Off-Line Coupling of High-Performance Liquid Chromatography and ^{1}H Nuclear Magnetic Resonance for the Identification of Filbertone in Hazelnut Oil

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ABSTRACT: A new procedure is presented for off-line coupling of high-performance liquid chromatography and proton nuclear magnetic resonance spectroscopy (^{1}H NMR) in hazelnut oil analysis. The optimization of some parameters affecting both the liquid chromatography preseparation step and the effective multiple-solvent suppression required for the NMR study enabled us to determine the presence in a hazelnut oil of (*E*)-5-methyl-hept-2-en-4-one (filbertone), a marker previously proposed to detect the adulteration of olive oil with hazelnut oil. The described procedure requires the filtration of the oil prior to its introduction into the chromatographic system and combines the advantages of providing sufficient sensitivity and selectivity with simple methodology and reduced sample handling.

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KEY WORDS: Filbertone, hazelnut oil, ^{1}H NMR, LC preseparation, off-line LC-^{1}H NMR.

Demand is strong for the development of new analytical methods to safeguard the quality and authenticity of olive oils in order to avoid their adulteration with other edible oils of lower commercial value.

Specifically, the detection of the adulteration of olive oil with hazelnut oil is quite difficult owing to the similar composition of their fatty acids. To this aim, several methods have previously been proposed, including, for example, the measurement of the $^{13}C/^{12}C$ ratios of fatty acids using gas chromatography coupled with isotopic ratio mass spectrometry (1), the study of linolenic acid content (2), or the determination of the esters of glycerol and fatty acids (3).

It has been reported that analysis of natural minor volatile components may also be relevant in determining the sensory and nutritional quality of virgin olive oil or in distinguishing between different commercial qualities (2,4,5). Recently, we proposed the presence or absence in a specific sample of *(E)*-5-methyl-hept-2-en-4-one (filbertone), a flavor compound in hazelnuts, as an adequate marker to distinguish between hazelnut oil and olive oil (6,7). This approach seems to be promising but must be confirmed for hazelnut oils of different characteristics and geographical origins. In this respect, it is mandatory to guarantee the reliable and rapid detection of filbertone in oil, preferably by direct analysis (i.e., with a minimum sample pretreatment).

On the other hand, in the last few years nuclear magnetic resonance (NMR) has played an ever-increasing role in the study of edible oils. Several authors have reported the advantages of NMR compared with conventional methods for the assessment of the authenticity of olive oil. The possibility of avoiding decomposition or isomerization reactions contributes to the reliability of the analysis, but overlapping signals in the spectrum may make the identification of some specific compounds difficult. Previous research mainly focused on the study of fatty acids and the triglyceride fraction (3, 8–12), but some authors have applied NMR to the study of the unsaponifiable fraction of the oil (13) as well as to the identification of volatile compounds, in particular, unsaturated and saturated aldehydes (2,9,14). However, no data have been published so far concerning the identification of filbertone in edible oils by NMR, although doing so would take into account both the potential of NMR for oil analysis and the previously reported usefulness of filbertone as a marker for distinguishing between hazelnut oil and olive oil. The aim of the present work was to evaluate the use of proton NMR spectroscopy to establish the presence of filbertone in hazelnut oil. Furthermore, we tested the possibility of using high-performance liquid chromatography coupled with NMR (LC–NMR) for the rapid screening of edible oils on the basis of the detection of the presence or the absence of filbertone.

EXPERIMENTAL PROCEDURES

Materials. Hazelnut oil was obtained from the commercial market. The filbertone used for identification purposes was acquired from Haarman & Reimer (Holzminden, Germany), and deuterated chloroform was purchased from Cortec (Paris, France). Methanol (HPLC grade) and water used as eluent in the LC step were obtained from Scharlau Chemie, S.A. (Barcelona, Spain) with a Milli-Q water purification system (Millipore, Milford, MA), respectively. In all cases, the oil was filtered through a 0.22-μm filter prior to its sampling into the HPLC equipment.

LC preseparation. A Hewlett-Packard (Wilmington, DE) model 1050 liquid chromatograph equipped with a manual

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injection valve (model 7125; Rheodyne, Cotati, CA) was used for the preseparation of the oil. All analyses were accomplished using a 5 cm × 4.6 mm i.d. Kromasil column (pore size, 100 Å; particle size, 10 µm; phase, C_4; Symta, Madrid, Spain) and an ultraviolet detector, methanol/water (65:35) being the eluant (2000 µL/min). To achieve the required sensitivity to obtain the NMR spectrum of filbertone, different sample loops having volumes ranging from 20 to 500 µL were tested.

Solid phase extraction (SPE). To achieve the enrichment required for the NMR study, an SPE step was also performed using C18 (octadecyl) as an adsorbent. Prior to its use, this adsorbent was pretreated with 3 mL of methanol and 3 mL of distilled water. The accumulated fractions from the LC preseparation, in a number that depends on the loop-type used in each case as detailed below, were concentrated by pouring them through the SPE cartridge. Once the adsorption was complete, the retained compounds were subsequently eluted with the lowest possible volume (1.0 mL) of $CDCl_3$.

NMR analysis. The ^{1}H NMR spectrum of filbertone standard was recorded from a solution of 10 mg of compound dissolved in a 0.7-mL volume of $CDCl_3$. All NMR spectra of filbertone in hazelnut oil were performed using a 5-mm tube in which a 0.7-mL volume of the SPE-extracts was placed. The spectra were recorded at 293 K on a Bruker DPX300 spectrometer using an inverse broad-band probehead equipped with shielded Z-gradient coil. Multiple solvent suppression was achieved by applying a recently proposed pulse sequence (15,16). This is a modification of the original H_2O excitation using the basic principles of the excitation sculpting methodology previously reported (17) and based on the double pulse field gradient echo DPFGSE principle. The spectra in Figure 2C was acquired with 3000 scans and 4 dummy scans. The gradient length was 1.5 ms and the gradient values were G1 = 10.7 and G2 = 18.2 G cm^{-1}. The selective 180° pulses were of 1.72 ms duration with a rectangular shape applied on resonance on each solvent signal.

Gas chromatography (GC) analysis. The recoveries, relative standard deviations, and detection limits resulting from the procedure, including both the LC preseparation performed under different experimental conditions and the subsequent SPE concentration, were estimated from the chromatograms obtained by GC analysis of filbertone. For the GC system, a gas chromatograph (PerkinElmer, Norwalk, CT) model 8500 fitted with a PTV-injector (programmed temperature vaporizer) operated in the splitless mode and a flame-ionization detection system (FID) was employed. A 25 m × 0.25 mm i.d. fused-silica capillary column coated with a 0.25-µm layer of Chirasil-β-Dex was used, and the GC oven was initially maintained at 45ºC for 5 min and then programmed to 160ºC at 4ºC/min.

RESULTS AND DISCUSSION

Initial attempts to identify filbertone in hazelnut oil by direct NMR analysis (i.e., without any workup) were unsuccessful, as the sensitivity (signal-to-noise ratio) achievable in the analysis was not sufficient to detect its presence, even after having optimized the oil concentration in the NMR tube. Moreover, even the use of both a Bruker AMX 500 and a Bruker AMX 600 operated under different experimental values for the acquisition parameters (i.e., time domain, spectral width, relaxation delay, and number of scans) did not enable us to obtain satisfactory results. As an example, Figures 1A and 1B show data obtained from a hazelnut oil with the mentioned spectrometers and include in each case the expansion of the spectral ranges of both 0.5–8.0 ppm and 5.80–7.10 ppm. For the sake of comparison, the NMR spectrum obtained for the standard of filbertone is also included (see Fig. 1C). As expected, typical resonances for filbertone were easily identified in the last spectrum, namely, a triplet centered at 0.90 ppm (H7, $J_{6,7}$ = 7.4 Hz), a doublet at 1.05 ppm (H8, $J_{5,8}$ = 6.8 Hz), and a double doublet centered at 1.90 ppm (H1, $J_{1,2}$ = 6.8 Hz; $J_{1,3}$ = 1.6 Hz) due to the methyl protons in positions 7, 8, and 1, respectively; a sextuplet at 2.67 ppm (H5, $J_{5,8} = J_{5,6}$ = 6.8 Hz) assigned to the methine proton in C5 and two multiplets centered at 1.40 and 1.70 ppm due to the methylenic protons in C6. The lower-field double quadruplets centered at 6.90 (H2, $J_{2,3}$ = 15.5 Hz; $J_{1,2}$ = 6.8 Hz) and 6.20 ppm (H3, $J_{2,3}$ = 15.5 Hz; $J_{1,3}$ = 1.6 Hz) were assigned to the *trans* olefinic protons in C2 and C3.

However, characteristic resonances for filbertone were not observed in the spectra shown in Figures 1A and 1B even when up to 10,000 scans were recorded for each tube (total acquisition time equal to 12 h) and a careful baseline correction was performed in both cases. Further attempts to increase the performance of the analysis with these spectrometers, for example, by recording a higher number of scans, were not successful as they resulted in an inadmissible increase of the time of analysis and a clear loss of resolution.

Therefore, we decided to perform both a cleanup and a concentration step and to carry out the experimentation with the spectrometer described in the Experimental Procedures section, operating at 300 MHz. According to our previous experience in the on-line LC–GC analysis of different edible oils (18,19), an HPLC preseparation was used to select the fraction containing filbertone, although the experimental conditions previously reported did not allow obtaining satisfactory results to be obtained. For that reason, different sample loops (i.e., 20, 100, 275, and 500 µL) were prepared to investigate their usefulness to achieve the required sensitivity for the

TABLE 1
Recovery, Relative Standard Deviation (RSD), Detection Limit (DL), and Number of Accumulated Fractions Obtained for the LC Preseparation of Filbertone with Different Loop Volumes

Loop volume (µL)	Recovery[a,b] (%)	RSD[a,b] (%)	DL[a,c] (ppm)	Number of fractions
20	—	—	—	25
100	88	4	0.52	5
275	76	9	0.40	2
500	100	32	0.40	1

[a]Estimated from the gas chromatographic analysis of the collected fractions.
[b]Average value of three replicates.
[c]Calculated as the amount of product giving a signal equal to five times the background noise (determined from the width of a baseline). LC, high-performance liquid chromatography.

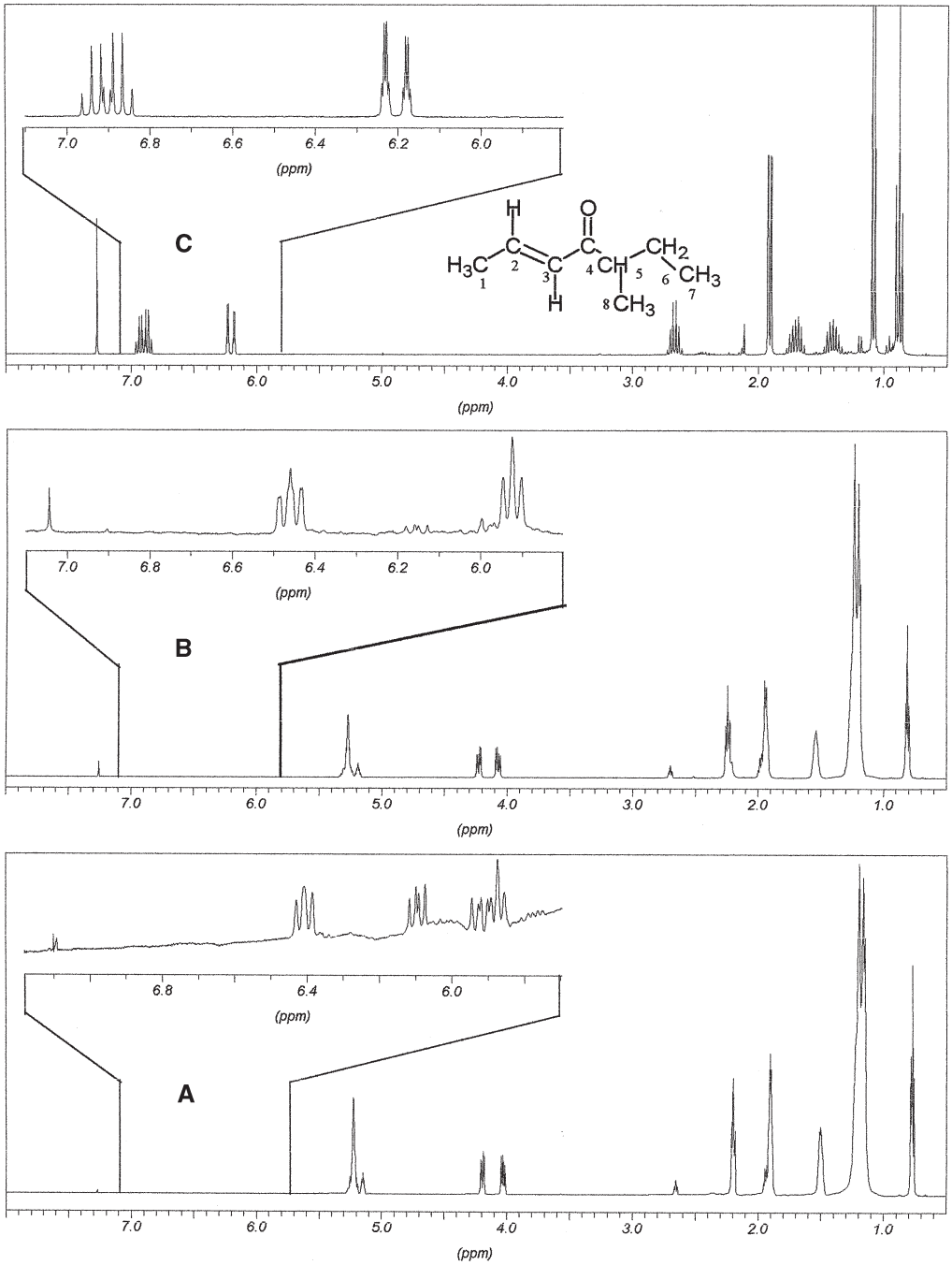

FIG. 1. ^{1}H Nuclear magnetic resonance (NMR) spectra of hazelnut oil obtained with two spectrometers operated at 600 MHz (A) and 500 MHz (B) showing the expansion of the spectral range 5.80–7.10 ppm and ^{1}H NMR spectrum of the standard of filbertone recorded with 300 MHz equipment (C).

NMR analysis. Data obtained by performing the LC preseparation with these mentioned sample loops were evaluated in terms of the recovery, relative standard deviation, and detection limits achieved after carrying out the GC analysis of the fraction resulting from a hazelnut oil. Moreover, the performance, speed of analysis, and ease of the overall procedure were considered. As can be seen in Table 1, the 20-µL loop was initially ruled out owing to the excessive time required for the sample preseparation, as such conditions involved the need to accumulate at least 25 fractions to reach the detection limit estimated for the NMR analysis of filbertone. With respect to analysis time, the best option clearly would be the use of the 500-µL loop, as only one fraction is then required but this option raises numerous experimental problems owing to the high amount of sample (mainly fat) entering the LC column and the subsequent overpressure caused in the system. Both irreversible damage to the column and poor repeatability of the retention times (and, thereby, of the limits of the fractions to

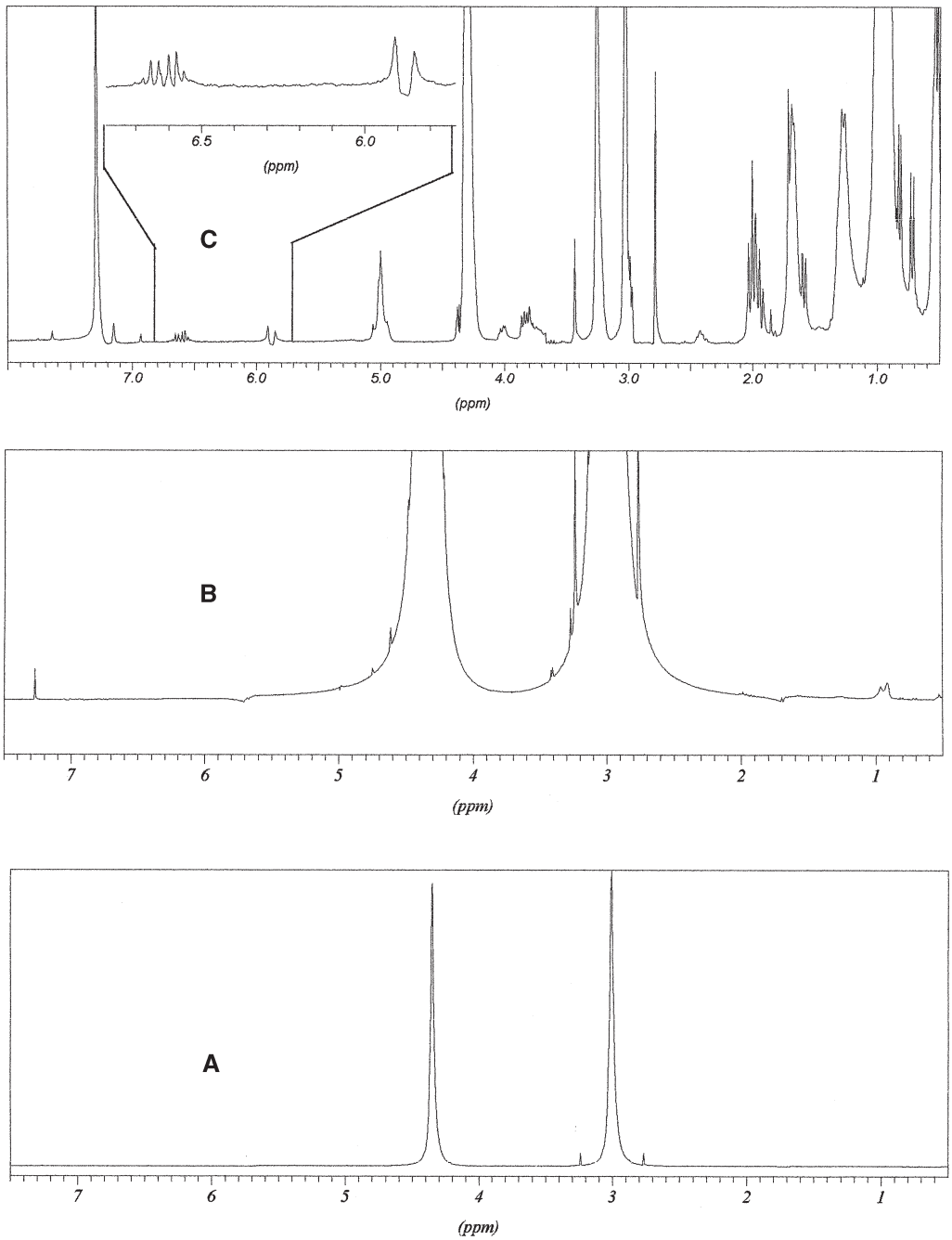

FIG. 2. Conventional ^{1}H 300 MHz spectrum of the fraction containing filbertone resulting from the liquid chromatography preseparation of a hazelnut oil (A) and its vertical expansion (×64) (B). The ^{1}H NMR spectrum obtained after applying the pulse sequence for solvent suppression is labeled as C. See Figure 1 for abbreviation.

be collected) result. Consequently, the relative standard deviations obtained are unacceptable, and the need to perform an exhaustive cleanup of the system after each experimental run increases the time required for the overall procedure.

The overpressure of the system may also eventually become a problem when using the 275-µL loop although in this case the consequences are less evident. However, the mentioned overpressure makes it difficult to establish the start and the end of the fraction to be collected and results again in the need to clean the system carefully after each analysis.

In summary, the conditions yielding the most suitable analyses involved the use of a 100-µL volume loop, as it represents the best alternative to obtain satisfactory recoveries, low relative standard deviation values, and an acceptable detection limit for filbertone in a reasonable analysis time. In this case, the collection of the required five fractions from the LC preseparation of the hazelnut oil represented approximately 2.5 mL, which was passed through an SPE cartridge prior to the NMR analysis, as described above. However, the spectra initially obtained (Figs. 2A and 2B) from the hazelnut

oil showed essentially nothing but the intense singlets of the solvents used during the LC preseparation (i.e., methanol and water). Both solvent resonances were efficiently suppressed to a satisfactory level (Fig. 2C) by applying a modified double pulsed field gradient echo scheme and using the parameters described in the Experimental Procedures section. In this way, we could finally detect the presence of filbertone in hazelnut oil. Even in this case, information on the terminal aliphatic region is not available from the NMR spectrum owing to overlapping with other signals with resonances in the 0.9–3.0 ppm region. However, the lower-field signals centered at 6.60 and 5.85 ppm could be unequivocally assigned to olefinic protons adjacent to both a terminal methyl and a carbonyl group, as are those existing in filbertone. Moreover, the increase observed for the 6.60 and 5.85 ppm signals when adding the standard to the oil and performing a new spectrum under identical experimental conditions confirmed the presence of filbertone in the hazelnut oil. The presence of significant amounts of methanol and water brings about small differences between the chemical shifts observed for filbertone in an oil and those obtained from the standard solution.

On the other hand, the reliability of the attribution of the mentioned resonances is supported by the fact that the experimental conditions for the LC preseparation of the oil were established in such a way that collection of sufficiently narrow fractions was ensured. Coelution of a number of nonpertinent and interfering compounds was precluded, and simultaneously the required selectivity for NMR analysis was obtained. As a result, the otherwise impossible detection of filbertone in edible oils was finally achieved.

On the basis of the results obtained concerning the effective suppression of the solvent used as the mobile phase in the LC preseparation, the on-line LC–NMR analysis of filbertone in oil is now possible. This feature makes the proposed method very attractive for the fast screening of large numbers of olive oils whose adulteration with hazelnut oil is suspected.

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