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Ultrahigh Performance Liquid Chromatography Analysis of Volatile Carbonyl Compounds in Virgin Olive Oils

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Supporting Information

ABSTRACT: The enzymatic and chemical oxidation reaction in olive oil produces many volatile carbonyl compounds that contribute to the complex flavor of olive oil. A novel ultrahigh performance liquid chromatography (UHPLC) method with dynamic headspace sampling and 2,4-dinitrophenylhydrazine (DNPH) derivatization were established to determine the volatile carbonyls in virgin olive oil. Quantification of nine characteristic carbonyls (acetone, hexanal, *E*-2-hexenal, octanal, *E*-2-octenal, nonanal, *E*-2-nonenal, *E*,*E*-2,4-nonadienal, and *E*,*E*-2,4-decadienal) was achieved using cyclopentanal as an internal standard. This method provides comparable linearity (R^2 = 0.9917−1.0000) and repeatability (less than 7.6% relative standard deviations) with solid phase microextraction gas chromatography (SPME-GC). The relative standard deviations (%RSD) of all applied carbonyl standards were lower than 7.6%. The limits of detection (LOD) and quantification (LOQ) were in the ranges of 1.6−150.1 and 4.8−906.1 μg/kg. The recoveries obtained for olive oil samples were in the range of 81.0−115.3%. To show the potential of this method on the quantification of other volatile carbonyls that were not included in this study, GC−electron ionization mass spectrometry (GC−EI/MS) was employed to identify the derivatized carbonyls (carbonyl (2,4-DNPH) hydrazones) while peak assignments were made on the basis of elution sequences and peak areas. This method provided feasibility of using LC to determine volatile carbonyls in oil matrices, which can be applied to exam the degree of lipid oxidation and evaluate the sensory properties of VOO and other edible oils.

KEYWORDS: *volatile carbonyls, DNPH, UHPLC/DAD, GC/MS, lipid oxidation, virgin olive oil*

INTRODUCTION

Virgin olive oils (VOOs) are mechanically extracted from healthy olive fruits with minimal changes in the oil composition. The desirable flavor and health benefits of VOO contribute to its important role in the Mediterranean diet and its expanding market in the United States and other countries.[1] Mechanical extraction of VOO conserves abundant minor plant compounds from the olive fruits, such as diacylglycerols (DAGs), monoacylglycerols (MAGs), free fatty acids, phenolic compounds, α-tocopherols, phospholipids, sterols, pigments, and volatile compounds.[2] It has been demonstrated that volatile compounds produced by biochemical pathways (C5 and C6 compounds) mainly contribute to the pleasant flavor of VOO;[3−5] the series of C6−C12 volatile compounds formed by autoxidation and photo-oxidation during processing and storage are responsible for off-flavors and degradation of sensory quality of olive oil.[6−8] Therefore, volatile compounds, especially carbonyl compounds, have been used to evaluate the freshness of VOO.[8−11]

Many analytical methods have been established to determine the flavor components of VOO using gas chromatography (GC).[12] Since most of the volatile compounds present have a low concentration in the headspace of VOO, GC analysis is usually carried out with a preconcentration process.[13] Dynamic headspace (DHS) is a solventless extraction technique that has been widely applied in the studies of VOO volatile compounds.[14−17] In the process of DHS sampling, volatile compounds are moved by a stream of gas blown from the headspace of the oil sample and absorbed onto a trap material. Later, the compounds are desorbed by solvent or high heat for GC analysis. Solid phase microextraction (SPME) is an alternative headspace extraction technique that relies on the enrichment and thermal desorption of volatile compounds on a fused-silica fiber with different stationary phase coating.[18−20] This affordable and rapid method has been used in several recent studies of VOO volatiles.[21−26] With the use of universal detectors, such as flame ionization detector (FID) and mass spectrometry (MS), GC can provide a complete profile of flavor compounds of the oil matrix.

However, since many of the volatiles have no ultraviolet (UV) or fluorescence absorption, high performance liquid chromatograph (HPLC) is not often used for flavor compound analysis. In order to detect flavor compounds on HPLC, derivatization is required to attach chromaphores or fluorophores onto analytes before analysis.[18] The use of 2,4-dinitrophenylhydrazine (DNPH) as a derivatization reagent to determine carbonyls has been applied on many different matrices.[27] Sampling from liquid materials, such as alcoholic beverages [28] and oils,[29,30] is often implemented by adding an acidic DNPH solution into samples and extracting the precipitated carbonyl (2,4-DNPH) hydrazones after reaction. Extracting from gaseous samples, including ambient air,[31−36] tobacco smoke,[36,37] and diesel emission,[38] usually employs a DNPH coated cartridge. In this case, the volatile carbonyls are

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carried onto the cartridge with the gaseous sample and react immediately with the DNPH coated on the cartridge sorbent. After sampling, the carbonyl-DNPH derivatives are eluted with solvent for HPLC analysis. Da Silva et al. used a DNPH coated cartridge to determine volatile carbonyl emissions of edible frying oils under high heat,[35] but to the best of our knowledge, no work has been done to determine volatile carbonyl compounds in fresh or aged VOO with HPLC.

To reduce the instrumental limitation on the determination of volatile carbonyls in oil matrix, which can be useful to evaluate degree of oxidation, HPLC was employed as an alternative technique to GC. The novel purge and trap-ultra-HPLC/diode array detector (P&T-UHPLC/DAD) method established here is suitable for the quantitative analysis of volatile carbonyl compounds in oil matrix. The quantification of nine critical carbonyl compounds of VOO[2,39] was achieved using internal standard and external standard curves. The quantification, accuracy, and repeatability of the P&T-LC method were evaluated through the analysis of oil samples spiked with carbonyl standards. The comparison of the new method and traditional SPME-GC method shows the possibility of using HPLC to determine volatile carbonyls when GC is not available. Moreover, the major unknown carbonyl-DNPH derivatives observed in LC profiles of VOO were further identified with the assistance of GC−electron ionization (EI)/MS to demonstrate the utility of the method on the analysis of more volatile carbonyls.

MATERIALS AND METHODS

Chemicals. All carbonyl standards hexanal, *E*-2-hexenal, nonanal, *E*-2-nonenal, *E*,*E*-2,4-nonadienal, octanal, *E*-2-octenal, *E*,*E*-2,4-decadienal, and cyclopentanal were obtained from Sigma-Aldrich (St Louis, MO) as well as the derivatization reagent 2,4-dinitrophenylhydrazine. The sampling cartridge, LpDNPH S10 cartridge, was purchased from Supelco (Bellefonte, PA). HPLC grade acetone, acetonitrile, formic acid, and reagent grade ethanol, phosphoric acid, hydrochloric acid were purchased from Fisher Scientific (Pittsburgh, PA). Type 1 ultrapure water was produced by Barnstead Nanopure system (Waltham, MA). The VOO samples, from 2010 and 2011 harvests, were provided by a local producer and stored in the dark at room temperature (22 °C).

DNPH Derivatization of Carbonyl Standards. The derivatives of carbonyl standards were prepared according to the method described by Seppanen et al.:[29] 0.15 g/L saturated DNPH solution was prepared in 10% (v/v) phosphoric acid in methanol. Then 25 mL of the saturated solution was added to 0.1 g of each carbonyl standard, respectively. The reaction vials were set in a 25 °C water bath and shaken for 30 min. The crystallized carbonyl-DNPH was then filtered and washed with 2 mol/L hydrochloric acid solution and ultrapure water. If necessary, the solution can be stored in a freezer to promote crystallization. The filtered solid derivatives were dried over silica gel in a vacuum oven at 50 °C. All carbonyl-DNPH standard solutions were prepared in acetonitrile and stored at −20 °C.

Quantification of Carbonyl Volatiles. Mixed carbonyl standards of cyclopentanal acetone, *E*-2-hexenal, hexanal, *E*-2-octenal, octanal, *E*-2-nonenal, nonanal, *E*,*E*-2,4-nonadienal, and *E*,*E*-2,4-decadienal were prepared in commercial stripped corn oil (Acros Organics, Morris Plains, NJ) to study the linearity, relative response factors (RRFs), and repeatability of P&T-LC and SPME-GC methods. No detectable volatile compound was observed in the stripped corn oil as determined by both methods. Five dilutions were tested in the concentration range of 0−10 μg/g. Each data point was analyzed in triplicate. The sample sizes of P&T-LC and SPME-GC method were 10 and 2 g, respectively.

P&T-DNPH Sampling. As shown in Figure S1 (see Supporting Information), the oil sampling setup was mounted as follows. An olive oil sample (10 g) spiked with internal standard was heated to 45 °C and continuously stirred in a 250 mL round-bottom glass flask. Then 3 μg of cyclopentanal was added as internal standard. As a stream of nitrogen was purged into the oil sample, the volatized carbonyl compounds were collected in a connected LpDNPH S10 cartridge at a 1 L/min flow rate for 60 min.[38] The derivatized carbonyl compounds were then eluted into an amber vial with 2 mL of acetonitrile using gravity feed. The eluent was stored in the dark at −20 °C until analysis.

UHPLC/DAD Separation and Detection of DNPH Derivatives. An Agilent 1290 Infinity UHPLC/DAD system was employed for carbonyl-DNPH analysis. The separation of carbonyl-DNPHs was achieved with a Poroshell 120 EC-C18 column (2.1mm × 100 mm, 2.7 μm) under the following conditions: injection volume, 6 μL; flow rate, 0.6 mL/min; gradient of 0.1% formic acid in ultrapure water (A) and acetonitrile/methanol (50:50) (B), 40−80% B in 55 min, 80−100% B in 1 min, remaining for 2 min, returning to 50% in 0.2 min, and remaining for 0.8 min. The total running time was 27 min. Absorbance was monitored at 360 nm. A typical chromatogram of carbonyl standards was shown in Figure S2 (see Supporting Information).

GC/MS Analysis of DNPH Derivatives. The GC/MS analysis of carbonyl-DNPHs was performed on a Varian 450-GC equipped with a Varian 220-MS ion trap (Agilent Technologies) following the method described by Dong et al.[40] A DB-5HT capillary column (30 m × 0.25 mm × 0.1 μm; Agilent Technologies) was employed to separate carbonyl-DNPHs with helium as carrier gas at a flow rate of 2.0 mL/min. The injector was held at 240 °C at a split ratio of 20. The GC oven was initially set at 100 °C and ramped at 18 °C/min to 330 °C and held for 10 min to bake off remaining high boiling entities. The injection volume was 1 μL. Electron impact ionization at 70 eV was used as the ion source, and ions in a m/z range from 40 to 400 were collected and analyzed. Other EI/MS parameters included automatic gain; target ion count, 2 × 104 ions; maximum ionization time, 25 000 μs; prescan ionization time, 100 μs; scan time, 1 s/scan; emission current, 80 μA. A total ion chromatogram (TIC) of DNPH-carbonyl standards is shown in Figure S3 (see Supporting Information).

SPME-GC/MS Analysis. Two grams of oil sample was weighted in an amber bottle sealed with a PTFE/silicon septum (Supelco). After 30 min of equilibration at 45 °C, a SPME fiber coated with divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS, Supelco) was exposed to the headspace for 30 min at 45 °C while the sample was continuously stirred with a magnetic stir bar.

After sampling, the fiber was thermally desorbed onto GC on the injection port for 5 min. The injector was held at 240 °C with splitless mode. The separation of volatile compounds was also carried out with a DB-5HT capillary column (30 m × 0.25 mm × 0.1 μm; Agilent Technologies) at flow rate of 1 mL/min. The temperature started at 40 °C for 5 min, was ramped at 5 °C/min to 220 °C, and then held for 10 min. EI/MS parameter setups were the same as those for carbonyl-DNPH derivatives analysis.

RESULTS AND DISCUSSION

P&T-LC Analysis. *Chromatographic Separation and Detection.* The LC chromatogram of carbonyl-DNPH standards indicated that the separation of carbonyl-DNPH derivatives was based on their carbon numbers and degrees of unsaturation (Figure S2 and Table S1 in Supporting Information). Generally, carbonyl-DNPHs with smaller carbon number were eluted before those with larger carbon numbers, and the derivatized carbonyls with higher degrees of unsaturation eluted earlier than the corresponding carbonyl-DNPHs with lower degrees of unsaturation. The instrumental detection limit and linear range of each carbonyl-DNPH standard were determined by injecting known concentrations of a carbonyl-DNPH standard mixture in acetonitrile (Table S1 in Supporting Information). The concentrations of carbonyl-DNPH derivatives in acetonitrile eluted can be calculated by external standard curves as shown in Table S1, which are

Table 1. Calibration Data for the Determination of Carbonyl Compounds in Oil Sample by P&T-UHPLC and SPME-GC Methods

	P&T-UHPLC						SPME-GC					
analyte	linear equation[a]	R^2	LOD[b] (μg/kg)	LOQ[c] (μg/kg)	%RSD[d]	RRF[e]	linear equation[a]	R^2	LOD[b] (μg/kg)	LOQ[c] (μg/kg)	%RSD[d]	RRF[e]
acetone	$y = 619.32x$	0.9980	1.6	4.8	2.6	5.72	$y = 25803x$	0.9924	0.02	0.08	11.5	1.90
cyclopentanal	$y = 108.16x$	0.9967	9.2	27.7	0.5	1.00	$y = 13606x$	0.9996	0.06	0.20	1.4	1.00
E-2-hexenal	$y = 403.94x$	0.9963	2.5	7.4	2.0	3.73	$y = 56585x$	0.9889	0.01	0.04	2.3	4.16
hexanal	$y = 634.5x$	1.0000	1.6	4.7	5.9	5.86	$y = 76637x$	0.9984	0.01	0.03	9.6	5.63
E-2-octenal	$y = 150.27x$	0.9999	6.7	20.0	3.6	1.39	$y = 16589x$	0.9970	0.05	0.17	0.1	1.22
octanal	$y = 263x$	0.9917	3.8	11.4	1.4	2.43	$y = 41359x$	0.9945	0.02	0.06	1.2	3.04
E,E-2,4-nonadienal	$y = 6.662x$	0.9934	150.1	450.3	0.4	0.06	$y = 1451.2x$	0.9843	0.66	2.18	2.5	0.11
E-2-nonenal	$y = 50.16x$	0.9990	19.9	59.8	2.4	0.46	$y = 5974.1x$	0.9863	0.16	0.52	8.1	0.44
nonanal	$y = 108.16x$	0.9924	9.2	27.7	1.5	1.00	$y = 22911x$	0.9849	0.04	0.14	6.7	1.68
E,E-2,4-decadienal	$y = 3.311x$	0.9980	302.0	906.1	3.7	0.03	$y = 634.03x$	0.9794	2.8	9.23	6.5	0.04

[a]All linear equations were determined in the range of 0−10 μg/g. *y*: peak area. *x*: concentration of carbonyl in oil sample (μg/g). [b]LOD: detection limit. [c]LOQ: quantification limit. [d]%RSD: relative standard deviation. [e]RRF: T, using cyclopentanal as an internal standard.

correlated with the amount of carbonyl compounds in the headspace.

Quantification. Since the collection efficiencies of carbonyl compounds were found to vary, in order to achieve quantification of carbonyls in VOO, the striped corn oil samples spiked with certain concentrations of each carbonyl standard were processed under the sampling conditions described below. The linearity and sensitivity of the method were studied in the concentration range of 0−10 μg/g (Table 1). The coefficients of determination (R^2) for the P&T-LC method applied to the 10 carbonyl standards were found to range from 0.9917 to 1.0000. The repeatability of five detections of 5 μg/g spiked oil samples was determined to be less than 7.6% relative standard deviations (%RSD) for all applied carbonyl standards. The limit of detection (LOD) was calculated by a signal-to-noise ratio of 3, and the limit of quantification (LOQ) was determined by a signal-to-noise ratio of 10. The LOD and LOQ of the P&T-LC method were in the ranges of 1.6−150.1 and 4.8−906.1 μg/kg, respectively. Cyclopentanal, a carbonyl compound that was not reported to be found in olive oils,[4] was used as an internal standard for this study. The RRF defined as the relative response of the DAD to an analyte on 360 nm compared to internal standard was calculated using following equation:

$$\mathrm{RRF} = (A_C C_{IS})/(A_{IS} C_C) \quad (1)$$

where A_C is the area of the target carbonyl analyte, A_{IS} is the area of the corresponding internal standard, C_{IS} is the concentration of the corresponding internal standard, and C_C is the concentration of the target carbonyl analyte.

To evaluate the performance of the P&T-HPLC method, calibration curves of the typical SPME-GC method were constructed with the same spiked strip oil samples (Table 1). The linearity of calibration curves and the repeatability of the SPME-GC method (evaluated by %RSD) were comparable to those of the DNPH-LC method. However, since the SPME fiber has stronger ability to trap volatile compounds and the GC−EI/MS has high response factors to all of the carbonyl strands, the sensitivity (LOD and LOQ) of the SPME-GC method was significantly higher than that of the P&T-LC method.

The RRFs of carbonyls corresponded to their collection efficiencies for both P&T-LC and SPME-GC (Table 1). Higher carbon number and degree of unsaturation of carbonyl compounds tend to yield lower collection efficiency. The decrease of RRFs of aldehyde standards with the same degrees of unsaturation was linearly related to the increase of carbon number (Figure 1). It has been demonstrated that compounds

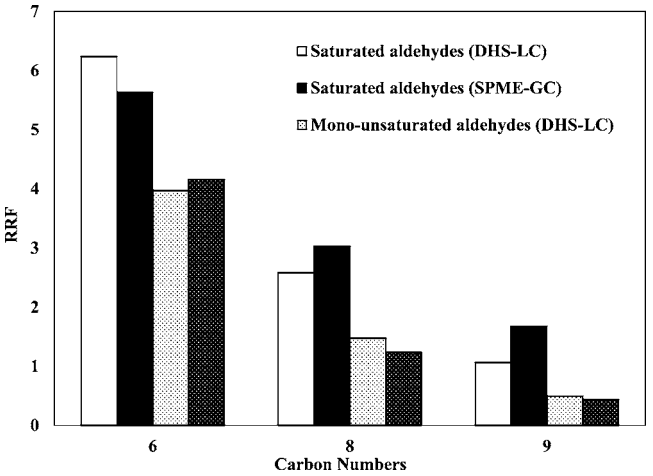

Figure 1. RRF of saturated aldehydes (hexanal, octanal, nonanal) and monounsaturated aldehydes (*E*-2-hexenal, *E*-2-octenal, *E*-2-nonanal) using P&T-UHPLC and SPME-GC methods.

with higher equivalent chain numbers (ECN) have lower degrees of volatilization from oil matrices.[41] Moreover, the low collection efficiencies of unsaturated carbonyls were reported to be associated with the instabilities of their DNPH derivatives, which could further react with DNPH and form undesired UV absorption products, in previous studies of air and smoke using DNPH cartridges.[42−44] However, no significant difference was observed for the collection efficiencies of unsaturated carbonyls with the P&T-LC and SPME-GC methods; therefore, the instability of unsaturated carbonyl-DNPH derivatives may not play a dominant role in this study.

While the P&T-LC method was not as sensitive as the SPME-GC method, it showed a comparable repeatability. In general, quantification of volatile carbonyl compounds in oil matrices can be achieved with the P&T-LC method using both external calibration curves and internal standards.

Analysis of VOO Samples. *Identification and Quantification by P&T-LC Method.* To illuminate the applicability of the proposed method, two VOOs were analyzed. Seven

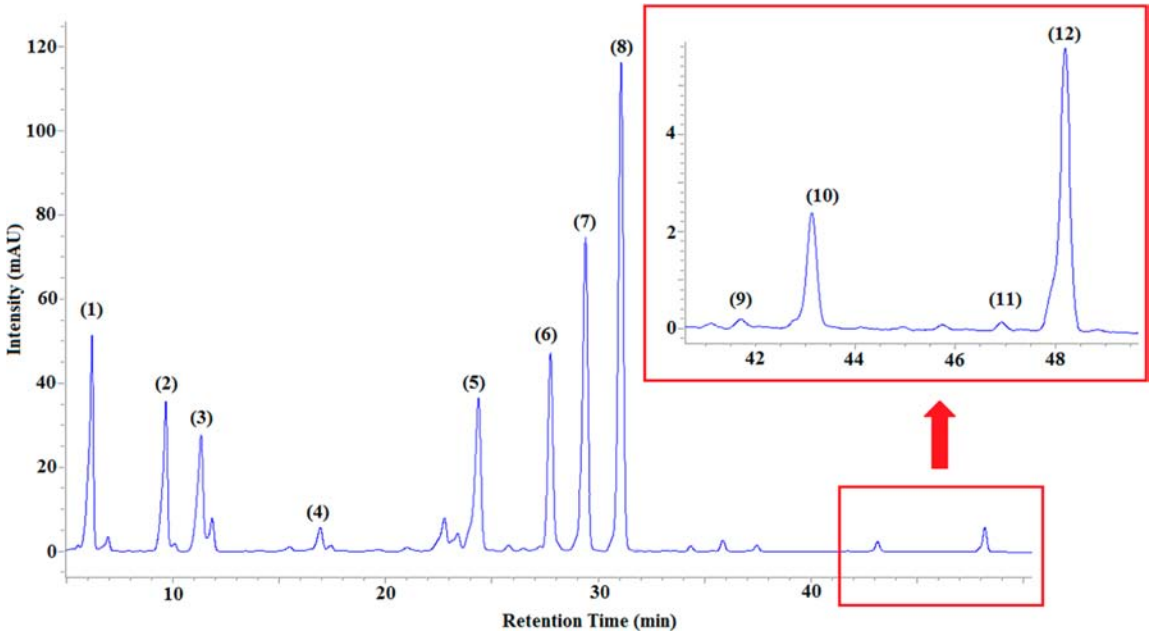

Figure 2. LC chromatogram of VOO (I) at 360 nm. The chromatogram in red frame is expanded to show peaks between 41 and 49 min. Peak identification by retention time is as follows: (2) acetone; (4) cyclopentanal; (7) *E*-2-hexenal; (8) hexanal; (9) *E*-2-octenal; (10) octanal; (11) *E*-2-nonenal; (12) nonanal. Peak identification by GC–MS is as follows: (1) acetaldehyde; (3) propanal; (5) pentanal; (6) *Z*-3-hexenal.

carbonyls were identified in the LC chromatogram of VOO samples according to retention times (Figure 2). An amount of 1 μg/g cyclopentanal was added as internal standard, and the concentrations of identified carbonyl compounds were calculated by eq 1 (Table 2). The accuracy (recovery) and

Table 2. Analysis of Carbonyl Compounds in VOOs: Recoveries and Repeatability of the Quantifiable Carbonyls

	VOO (I)			VOO (II)		
analyte	average (μg/kg)	recovery (%)	%RSD	average (μg/kg)	recovery (%)	% RSD
acetone	904.8	91.7	2.7	613.4	83.8	2.4
E-2-hexenal	3510.5	115.3	4.7	8044.2	105.8	4.7
hexanal	2960.6	107.8	1.8	1143.6	104.3	1.5
E-2-octenal	25.4	91.7	8.2	23.6	92.5	3.6
octanal	139.3	102.7	2.2	214.0	91.8	1.6
E-2-nonenal	N/Q	N/A	N/A	180.2	95.6	8.6
nonanal	758.9	83.3	2.6	1159.3	84.7	2.5

precision (%RSD) of this method for the analysis of VOO samples were evaluated by the spiking experiment. Particular amounts of carbonyl standards were spiked in VOO samples to determine the recoveries by comparing the calculated spiked amounts with the corresponding amounts actually added. All samples were analyzed in triplicate. The recoveries of targeted carbonyls were in the range of 81.0–115.3%, and the %RSD values were lower than 8.6% (Table 2). The results indicate that the method is acceptable for the determination of volatile carbonyl compounds in VOOs.

GC–MS Identification of Carbonyl-DNPH derivatives. Some of the peaks presented in the LC chromatogram of VOO were not identifiable by retention times because of a lack of standards (Figure 2). In order to show a more complete carbonyl profile and the ability of this method on the analysis of other volatile carbonyls, GC–EI/MS was utilized to assist with peak identification. DNPH derivatized carbonyl standards were first injected into the GC/MS to study the elution sequence and fragmentation patterns. As shown in Figure S3 (see Supporting Information), the DNPH-carbonyl standards with smaller carbon number eluted earlier than those with larger carbon numbers, and those with higher degrees of unsaturation eluted after the corresponding saturated DNPH-carbonyls. The representative mass spectra of hexanal and *E*-2-hexenal are shown in Figure 3. The molecular ions, M^+, were observed in all of the mass spectra of carbonyl-DNPH standards; the *m*/*z* 235 ions produced by allylic bond cleavage were only found in the spectra of *E*-2-hexenal, *E*-2-octenal, and *E*-2-nonenal. Therefore, the identification of saturated and monounsaturated carbonyls can be achieved by the observation of M^+, and the existence of double bonds can be determined by observation of *m*/*z* 235 ions or the equivalent fragment ions.

Four additional peaks were identified in the TIC of VOO sample (I) according to the pattern of fragmentation (Table 3). Some of the peaks had very similar mass spectra, such as peak b vs peak c, and peak f vs peak g (Figure 4). Known standards were used to help identify the compounds. For example, spectra of both peaks f and g have M^+ ions at *m*/*z* 278 and base peaks at *m*/*z* 235, which indicates that the two original carbonyls share the same chemical formula and degree of unsaturation. As peak g can be identified as *E*-2-hexenal-DNPH based on retention time, peak f was assigned to be the DNPH derivatized *Z*-3-hexenal, which was reported to be present in VOO.[4,9,25] Afterward, the new peak assignments on TIC (Figure 4) were matched up with the unidentified peaks in Figure 2 based on elution sequences and peak sizes. Therefore, four more peaks were identified in the LC chromatogram of VOO.

In conclusion, a suitable P&T-LC method for the quantitative determination of volatile carbonyl compounds in VOO was established in this study. In comparison with the typical SPME-GC method, the proposed method achieved good linearity (R^2 = 0.9917–1.0000) and repeatability (%RSD < 7.6%). Although the sensitivity of this method may limit its application to the quantification of polyunsaturated carbonyls,

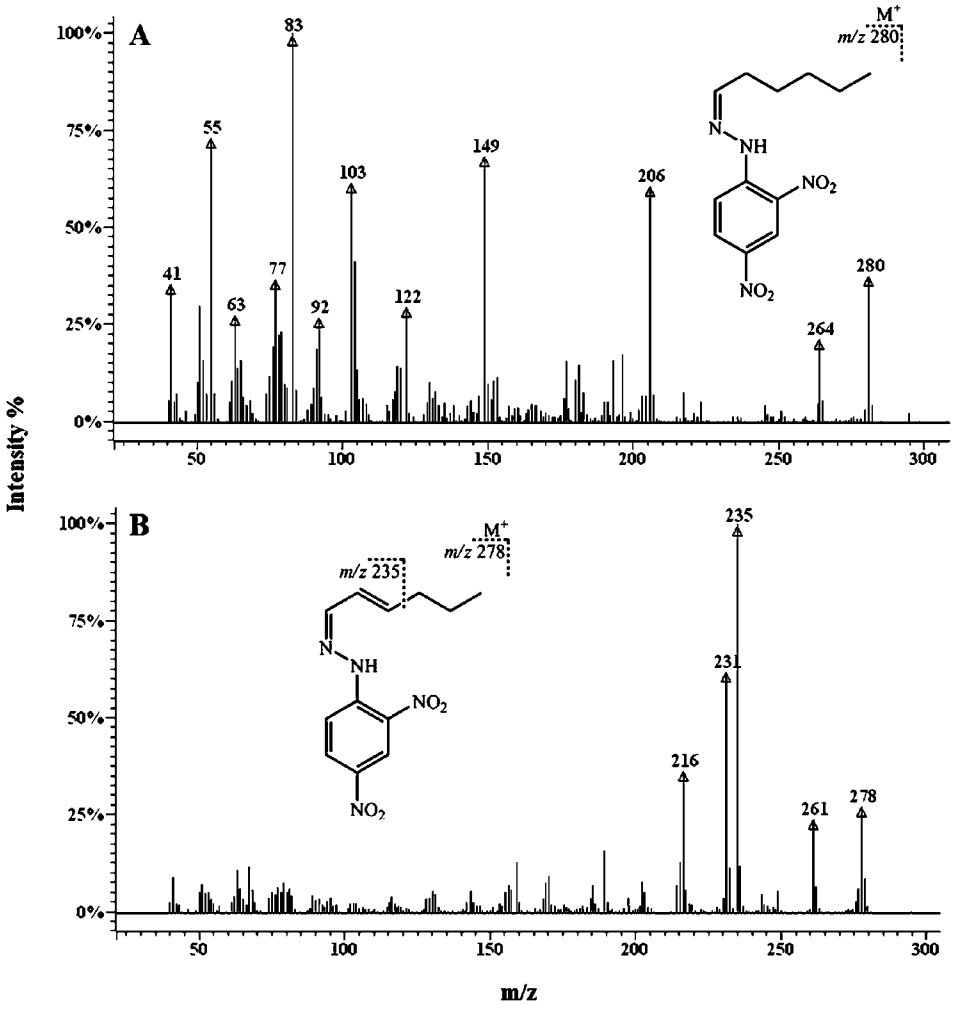

Figure 3. EI-MS spectra of hexanal (A) and *E*-2-hexenal (B). The characteristic sites of cleavage and detectable ion masses are shown on the chemical structures.

Table 3. Carbonyl Compounds Identification by GC−MS and Their Corresponding Peak No. in Figure 2 and Figure 4

peak no. in Figure 3	M^+ (m/z)	diagnostic ion for double bond locating (m/z)	standard available	peak identification	corresponding peak no. in Figure 2
a	224	N/A	no	acetaldehyde	1
b	238	N/A	yes	acetone	2
c	238	N/A	no	propanal	3
d	266	N/A	no	pentanal	5
e	280	N/A	yes	hexanal	8
f	278	235	no	*Z*-3-hexenal	6
g	278	235	yes	*E*-2-hexenal	7

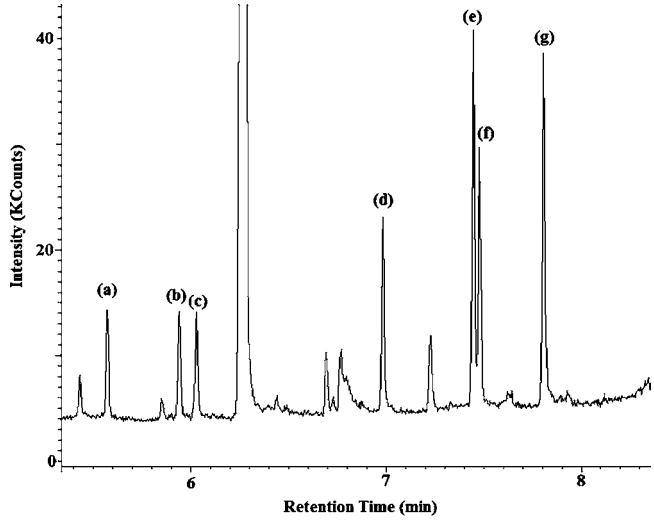

Figure 4. GC/MS total ion chromatogram of VOO (I). Peak identification is as follows: (a) acetaldehyde; (b) acetone; (c) propanal; (d) pentanal; (e) hexanal; (f) *Z*-3-hexenal; (g) *E*-2-hexenal.

we find that this P&T-LC method is acceptable for the analysis of saturated and monounsaturated carbonyl compounds present in oil matrices. Peak identification assisted by GC/MS not only revealed a more complete carbonyl profile of VOO but also demonstrated the potential of this method for the quantitative analysis of other volatile carbonyls not included in this study. Overall, this method provided feasibility of using an LC technique to determine volatile carbonyls in oil matrices, which may be applied to monitor the occurrence of lipid oxidation and to evaluate the sensory properties, especially rancidity of VOO and other edible oils.

■ ASSOCIATED CONTENT

Supporting Information

Oil sampling setup, LC chromatogram of standards, analytical parameters carbonyl compounds, GC-EI/MS chromatogram of standards. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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