Rapid Separation of Free Sterols in Edible Oils by On-Line Coupled Reversed Phase Liquid Chromatography−Gas Chromatography

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A rapid analysis of the sterol fraction of oils by on-line reversed phase liquid chromatography−gas chromatography (LC−GC) is described. Methanol:water is used as eluent in the LC preseparation step, 2000 μL/min being the speed of sample transfer. A programmed temperature vaporizer is used as an interface for the LC−GC equipment, and the solvent elimination is performed simultaneously in both the evaporative and nonevaporative modes so that a 3000-μL LC fraction can be transferred into the GC. The proposed procedure only demands previous dilution of the oil sample with CH_2Cl_2 and allows the analysis of the sterol fraction in only 20 min, including the oil sample preparation and the LC−GC analysis itself.

Keywords: *Reversed phase LC−GC; on-line coupling; sterols; direct injection; edible oils*

INTRODUCTION

On-line liquid chromatography−gas chromatography (LC−GC) analysis is advantageous over conventional methods due to the possibility of removing most of the undesired constituents from the sample, thus achieving the fractionation of different classes of closely related compounds in the LC step, so that more complete separation is finally achieved in GC (Grob, 1991, 1995). Different transfer techniques (i.e., the concurrent eluent evaporation, the retention gap technique, and the partially concurrent eluent evaporation) and types of interface (i.e., the loop-type interface and the on-column interface) have been reported, but nearly all LC−GC applications involve normal phase LC conditions (Cortes et al., 1985; Cortes, 1990; Davies et al., 1988, 1989; Grob et al., 1984; Grob, 1991; Riekkola, 1989). The coupling of reversed phase LC (RPLC) with capillary GC brings about a number of problems due to water properties which cause greater difficulty with interfacing the LC to the GC inlet.

Several authors have reported different approaches for on-line RPLC−GC analysis including direct introduction of the eluent from a micro-LC system into the GC (Cortes et al., 1989; Duquet et al., 1988), the use of retention gaps (Goosens et al., 1991, 1994; Grob and Artho, 1991), employment of phase switching from the aqueous phase to an organic solvent (Mol et al., 1993; Staniewski et al., 1992; Vreuls et al., 1991a), or intermediate trapping of analytes in a solid phase followed by thermal desorption (SPE-TD) (Vreuls et al., 1991b, 1993). However, various aspects mainly related to (a) the increase of the volume transferred from LC into GC and (b) the speed of sample evaporation have avoided the development of the technique.

On the other hand, it is widely known that the analysis of major components of fats and oils often does not enable their identification or the distinction between different qualities and oil treatments. Consequently, trace components analysis is strongly required. Specifically, the sterol composition is commonly used (Itoh et al., 1981; Brumley et al., 1985; Grob et al., 1990; Firestone, 1995; Biedermann et al., 1996), although several problems concerning the small concentration of these compounds (i.e., the sterolic fraction of a sunflower oil accounts for approximately 0.3% of the oil composition) make the analysis difficult. Moreover, a number of problems concerning effective separation of different oil fractions and interferences in the subsequent chromatographic analysis usually demand the performance of laborious (and unreliable) saponification of the triglycerides and subsequent extraction of the unsaponifiable which involves the use of relatively high volumes of hazardous solvents (Hubbard et al., 1977; Itoh et al., 1981; *Official Methods of Analysis*).

In the present work, we propose a new method for the rapid determination of the sterolic fraction by on-line RPLC−GC coupling which is applied to the direct analysis of an edible oil. This procedure was developed to fulfill the following requirements: (a) elimination of the conventional sample preparation step (including saponification of the triglycerides), (b) reducing solvent use, (c) possibility of coupling RPLC with GC, (d) capability of working under experimental conditions adequate to a variety of column types and internal diameters, and (e) possibility of transferring high-volume fractions to improve the detection limits achievable with the analysis.

EXPERIMENTAL PROCEDURES

Preparation of Samples. Prior to the RPLC−GC determination of sterols, different organic solvents (i.e., methanol, acetonitrile, ethanol, 2-propanol, *n*-hexane, acetone, chloroform, methyl *tert*-butyl ether, and dichloromethane) were tested for diluting the sample to establish the most convenient conditions for the subsequent analysis of the oil sample. To this aim, two different aspects were considered, namely, the compatibility of the solvent to be selected with the mobile phase used in the LC preseparation and the adjustment of the amount of triglycerides to be introduced into the LC column to avoid its overloading with fat. Initially, *n*-hexane, acetone, chloroform, methyl *tert*-butyl ether, and dichloromethane seemed to be adequate, although the first three are not compatible enough with the eluent to be used in the LC step. Also, different sample weights of the oil sample were diluted in various solvent volumes so that dilutions of 1/40, 1/30, 1/20, 1/15, 1/10, 1/7, 1/3, 1/2, and 1.5/1 (w:v) were considered. Finally, a 300-mg sample weight of the oil to be analyzed (i.e., a refined sunflower oil obtained from the local market) was diluted with 200 μL of CH_2Cl_2 in a 5-mL screw-capped flask.

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In this way, the amounts of triglyceride injected did not affect significantly the performance of the LC column as it could be observed after having carried out more than 100 injections. The sample was then filtered through a 0.22-μm Pro-X filter (Teknokroma, Madrid, Spain) and subsequently injected into the HPLC.

Chromatographic Equipment. The analyses were performed using an on-line coupled LC−GC equipment. The HPLC system, a Hewlett-Packard Model 1050 chromatograph (Wilmington, DE), was fitted with a manual injection valve (Model 7125, Rheodyne, Cotati, CA) having a 20-μL sample loop and a ultraviolet (UV) detector. The column (5 cm × 4.6 mm i.d.) was slurry packed using Vydac 214 TPB 10 according to a previously reported procedure (de Frutos et al., 1992). Methanol:water was used as mobile phase. Initial conditions (methanol:water, 68:32) were maintained for 12 s and then followed by a linear gradient up to 22% water. After elution of the sterol fraction, the linear gradient was modified up to 14% water within 2 min and maintained there for 4 min. Subsequently, the gradient was again modified within 4 min up to 0% water. Separations were carried out at 45 °C, 2.0 mL/min being the flow rate. All throughout the experimentation, the UV detection was performed at 205 nm. The water used was collected from a Milli-Q water purification system (Millipore, Milford, MA), and methanol (HPLC grade) was purchased from Lab-Scan (Dublin, Ireland).

A Perkin-Elmer (Norwalk, CT) programmed temperature vaporizer (PTV) (Poy et al., 1981; Schomburg, 1981; Vogt et al., 1979a,b) was used as the interface for the LC−GC system. The transfer from the LC fraction into the gas chromatograph was performed using a multiport valve positioned between the UV detector of the HPLC system and the PTV injector of the GC, as described elsewhere (Señoráns et al., 1995a).

The choice of transfer conditions was based on our preliminary work concerning the use of different experimental designs (Villén et al., 1992; Señoráns et al., 1993, 1995b). On this basis, the following conditions were used: packing material in the glass liner of the PTV, Tenax TA, 80−100 mesh (Chrompack, The Netherlands); plug length of packing material, 4 cm; initial PTV temperature, 30 °C; helium flow rate during transfer, 700 mL/min.

Once the transfer step was completed, both the PTV temperature and the helium flow rate were kept constant for 15 s to eliminate the remaining solvent from the glass liner. The thermal desorption of the trapped material was achieved by heating the PTV at 14 °C/s to 350 °C. The GC analyses were performed on a Perkin-Elmer Model 8310 instrument with FID operated at 310 °C, and the 2600 Chromatography software (Perkin-Elmer Nelson Systems) was used for acquisition of data. A DB-5 (Chrompack) fused silica column (9 m × 0.105 mm i.d., 0.4-μm film thickness) was used, helium (21 cm/s) being the carrier gas. The temperature was programmed from 130 to 260 °C (20 °C/min) and then to 300 °C (6 °C/min). The final temperature was maintained for 3 min.

RESULTS AND DISCUSSION

Our previous research concerning the RPLC−GC analysis did not allow the transfer of LC fractions larger than 1600 μL, the speed of sample introduction being up to 1500 μL/min. Under these experimental conditions, several limitations concerning the analysis of minor compounds occurring in real samples were observed, as detection limits achievable are not low enough to make their analysis possible. In this respect, the experimental procedure proposed in this work allows a significant improvement concerning the direct analysis of real samples.

Figure 1 shows both the liquid chromatogram obtained by direct injection of a diluted sunflower oil (see Experimental Procedures) and the GC analysis resulting from transferring a 3000-μL fraction into the GC system, 2000 μL/min being the HPLC flow rate. Tentative identifications of some of the constituents show that

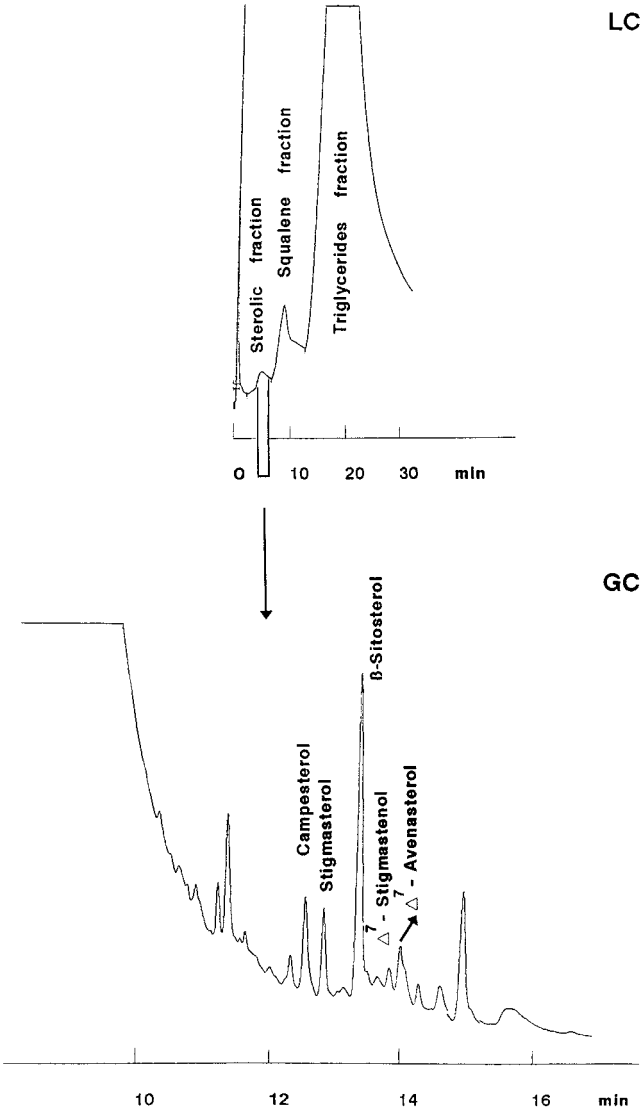

Figure 1. LC preseparation and GC separation of a sunflower oil by reversed phase LC−GC direct coupling via PTV (HPLC flow rate, 2000 μL/min; transferred volume, 3000 μL).

beside the sterolic fraction, squalene and triglycerides are also obtained in the LC preseparation.

It is worth emphasizing that the characteristics of the method proposed are advantageous over other procedures which only admit small LC fraction volumes in order to shorten solvent evaporation times thus making the transfer easier. In this work, however, solvent elimination is carried out simultaneously in both the evaporative and nonevaporative modes. Consequently, solvent elimination is achieved by using (a) the PTV injector operated in the solvent split mode (Poy et al., 1982) and (b) the sorption properties of the packing material placed in the glass liner (solid phase extraction mode) (Vreuls et al., 1991b; Señoráns et al., 1995a).

The possibility of transferring the sample at rates as high as 2000 μL/min permits transfer of 3000 μL in only 90 s. Under these conditions, a LC column having an internal diameter as big as 4.6 mm (i.e., high sample capacity) has proved to be well suited for the LC−GC procedure proposed in this work. As can be seen in Figure 1, the obtained results clearly show the abundance of β-sitosterol and the four much smaller campesterol, stigmasterol, Δ^7-stigmastenol, and Δ^7-avenasterol peaks typical of sunflower oil. Other peaks in Figure 1 have not been identified, but some of them probably

Table 1. Relative Standard Deviations ($n = 5$) for the Absolute Peak Areas for the GC Analysis of a 3000-μL Volume Fraction Resulting from On-Line RPLC−GC Transfer of a Sunflower Oil

RRT[a]	compound	average	RSD ($n - 1$)
0.93	campesterol	2988.8	3.12
0.96	estigmasterol	2302.6	2.43
1.00	β-sitosterol	9641.4	2.90
1.05	Δ^7-avenasterol	2215.2	3.59

[a] RRT, ratio of the retention time of each peak to that for β-sitosterol.

represent other minor sterols (only accounting for approximately 5% of the sterol fraction of a sunflower oil) (Gunstone et al., 1986).

Table 1 gives the relative standard deviation (RSD) values resulting from the absolute peak areas of the major peaks obtained in five replicate analyses of a sunflower oil. As can be seen, RSD's for identified sterols range between 2.4 and 3.6%. It should be underlined that the low RSD value obtained (2.3%) for the sum of peak areas transferred from LC into GC suggests the stability of the system over an extended period of time for the direct transfer of a specific fraction. It should be also mentioned that under the experimental conditions described, the lifetime of both the liquid and gas chromatographic columns was satisfactory as the injection of more than 100 oil samples did not affect their performances.

Summarizing, we have developed the basis of an analytical procedure which allows the rapid on-line coupled RPLC−GC analysis of edible oils without the need for pretreatment of the sample. Although many aspects require a more precise study, we believe that these preliminary results demonstrate the advantages of the proposed method compared to conventional methods for the analysis of the sterolic fraction of edible oils.

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