterpene dialcohols can be separated in one chromatographic run, which represents a great simplification compared with conventional methods, which require laborious sample preparation.

3.3. Peak Identification. Peak identification was carried out by separately injecting the standard solutions with the available minor compounds (50 mg/L) in methanol. Some additional peaks, which remained unidentified since it was not possible to obtain standards for all the sterols and tocopherols, were identified by comparing the GC chromatograms of different oils. To this end, the described composition of minor compounds of each oil was considered. The peaks that were identified agreed with those obtained by other authors (*17*, *29*, *30*). The GC chromatogram of **Figure 1a** clearly shows the abundance of β-sitosterol and the smaller campesterol and stigmasterol peaks typical of olive oil mentioned by other authors (*15*).

3.4. Application to Different Oils. The minor components of the unsaponifiable fraction are useful for recognizing the origin of the lipids from which they were extracted, since the qualitative and quantitative distribution of many compounds is characteristic of the lipid source. **Figure 2** shows the GC chromatograms obtained in the analyses of four different oils: virgin sesame, virgin sunflower, virgin soybean, and virgin wheat-germ oil. The different compositions of minor components among the oils can be observed.

3.5. Split in Different Fractions. When only one class of compounds is to be analyzed, the LC fraction to be transferred to the gas chromatograph must be adjusted to include only this class of compound. **Figure 3** shows both the LC chromatogram of an olive oil where two different fractions are indicated and the GC chromatograms obtained after transfer of both LC fractions to the gas chromatograph (in two different runs), as

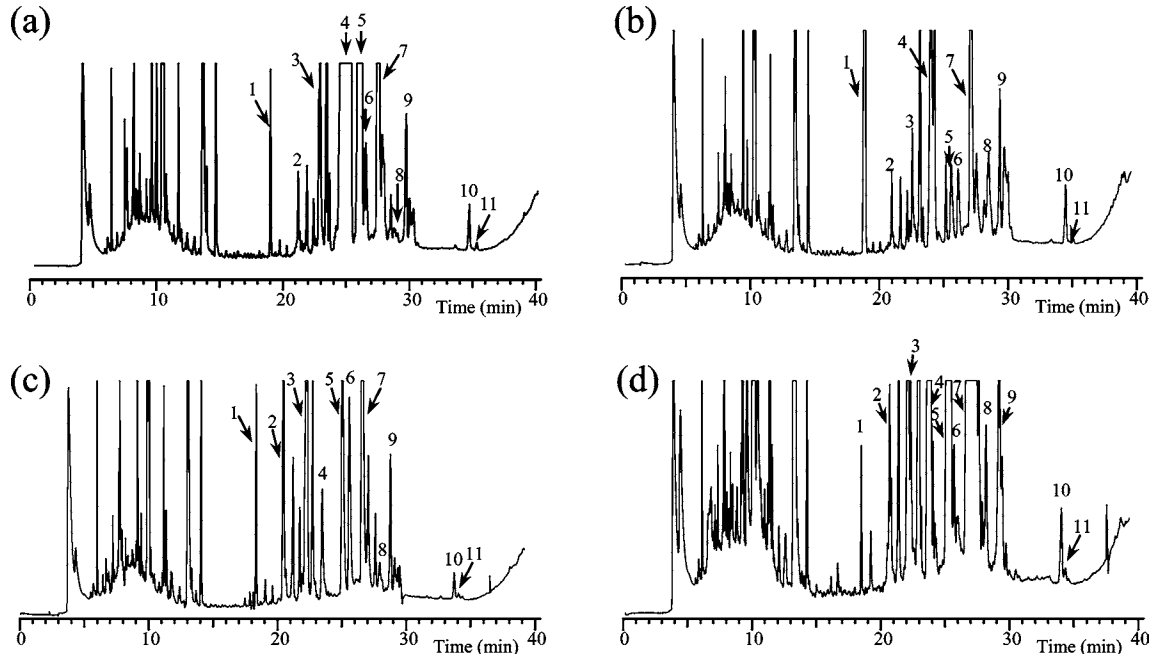

Figure 2. GC chromatograms obtained from the direct RPLC–GC–FID analysis of different edible oil samples: (**a**) virgin sesame oil, (**b**) virgin sunflower oil, (**c**) virgin soybean oil, and (**d**) virgin wheat-germ oil. Conditions and identification of peaks like those for **Figure 1**.

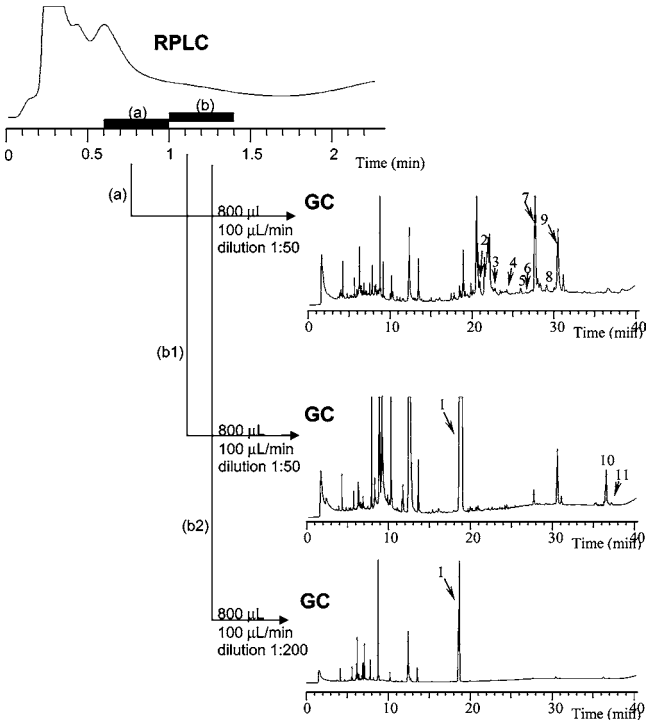

Figure 3. GC chromatograms obtained from the direct RPLC–GC–FID analysis of the two fractions indicated in the LC chromatogram. Identification of peaks like that for **Figure 1**: (**a** and **b1**) conditions like those for **Figure 1a** and (**b2**) olive oil diluted 1:200 with 2-propanol.

described in the Experimental Procedures. The first fraction, from 0.6 to 1 min, corresponds to the sterol and tocopherol (**Figure 3a**), and the second fraction, from 1 to 1.4 min, corresponds to squalene and the triterpene dialcohols **(Figure 3b1**). Since squalene is present in olive oils in much larger quantities than in other edible oils, it is useful to be able to identify this terpenoid hydrocarbon to detect possible adulterations of olive oils. As seen in **Figure 3b1**, the squalene peak overloaded. To obtain a more reliable quantification of squalene, the olive oil was diluted 1:200 in 2-propanol and the second fraction was analyzed. In this case, the peaks of erythrodiol and uvaol did not appear due to the dilution, but squalene can be quantified more accurately **(Figure 3b2**).

Table 1. Coefficients of Variation (CV) for Absolute Peak Areas and Retention Times Resulting from the Analysis of the Total Fraction (0.6–1.4 min) of Minor Components ($n = 5$) and Coefficients of Variation (CV) for Absolute Peak Areas for Delimited Fraction a (from 0.6 to 1.0 min) and b (from 1.0 to 1.4 min) ($n = 3$)[a]

		total		CV (area)		
peak	minor component	CV (area)	CV (t_r)	fraction a	fraction b1	fraction b2
1	squalene	3	0.1	–	4	0.9
2	**δ-tocopherol**	4	0.1	6	–	–
3	**γ-tocopherol**	9	0.1	7	–	–
4	**α-tocopherol**	12	0.2	11	–	–
5	campesterol	6	0.2	6	–	–
6	stigmasterol	8	0.1	9	–	–
7	**β-sitosterol**	5	0.2	8	–	–
8	Δ^7-stigmasterol	10	0.2	10	–	–
9	Δ^7-avenasterol	3	0.1	3	–	–
10	erythrodiol	11	0.3	–	6	–
11	uvaol	9	0.2	–	6	–

[a] Twenty microliters of olive oil diluted 1:50 in 2-propanol was injected for the total fraction and fractions a and b1. Twenty microliters of olive oil diluted 1:200 in 2-propanol was injected for fraction b2.

3.6. Method Validation. The precision obtained with the RPLC−GC analytical method was evaluated. **Table 1** lists the coefficient of variation (CV) values calculated from the absolute peak areas and from the retention times for the different fractions analyzed and for the overall analysis. As one can see, no variability in retention time was observed. The CV of the absolute peak areas was less than 12%. It should be stressed that these CV values correspond to the full analysis, as no sample pretreatment was carried out.

3.7. Increase in the Rate of Transfer Flow. The effect of the flow rate during the transfer step on the sensitivity is shown in **Figure 1**. As one can see, a higher flow rate caused a lower

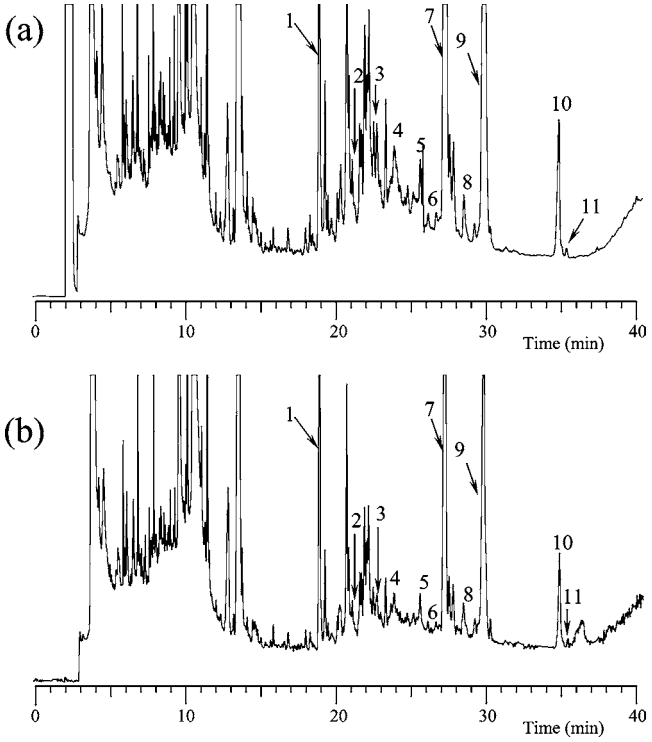

Figure 4. GC chromatograms obtained from the direct RPLC–GC–FID analysis of a nondiluted virgin olive oil sample transferred at a rate of 2 mL/min and at different helium flow rates through the sorbent: (**a**) 500 and (**b**) 1600 mL/min. Identification of peaks like that for **Figure 1**.

sensitivity and poorer solvent elimination, although solvent elimination can be considered good in any case. At the highest flow rate used during the transfer step, only squalene could be clearly identified when 20 μL of virgin olive oil diluted 1:50 in 2-propanol was injected. It is clear that a lower flow rate during the transfer step implies a longer overall analysis time. Bearing in mind that olive oil was diluted in 2-propanol because some compounds saturated the GC signal, we were able to achieve a similar sensitivity at the highest transfer step flow rate (2 mL/min), which represents less sensitive conditions, by sampling 20 μL of nondiluted virgin olive oil, as shown in **Figure 4**.

This increase in the transfer flow rate led us to test the flow rate of the helium pushing the transferred fraction through the sorbent. Two different rates, 500 and 1600 mL/min, were tested in an effort to improve solvent elimination. As shown in **Figure 4**, solvent elimination at 500 mL/min is sufficiently good even with a 2 mL/min flow rate during the transfer step. Furthermore, better sensitivity can be achieved and the amount of gas saved substantial if many samples have to be analyzed. One recommendation that might be made is that if the analyst has not worked with the TOTAD interface before, it would be advisable to dilute the oil and to carry out the transfer at 0.1 mL/min. Under such conditions, any mistake that caused flooding of the GC system would not significantly damage the system. Once the analyst has gained experience, undiluted oil could be used and the rate of transfer increased to 2 mL/min.

3.8. Conclusions. These experiments demonstrate the flexibility of the TOTAD interface, which allows the transfer from the reversed-phase liquid chromatograph to the gas chromatograph of different groups of compounds, the volume of the eluent transferred being as large as 1.6 mL (it is possible to transfer larger volumes, although it is not necessary in this case), while good performance is maintained at different eluent and helium flow rates during the transfer step.

The proposed method allows the automated and simultaneous analysis of different groups of minor components in edible oils in only one run, because the whole procedure (LC preseparation, LC−GC transfer, and GC analysis) takes ~60 min. In addition, it is possible to analyze one group of compounds, by including in the LC fraction only the class of compounds to be considered. The described method shows good repeatability and sensitivity. Filtered oil, diluted or not, is directly injected, preventing saponification and derivatization. The TOTAD interface is shown to be highly suitable for the automation of RPLC−GC systems, an advantage that practically eliminates any time-consuming sample preparation step and prevents errors caused by sample manipulation.

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