Rapid and Simultaneous Analysis of Free Sterols, Tocopherols, and Squalene in Edible Oils by Coupled Reversed-Phase Liquid Chromatography−Gas Chromatography

Jesús Villén,[†] Gracia P. Blanch,[‡] María Luisa Ruiz del Castillo,[‡] and Marta Herraiz*[,‡]

Escuela Técnica Superior de Ingenieros Agrónomos, Universidad de Castilla−La Mancha, Campus Universitario s/n, 02076 Albacete, Spain, and Instituto de Fermentaciones Industriales, CSIC, c/Juan de la Cierva 3, 28006 Madrid, Spain

On-line coupling of reversed-phase liquid chromatography and gas capillary chromatography (RPLC-GC) using a programmed temperature vaporizer (PTV) as interface is used for the direct and simultaneous analysis of free sterols, tocopherols, and squalene of edible oils by direct injection (that is, without the need for prior enrichment of the sample). The proposed method allows large volume fractions of water methanol eluents to be transferred into the GC system, 2000 μL/min being the speed of sample introduction. The overall procedure including LC preseparation, transfer from LC to GC, and GC analysis takes <1 h.

Keywords: *On-line coupled RPLC-GC; sterols; tocopherols; squalene; direct analysis; edible oils*

INTRODUCTION

Squalene (2,6,10,15,19,23-hexamethyl-2,6,10,14,18,-20-tetracosahexaene) is a terpenoid hydrocarbon occurring in high concentrations (60−75%) in the unsaponifiable fraction of olive oil. The distilled fraction obtained from the deodorization step included in the raffination process ("olive oil deodorization distillation") also contains squalene (Bondioli et al., 1993). As this compound is present in olive oils in much larger quantities than in other vegetable oils, its determination may be used in detecting adulterations of olive oils. Specifically, rapid analysis of squalene is of great interest for determining whether oils labeled as nonrefined "virgin" contain refined products due to the fact that refining treatments remove or transform flavor components and antioxidants (Grob et al., 1992); consequently, oils marketed as refined olive oils are of considerably lower price than nonrefined (virgin) oils.

Further interest in squalene analysis arises because it is a precursor in cholesterol biosynthesis (Strandberg et al., 1989) and it is widely used in cosmetic preparations. Moreover, the influence of squalene as a potential oxidation inhibitor related to the stability of different vegetable oils has been previously investigated (Malecka, 1994).

On the basis of these considerations is evident the interest in developing analytical methods suitable for detecting squalene in edible oils. Also, analysis of other minor compounds present in the unsaponifiable matter (for example, sterols and tocopherols) has proved its usefulness for the characterization of different edible oils (Firestone, 1995).

The methods normally used for the analysis of different fractions of interest in edible oils involve several steps, namely the removal of triglycerides, the fractionation of the unsaponifiable matter into several classes of compounds, and their subsequent analysis by gas chromatography (*Official Journal of the European Communities*, 1988). However, traditional methods are not only time-consuming and laborious but also disadvantageous with respect to their use of toxic organic solvents and the loss of target analytes by saponification (Grob et al., 1990; Park et al., 1996).

On-line coupling of high-performance liquid chromatography and high-resolution capillary gas chromatography (LC-GC) provides a very interesting approach to integrate sample preparation into the chromatographic procedure and, consequently, offers a new and practical alternative to traditional methods of sample preparation (Grob, 1991, 1995; Vreuls et al., 1994). However, previous works concerning on-line LC-GC methods for the analysis of the unsaponifiable matter of edible oils mainly refer to the use of normal phase in the LC separation. Under these circumstances, some variability in retention times can be observed if triglyceride concentrations vary from one sample to another (Grob et al., 1991). This is due to the fact that triglycerides deactivate the silica gel LC columns; consequently, use of normal phase in the LC step may cause several problems affecting performance.

Generally speaking, it is also evident that the possibility of using reversed phase in the LC step instead of normal phase will enlarge the field of LC-GC applications and, simultaneously, allow the development of new methods ideally suited for analyzing aqueous samples. In this respect, however, it must be taken into account that transfer to GC of polar solvents is rather difficult mainly because of the very large volumes of vapor that are produced per unit volume of liquid.

Different aspects concerning the design of robust and reliable interfaces that enable direct transfer of aqueous eluents have been previously investigated (Goosens et al., 1994; Mol et al., 1993; Staniewski et al., 1992; Vreuls

* Author to whom correspondence should be addressed [telephone (341) 562 29 00; fax (341) 564 48 53; e-mail mherraiz@fresno.csic.es].

[†] Universidad de Castilla−La Mancha.

[‡] CSIC.

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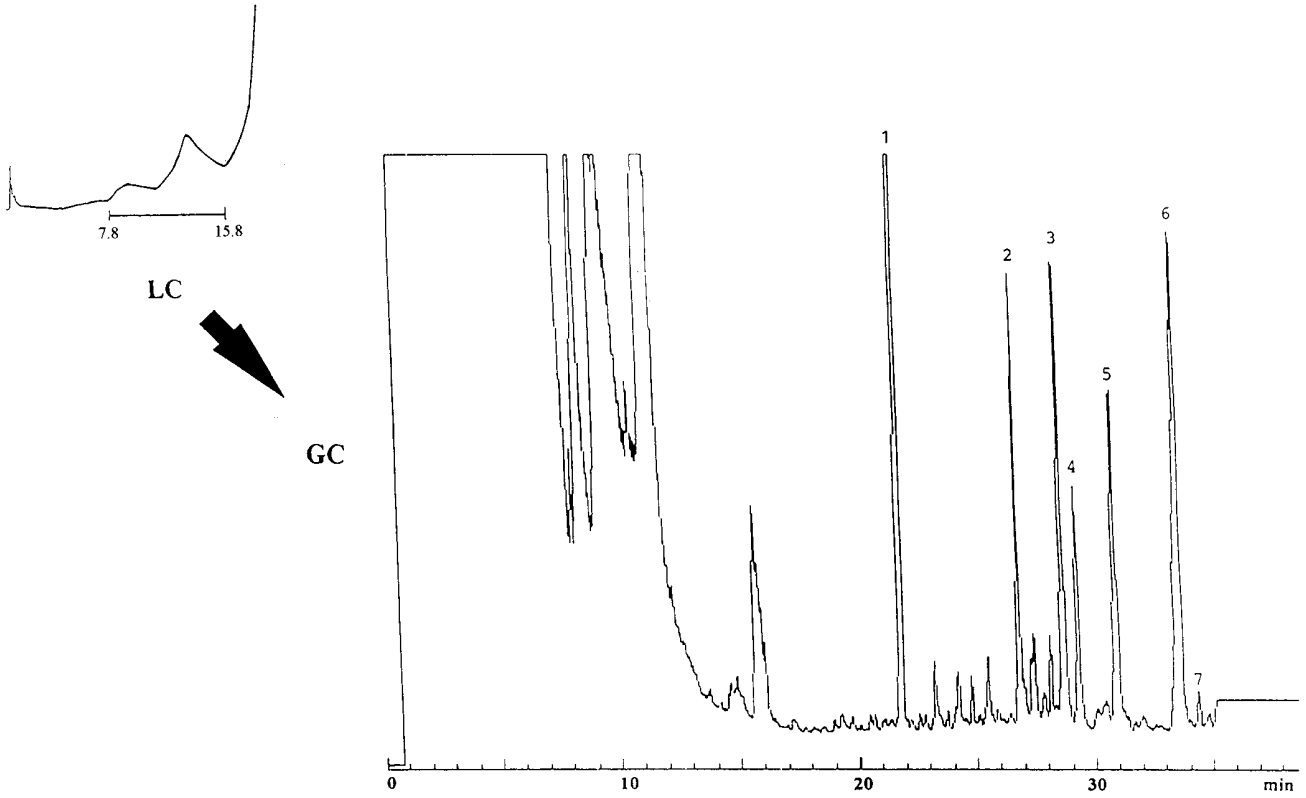

Figure 1. Liquid chromatogram of a rapeseed oil and gas chromatogram (full range, 7 mV) resulting from transfer of the indicated fraction. HPLC flow rate was 2000 μL/min. Peaks: 1, squalene; 2, δ-tocopherol; 3, $\beta+\gamma$-tocopherol; 4, brassicasterol; 5, campesterol; 6, β-sitosterol; 7, Δ^7-avenasterol. See text for further details.

et al., 1991). Specifically, our previous research in RPLC-GC coupling using a programmed temperature vaporizer (PTV) as interface of the system has shown its usefulness for the direct analysis of minor components, for example, free sterols of edible oils (Señoráns et al., 1996).

The purpose of this work was to develop a qualitative screening method for the direct analysis of squalene in olive oils suitable not only to make the routine analysis both easy and rapid but also to allow the simultaneous analysis of other fractions of interest of the unsaponifiable matter of edible oils. To this aim, off-line sample preparation techniques are precluded and an on-line coupled reversed-phase LC-GC method is proposed.

EXPERIMENTAL PROCEDURES

Samples and Materials. Oil samples were either purchased in the local market (rapeseed oil) or obtained from an oil mill (residue olive oil). Oil samples were directly injected into the HPLC after having been filtered through a 0.22-μm Pro-X filter (Teknokroma, Madrid, Spain). No further pretreatment was required prior to RPLC-GC analysis. Methanol (HPLC grade) was purchased from Lab Scan (Dublin, Ireland), and the water used was collected from a Milli-Q water purification system (Millipore, Milford, MA). The silylated glass inserts (85 mm × 1 mm i.d. × 2 mm o.d.) of the PTV injector were obtained from Gerstel (Mülheim/Ruhr, Germany) and were partly packed with a 4-cm plug length of Tenax TA, 80−100 mesh (Chrompack, Middelburg, The Netherlands), which was held in place by two plugs of silanized glass wool. The selection of Tenax TA as packing material was based on its low levels of potentially interfering substances. Both the adsorbent and its plug length in the glass liner were chosen according to our previous experience concerning large volume sample introduction into capillary GC and LC-GC transfer (Señoráns et al., 1995a,b).

Instrumentation. The on-line RPLC-GC analyses were performed using coupled equipment consisting of a Hewlett-Packard model 1050 liquid chromatograph (Wilmington, DE) provided with a manual injection valve (model 7125, Rheodyne, Cotati, CA) having a 20-μL loop and a Perkin-Elmer model 8500 gas chromatograph (Norwalk, CT) equipped with a PTV injector (Perkin-Elmer) and a flame ionization detector (FID). The PTV was used as interface of the system as described below.

LC Conditions. All analyses were carried out by using methanol/water as mobile phase and injecting 20 μL of the filtered oil onto a 50 × 4.6 mm i.d. column slurry packed with 10-μm silica, C_4, Vydac 214 TPB as previously reported (de Frutos et al., 1992). Throughout the experimentation, the UV detection was performed at 205 nm and the LC column was maintained at 45 °C, 2000 μL/min being the flow rate during both the LC preseparation and the LC-GC transfer. Initial conditions (methanol/water, 70:30) were followed from the third minute on by a linear gradient up to 22% water within 3 min. The latter water percentage was kept for 4 min, then was again modified up to 14% within 2 min, and maintained there for 3.5 min. Finally, the gradient was varied up to 0% within 3.5 min. Acquisition of data from the UV detector was performed with a HP ChemStation software, revision A.04.01 (Hewlett-Packard).

LC-GC Transfer. Transfer of the fraction of interest from LC to the GC was performed by means of both a multiport valve placed immediately after the ultraviolet detector of the HPLC and the PTV injector of the GC. Two ports of the valve were occupied by a waste line and a transfer line so that the LC effluent could be either wasted or transferred into the GC via PTV. The waste line (made from a stainless steel tube) allows the LC effluent to be fed to waste; the transfer line (consisting of a 60 cm × 0.32 mm fused silica tube) allows the sample to flow from the LC system to the GC injector simply by its insertion through the septum of the PTV body. The beginning and end times of the fraction to be transferred from LC into GC must be previously selected in the LC chromato-

gram. Upon detection of the beginning of the selected fraction, the multiport valve was switched from the waste position to the transfer position, and once the transfer time was complete, the valve was switched back to the waste position and the solvent led to waste. According to our previous experience in RPLC-GC analysis, a helium flow rate equal to 800 mL/min was passed through the PTV injector (kept at 21 °C) during LC-GC transfer, and an additional purge time (0.32 min) was established to efficiently eliminate the remaining solvent from the glass liner before transfer of the compounds to the GC column. Moreover, to facilitate solvent elimination through the injector bottom, the GC column end was removed from the injector body prior to the LC-GC transfer. After solvent elimination, the helium flow was switched off, the GC column end was again connected to the injector body, and the transfer line was removed from the PTV. The carrier gas was then turned on, and the PTV temperature was increased to 350 °C (at 14 °C/s) to achieve the thermal desorption of the solutes trapped in the adsorbent placed into the glass liner and its subsequent transfer to the capillary column. The final temperature was maintained for 6.5 min to promote the elimination of the remaining solvent from the glass liner.

GC Analysis. GC separations were performed on a 5% diphenyl/95% dimethyl polysiloxane fused silica column (30 m × 0.250 mm i.d., 0.25-μm film thickness, Sugelabor, Madrid, Spain) with helium as the carrier gas. The GC temperature was programmed at a rate of 20 °C/min from 130 to 230 °C (2 min) and then to 290 °C (30 min) at 3 °C/min. In all analyses, the detector temperature was set at 320 °C and the 2600 Chromatography software (Perkin-Elmer Nelson Systems) was used for data acquisition.

RESULTS AND DISCUSSION

According to our previous research on RPLC-GC, efficient elimination of the extremely large volume of vapor resulting from the aqueous eluent used as mobile phase in the RPLC step can be achieved not only in the evaporative mode but also in the solid-phase extraction mode. To this aim, experimental conditions were selected to be ideally suited, as far as possible, to both modes of solvent elimination (Señoráns et al., 1995a,b, 1996; Blanch et al., 1997). Moreover, although the aim of the work was, as previously mentioned, to develop a qualitative screening method suitable to perform the rapid and simple analysis of squalene in edible oils by RPLC-GC, it is also interesting to consider the eventual possibility of enlarging the field of application of the method to the analysis of other minor compounds occurring in edible oils. Consequently, experimental conditions were established to make possible the simultaneous RPLC-GC analysis of different groups of compounds. For that reason, previous results obtained concerning analysis of sterols and tocopherols were considered (Señoráns et al., 1996, 1998), and the requirements needed to simultaneously carry out the analysis of squalene present in edible oils were carefully evaluated. In this respect, it is clear that 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 one another in the gas chromatographic analysis.

Precision achievable with the method was evaluated from RPLC-GC analysis performed under identical experimental conditions of squalene present in a residue olive oil. Relative standard deviation ($n = 3$) obtained from the absolute peak areas was 6%. The detection limit (calculated as the amount of product giving a signal equal to three times the background noise) was 0.3 ppm.

To illustrate the potential of the method for the on-line transfer of very large fractions preseparated by LC, Figure 1 shows both the LC chromatogram resulting from the analysis of a rapeseed oil and the GC separation obtained after transfer of an LC fraction as large as 8 min. As a result, free sterols, tocopherols, and squalene present in the rapeseed oil can be separated in only one chromatographic run so that the proposed analytical procedure represents a significant simplification in comparison to conventional methods which usually require a laborious sample preparation. Tentative identification of the mentioned compounds is also given in Figure 1.

In conclusion, the proposed method may allow not only the rapid and reliable analysis of squalene in edible oils by direct injection (that is, without the need for prior enrichment of the sample) but also the simultaneous RPLC-GC analysis of other groups of compounds (that is, free sterols and tocopherols) of the unsaponifiable matter of edible oils, which are currently used to facilitate the identification or the distinctions among different qualities and oil treatments. The overall procedure (including LC preseparation, LC-GC transfer, and GC analysis) required ≈50 min.

LITERATURE CITED

Blanch, G. P.; Ruiz del Castillo, M. L.; Herraiz, M. Evaluation of a transfer technique for direct coupling of reversed phase liquid chromatography and gas chromatography (RPLC-GC). *J. Chromatogr.* **1997**, submitted for publication.

Bondioli, P.; Mariani, C.; Lanzani, A.; Fedeli, E.; Muller, A. Squalene recovery from olive oil deodorizer distillates. *J. Am. Oil Chem. Soc.* **1993**, *70*, 763−766.

de Frutos, M.; Cifuentes, A.; Amigo, L.; Ramos, M.; Diez-Masa, J. C. Rapid analysis of whey proteins from different animal species by reversed-phase high-performance liquid chromatography. *Z. Lebensm.-Unters. Forsch.* **1992**, *195*, 326−331.

Firestone, D. Fats and oils. *J. AOAC Int.* **1995**, *78*, 150−155.

Goosens, E. C.; de Jong, D.; de Jong, G. J.; Brinkman, U. A. Th. Reversed-phase liquid chromatography coupled on-line with capillary gas chromatography. II. Use of a solvent vapor exit to increase introduction volumes and introduction rates into the gas chromatograph. *J. Microcolumn Sep.* **1994**, *6*, 207−215.

Grob, K. *On-Line Coupled LC-GC*; Hüthig: Heidelberg, 1991.

Grob, K. Development of the transfer techniques for on-line high performance liquid chromatography-capillary gas chromatography. *J. Chromatogr. A* **1995**, *703*, 265−276.

Grob, K.; Lanfranchi, M.; Mariani, C. Evaluation of olive oils through the fatty alcohols, the sterols and their esters by coupled LC-GC. *J. Am. Oil Chem. Soc.* **1990**, *67*, 626−634.

Grob, K.; Kaelin, I.; Artho, A. Coupled LC-GC: the capacity of silica gel (HP)LC columns for retaining fat. *HRC* **1991**, *14*, 373−376.

Grob, K.; Artho, A.; Mariani, C. Determination of raffination of edible oils and fats by olefinic degradation products of sterols and squalene, using coupled LC-GC. *Fat Sci. Technol.* **1992**, *94*, 394−400.

Malecka, M. The effect of squalene on the thermostability of rapeseed oil. *Nahrung* **1994**, *38*, 135−140.

Mol, H. G. J.; Staniewski, J.; Janssen, H. G.; Cramers, C. A.; Ghijsen, R. T.; Brinkman, U. A. Th. Use of an open tubular trapping column as phase-switching interface in on-line coupled reversed-phase liquid chromatography-capillary gas chromatography. *J. Chromatogr.* **1993**, *630*, 201−212.

Official Journal of the European Communities **1988**, *L 22* (Jan 29), 58 (EEC Regulation 183/93 of the Jan 29, 1993).

Park, P. W.; Guardiola, F.; Park, S. H.; Addis, P. B. Kinetic evaluation of 3β-hydroxycholest-5-en-7-one (7-ketocholesterol) stability during saponification. *J. Am. Oil Chem. Soc.* **1996**, *73*, 623−629.

Señoráns, F. J.; Herraiz, M.; Tabera, J. On-line reversed-phase liquid chromatography-capillary gas chromatography using

a programmed temperature vaporizer as interface. *HRC* **1995a**, *18*, 433−438.

Señoráns, F. J.; Reglero, G.; Herraiz, M. Use of a programmed temperature injector for on-line reversed-phase liquid chromatography-capillary gas chromatography. *J. Chromatogr. Sci.* **1995b**, *33*, 446−450.

Señoráns, F. J.; Tabera, J.; Herraiz, M. Rapid separation of free sterols in edible oils by on-line coupled reversed phase liquid chromatography-gas chromatography. *J. Agric. Food Chem*. **1996**, *44*, 3189−3192.

Señoráns, F. J.; Villén, J.; Tabera, J.; Herraiz, M. A simplex optimization of the direct analysis of free sterols in sunflower oil by on-line coupled reversed phase liquid chromatography-gas chromatography. *J. Agric. Food Chem*. **1998**, in press.

Staniewski, J.; Janssen, H. G.; Cramers, C. A.; Rijks, J. A. Programmed-temperature injector for large-volume sample introduction in capillary gas chromatography and for liquid chromatography-gas chromatography interfacing. *J. Microcolumn Sep*. **1992**, *4*, 331−338.

Strandberg, T. E.; Tilvis, R. S.; Miettinen, T. A. Effects of cholestyramine and squalene feeding on hepatic and serum plant sterols in the rat. *Lipids* **1989**, *24*, 705−708.

Vreuls, J. J.; Brinkman, U. A. Th.; de Jong, G. J.; Grob, K.; Artho, A. On-line solid-phase extraction-thermal desorption for introduction of large volumes of aqueous samples into a gas chromatograph. *HRC* **1991**, *14*, 455−459.

Vreuls, J. J.; de Jong, G. J.; Ghijsen, R. T.; Brinkman, U. A. Th. Liquid chromatography coupled on-line with gas chromatography: state of the art. *J. AOAC. Int.* **1994**, *77*, 306−327.

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