On-line derivatization with on-line coupled normal phase liquid chromatography–gas chromatography using the through oven transfer adsorption desorption interface: Application to the analysis of total sterols in edible oils

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ABSTRACT

In the present work on-line derivatization is combined with on-line normal phase liquid chromatography–gas chromatography (NPLC–GC) to analyze total sterols in edible oils. The method uses the TOTAD interface with an additional LC injection valve to automatically introduce the derivatization reagent. The derivatization reaction takes place in the adsorbent material located inside the liner of the TOTAD interface. The samples were saponified with potassium hydroxide in an ethanolic solution and the unsaponifiable fraction was extracted with diethyl ether. The extract was then analyzed by on-line derivatization with on-line NPLC–GC, avoiding the laborious thin layer chromatography and off-line derivatization steps used in the Official European Union (EU) method. The relative standard deviations (RSDs) from the absolute peak area were lower than 14% except for campestanol. No variability in retention time was observed. Limits of detection (LODs) were less than 8.82 mg/kg. Different edible oils were analyzed using the proposed method and the results obtained were compared with those obtained using the Official EU method. There was good agreement between both methods for all the sterols analyzed.

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1. Introduction

In edible oils, sterols are primarily present in their free and esterified forms and provide rich information about the oil quality. The analysis of total sterols in edible oils is common laboratory practice to control oil quality. The sterol profile can be used to detect the adulteration of some vegetable oils with other cheaper ones, for instance the EU has established an upper limit in campesterol of 4% of total sterols [1,2]. The analyses are usually based on the official methods and involve saponification of the lipids, extraction of the non-saponifiable matter with diethyl ether and washing the extract with water, separation by thin layer chromatography (TLC) on silica gel plates and derivatization of the sterols and subsequent chromatographic analysis [3]. These methods have two drawbacks: they are laborious and time-consuming and are subject to error due to sample manipulation. On-line liquid chromatography–gas chromatography (LC–GC) greatly reduces the manual operations involved in the analysis of both free and esterified sterols. Grob

et al. [4–6] described different on-line LC–GC methods using normal phase after derivatization of free sterols. An LC–GC method using reverse phase to determinate free sterols was described by Villén et al. [7] and Señorans et al. [8] using a PTV injector to transfer the LC fraction to the GC. However, with this procedure, the GC column has to be removed and installed in each run and it is not possible to automate the system. Cortés et al. [9] described an automated on-line reversed phase liquid chromatography–gas chromatography (RPLC–GC) method using the TOTAD interface, which involved directly injecting the oil with no sample pre-treatment step other than filtration, although this method did not permit the detection of esterified sterols, but only of free sterols. A subsequent modification of this method proposed by Toledano et al. [10] allowed both free and esterified sterols to be analyzed. To determine free sterols the diluted oils are injected into the liquid chromatograph, where they are separated from triglycerides and the sterol fraction is automatically transferred to the gas chromatograph to be analyzed. To determine total sterols the samples were saponified with potassium hydroxide in ethanolic solution and the unsaponifiable fraction was extracted with diethyl ether. The extract was then analyzed by RPLC–GC. However, it presents two drawbacks: the first is that the GC chromatogram obtained does not show good resolution

and the second drawback is that does not provide good values for campesterol and campestanol. Having in mind that there was no derivatization, some campesterol might be degraded during the GC analysis. In order to increase its thermal stability, a derivatization reaction is needed. Typically, derivatization is performed off-line in the sample preparation before LC–GC analysis [6,11]; but on-line derivatization offers numerous advantages over off-line methods. The on-line derivatization needed in the LC–GC can be carried out in the LC system, between LC and GC or in the inlet of the GC column [12]. One advantage of on-line derivatization after the LC step is that the enrichment or pre-separation of the analytes is based on underivatized functional groups. Hyötyläinen et al. [13] used on-line derivatization before GC for the determination of morphine and its analogs in urine by on-line LC–GC. A loop-type interface and concurrent eluent evaporation technique were used in the coupling, and the aqueous phase was substituted by organic solvent before transfer to the GC. Chappell et al. [14] used on-line derivatization of stilbene hormones analyzed by on-line LC–GC and LC–GC–MS. Tzing et al. [15] used a PTV with a microvial placed inside the injector body where the derivatization reaction takes place. As far as we know, sterols have not previously been derivatized on-line. The objective of this work is to incorporate the on-line derivatization in the on-line LC–GC analysis of sterols in edible oils by using the TOTAD interface. To this end, a new method to analyze sterols in edible oils has been developed. The analytical method previously developed by Cortés at al. [9] used in the LC step methanol/water as eluent to separate free sterols from other constituents of the oil, mainly triglycerides. Toledano et al. [10] also used reversed phase in the LC step. Water is not an appropriate eluent for the derivatization reaction, which should be carried out in an organic solvent such as normal phase eluent LC. For this reason, normal phase was used in the on-line LC–GC analysis to incorporate the derivatization reaction in the method. Furthermore, the TOTAD interface was modified to incorporate an extra LC injection valve after the LC chromatograph, for the derivatization reagent to be introduced.

2. Experimental procedures

2.1. Materials

The edible oil samples were certified reference materials supplied by the Ministry of Agriculture and Fisheries of the Regional Government of Andalusia (Spain). Sample A was a mixture of extra virgin olive oil (60%) and sunflower oil (40%); sample B was a lampante olive oil and sample C a refined pomace olive oil.

Standards of stigmasterol and cholesterol were purchased from Sigma–Aldrich, (Steinheim, Germany). The Internal standard, 5α-cholestan-3β-ol (cholestanol) at 0.2% (w/v) in chloroform was from Supelco (Bellefonte, USA). Two different trimethylsilylating reagents were tested. N-methy-N-(trimethylsilyl) trifluoroacetamide (MSTFA) prepared dissolving 12 mg of dithioerythritol and 60 mg of ammonium iodide in 30 mL of MSTFA, were purchased from Sigma–Aldrich (Steinheim, Germany) and Silan-Sterol-1 CG prepared as pyridine–hexamethyldisilazane–trimethylchlorosilane (9:3:1, v/v/v), was purchased from Panreac (Castellar del Vallés, Spain). Silan-Sterol-1 CG was chosen because it provided better results. Different reagent volumes were evaluated (2, 3, 5, 10, 20 and 100 μL). 10 μL was chosen as the optimum injection volume. Ethanol, water, n-hexane and ethyl acetate, all HPLC grade, were purchased from LabScan (Dublin, Ireland). Potassium hydroxide was from Merck (Darmstadt, Germany). Tenax TA, 80-100 mesh (Chrompack, Middelburg, Netherlands), was used as packing material in the liner of the modified PTV (TOTAD interface). The glass-liner was packed with a 1 cm length of Tenax TA between two plugs of glass wool to keep it in place and was then conditioned under a helium stream by heating from 50 °C to 350 °C at 50 °C/10 min, where it was maintained for 60 min.

2.2. Sample preparation

50 μL of the internal standard was added to 0.5 g of the sample. Then, it was saponified with 5 mL of 2 N potassium hydroxide in ethanolic solution and the unsaponifiables were then extracted once with 8 mL of diethyl ether and two more times with 6 mL of the same solvent. The organic layer was washed with 5 mL of water until neutral pH was reached; the extract was filtered through a 0.20 μm (Millex-GN SLGN 013 NL) filter. Then, 2.5 μL of the extract was injected into the LC–GC system to be analyzed.

2.3. On-line NPLC–GC with on-line derivatization

2.3.1. 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) [16–18]. The HPLC system comprised a manual injection valve (model 7125, Rheodyne) with a 2.5 μL loop, a quaternary pump (HP model 1100), a column oven (HP model 1100) and a UV-Vis detector (Konik 550). The gas chromatograph (Konik model HRGC 4000B) was equipped with a TOTAD interface and an FID detector. The derivatization reagent was delivered by an additional injection valve (model 7125, Rheodyne) positioned between the HPLC detector and the TOTAD interface (Fig. 1). Data acquisition and processing were performed with KoniKrom 32 (Konik, Sant Cugat Del Vallés, Barcelona) software.

2.3.2. LC conditions

LC pre-separation was carried out on a Lichrospher 5 μm Si 60 250 mm × 4.0 mm column (Hichrom, Berks, U.K.) maintained at 20 °C. To ascertain the elution time of the fraction to be transferred to the gas chromatograph 20 μL solutions of stigmasterol and cholesterol in methanol at 1000 mg/L were injected. The initial composition of the eluent (n-hexane/ethyl acetate, 80:20, v/v) at a flow rate of 2 mL/min was maintained for 20 min, then the gradient was varied to reach 100% ethyl acetate within 1 min and maintained for 20 min. UV detection was performed at 205 nm.

In the sample analyses, 2.5 μL of the extract obtained as indicated above was injected, with a flow rate of 2 mL/min until the fraction of interest began to be eluted. During the transfer step, the flow was changed to 0.5 mL/min and maintained constant until the transfer step had finished. After the transfer, the flow was increased to 2 mL/min again, and the gradient was changed to 100% of ethyl acetate within 1 min and maintained for 20 min to ensure complete elimination of the retained compounds.

2.3.3. LC–GC transfer and on-line derivatization

Initially, the TOTAD interface and GC oven temperature were stabilized at 125 °C and 80 °C, respectively. The carrier gas (helium) stream entered the packed liner through the oven side (B) and through the opposite side (A), both at 500 mL/min. At the outset, the eluent from the HPLC was sent to waste. When the front of the fraction to be transferred reached the six port valve (at 3.5 min) it was automatically switched, transferring the LC fraction to the GC (Fig. 1). The solution reached the glass liner at 0.5 mL/min. The helium pushed the solution through the adsorbent. During the LC–GC transfer the analytes were retained on the packed material in the liner and the solvent was vented to waste through the WT tubing. At 15 min 30 s, 10 μL of the derivatization reagent was injected and propelled to the liner by the eluent from the HPLC. Once the transfer step was finished, the six port valve was automatically
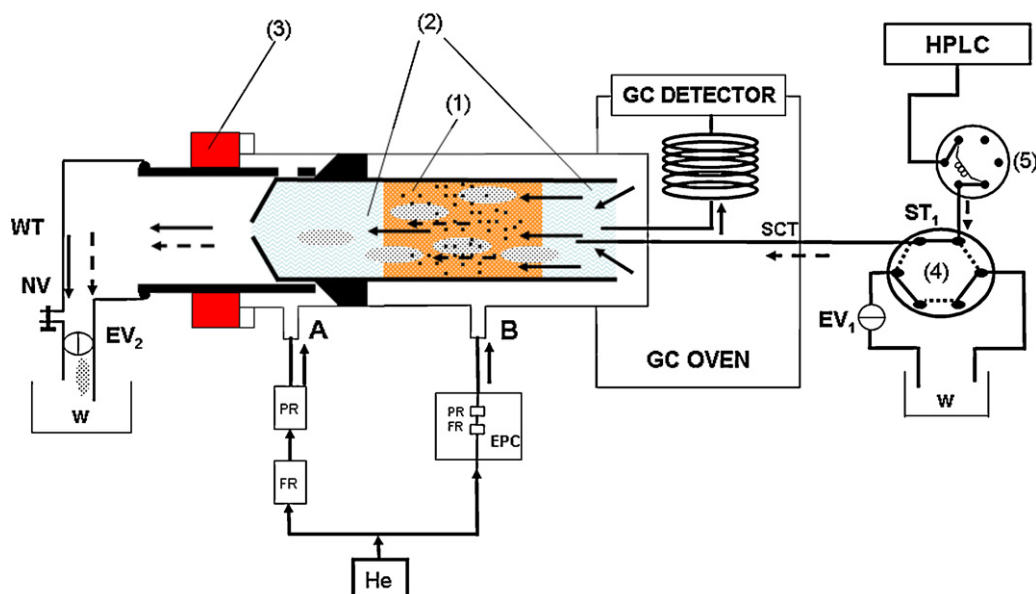
Fig. 1. Automated TOTAD interface during the transfer step. Symbols: (1) sorbent (Tenax TA); (2) glass wool; (3) heated cover; (4) six-port valve; (5) derivatization injection valve; (EV1 and EV2) electrovalves 1 and 2; (EPC) electronic pressure control; (PR) pressure regulator; (FR) flow regulator; (solid arrows) gas flow; (dotted arrows) liquid flow; (ST1) stainless steel tubing, 0.25 mm I.D., to transfer eluent from LC to six-port valve; (WT) waste tubing, to allow the exit of liquids and gases; (SCT) silica capillary tubing, 0.32 mm I.D.; (W) waste; (⬤) solvent; (⦙⦙) analytes; NV (⊥) needle valve.

switched so that the eluent coming from the LC was sent to waste. EV_1 was opened and the temperature was maintained at 125 °C for 1 min to allow the derivatization reaction to take place while the remaining solvent in the glass-liner and the SCT tubing was being eliminated. Afterwards (at 17 min 12 s), the TOTAD interface was quickly heated to 200 °C to remove volatile impurities from the derivatization reaction. Then (at 19 min) EV_1 and EV_2 were closed and the flow through B was interrupted while the flow through A was changed to 1 mL/min. At the same time, the TOTAD interface was quickly heated to 350 °C, leading to the thermal desorption of the trimethylsilyl ether derivatives of the analytes, which were transferred to the GC column, propelled by the helium. GC analysis was then carried out, after which EV_2 was opened and the interface was cleaned by maintaining the helium stream for 5 min at 350 °C. Finally, it was cooled to 125 °C so that another analysis could be carried out. Table 1 summarizes the timetable and events of the process.

2.3.4. GC conditions

A fused-silica column (60 m × 0.25 mm I.D.) coated with 5% phenyl methyl silicone (film thickness of 0.25 μm) from HiChrom (Berks, U.K.) was used for the gas chromatography separations. During the transfer and the solvent elimination steps, the oven temperature was kept at 80 °C. During GC-FID analysis, the temperature oven was programmed as follows; initially 80 °C; at 40 °C/min to

220 °C and hold for 1 min; 30 °C/min to 295 °C and hold for 30 min. The FID temperature was kept at 330 °C.

2.4. On-line NPLC–GC without on-line derivatization

Sample A was also analyzed using the conditions indicated in Section 2.3 but without addition of the derivatization reagent.

2.5. Official EU method

The Official EU method [1] and later modifications [2] were also applied in the analytical determinations of the samples in order to compare the obtained data with the proposed method. The certified value is reached from the median of the analytical results of the inter-lab certification studies carried out by fourteen laboratories chosen from among those accredited by the International Olive Oil Council (IOOC) and recommended by the same institution.

3. Results and discussion

In the present method sample preparation includes saponification and extraction of the unsaponifiable fraction with diethyl ether, as in the Official EU method, but the tedious and time consuming TLC step used to separate and purify the total sterols is avoided by using an automated on-line coupling LC–GC system. The saponification reaction releases the esterified sterols, and total

Table 1
Timetable and events in the analysis of total sterols by on-line NPLC–GC with on-line derivatization.

| Time	Event	HPLC	TOTAD	GC oven	Valves
0′00″–3′30″	LC analysis	LC eluent sent to waste	125 °C	80 °C	EV1 closed EV2 opened
3′30″–15′30″	LC transfer	LC eluent sent to TOTAD	125 °C	80 °C	EV1 closed EV2 opened
15′30″–16′12″	Derivatization reagent introduction/derivatization reaction	Derivatization reagent propelled to TOTAD by LC eluent	125 °C	80 °C	EV1 closed EV2 opened
16′12″–17′12″	Remaining solvent elimination	LC eluent sent to waste	125 °C	80 °C	EV1 opened EV2 opened
17′12″–19′	Volatiles impurities elimination	LC eluent sent to waste	200 °C	80 °C	EV1 opened EV2 opened
19′–24′	Analyte desorption	LC eluent sent to waste	350 °C	GC analysis	EV1 closed EV2 closed
19′–55′	GC analysis	LC eluent sent to waste	125 °C	GC analysis	EV1 closed EV2 closed
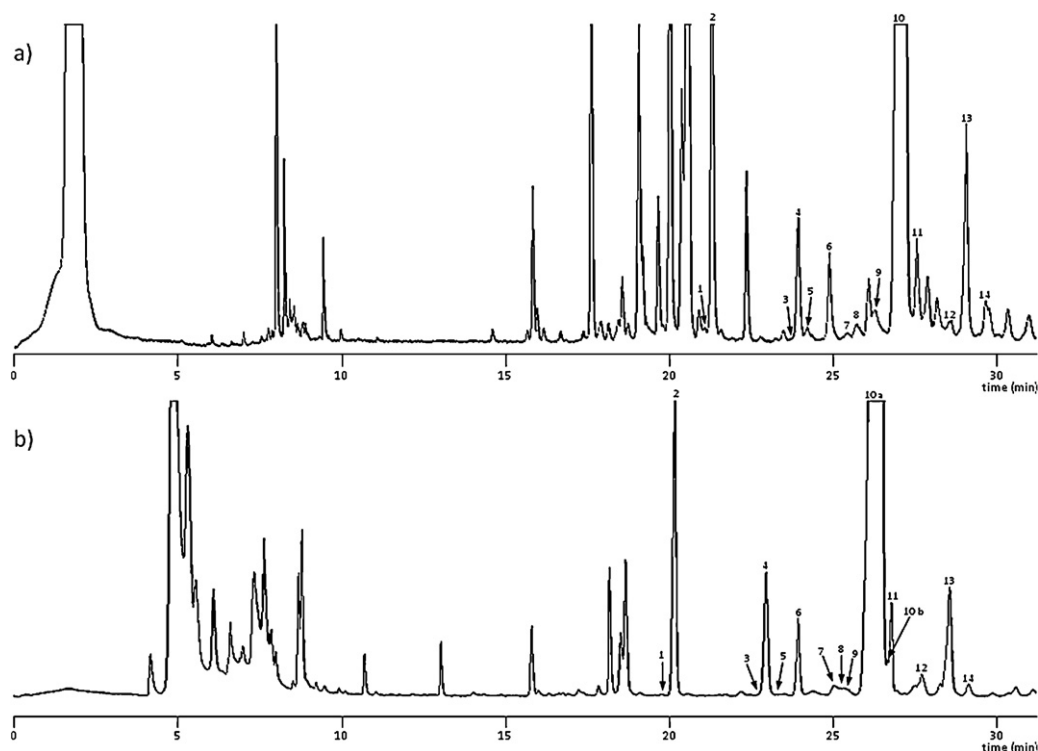
Fig. 2. (a) NPLC–GC-FID chromatogram from an extract of sample A without derivatization (1. Cholesterol, 2. Cholestanol (I.S.), 3. 24-Methylencholesterol, 4. Campesterol, 5. Campestanol, 6. Stigmasterol, 7. Δ7-Campesterol, 8. Δ5,23-Stigmastadienol, 9. Clerosterol, 10. β-Sitosterol + Sitostanol, 11. Δ5-Avenasterol, 12. Δ5,24-Stigmastadienol, 13. Δ7-Stigmastenol, 14. Δ7-Avenasterol). (b) NPLC–GC-FID chromatogram from an extract of sample A with on-line derivatization (1. Cholesterol, 2. Cholestanol (I.S), 3. 24-Methylencholesterol, 4. Campesterol, 5. Campestanol, 6. Stigmasterol, 7. Δ7-Campesterol, 8. Δ5,23-Stigmastadienol, 9. Clerosterol, 10a. β-Sitosterol, 10b. Sitostanol, 11. Δ5-Avenasterol, 12. Δ5,24-Stigmastadienol, 13. Δ7-Stigmastenol, 14. Δ7-Avenasterol).

sterols are quantified in the form of free sterols. The extraction with diethyl ether results in an extract free of triglycerides and other compounds from the saponifiable fraction. Most conventional methods use a 5 g sample and huge amount of organic solvents in the saponification and extraction process. The use of miniaturized techniques is very useful in food analysis since they reduce consumption of both solvent and sample. For this reason by using the method developed in the present work, the amount of sample and solvent used is reduced by approximately 90%. In the Official EU method 300 μL of the extract is subjected to TLC instead only 2.5 μL is injected into the LC–GC system in the proposed method. Fig. 2a shows GC chromatogram obtained when sample A was analyzed by on-line NPLC–GC without derivatization. As it can be observed in the figure, the use of a longer GC column provided a better resolution of the sterol peaks than the resolution obtained by Toledano et al. [10], achieving the complete separation of 24-methylencholesterol and campesterol, although β-sitosterol and sitostanol are still overlapped. Regarding the quantification, the analysis provided good values for all the sterols except campesterol and campestanol. 5.8% and 0.5% were obtained for campesterol and campestanol, respectively, while the values obtained by the Official EU method were 6.40% and 0.1%. The percentage of each sterol was calculated as the ratio of peak area corresponding to the sum of the areas under all the peaks of sterols. It can be observed that the sum of both sterols obtained with both methods is similar. This led us to think that when the on-line NPLC–GC without derivatization method is used some campesterol might be reduced to campestanol in the GC analysis because there is no derivatization step. It is very important to obtain an accurate value for campesterol because this sterol is used as an indicator of virgin olive oil quality. A derivatization reaction is obviously needed to avoid the degradation of campesterol. To this aim, we introduce in the present work the on-line derivatization in the on-line LC–GC method. The

derivatization reaction is performed after the LC step, which is the cleaning step and, of course, before the GC analysis. This reaction should be carried out after the cleaning step as in the Official EU method, in which the derivatization reaction is carried out after cleaning of the sample in the TLC step.

The pre-separation process of sterols from other matrix compounds, which occurred in the LC step carried out in normal phase, is based on underivatized compounds. The total sterols elute in the LC chromatogram from 3.5 to 6.5 min, providing a volume to be transferred of 6 mL since the LC flow was 2 mL/min. LC flow in the transfer step was 0.5 mL/min so the complete transfer took 12 min. The derivatization reagent was delivered after the transfer of the LC sterol fraction to the TOTAD interface. An additional LC injection valve situated between the HPLC equipment and the six port valve (Fig. 1) allowed the automated introduction of the derivatization reagent (during 42 s) which was propelled by the LC eluent. It can be assumed that the derivatization takes places in the adsorbent located inside the liner of the TOTAD interface; before the solvent had been completely eliminated. The derivatization reaction takes places on the surface of the Tenax and rapidly converts the sterols into trimethylsilyl ethers.

The variables studied for the optimization of the derivatization reaction were the silylation reagent and its volume, temperature and reaction time. Two silylation reagents were tested: MSTFA and Silan-Sterol 1 CG. The latter was chosen as it provided the best results. The volume of the derivatization reagent must be optimized because a large excess of derivatization reagent may disturb the separation of the analytes. The volume of derivatization reagent was varied between 2 and 100 μL. When 2, 3 and 5 μL were used, derivatized and non-derivatized sterol peaks appeared in the GC chromatograms. When 10, 20 or 100 μL were used, non-derivatized sterol peaks did not appear and the derivatized sterol peaks showed the same area indicating sterols were totally derivatized. So we

Table 2
Values (%, w/w) obtained with the present method versus certified values obtained with the olive oil analytical methods described in Regulation (EC) N° 2568/91. Relative standard deviation (RSD) from the absolute peak area and from the retention time ($n = 5$). Sample studied was a certified reference material, named as A. Detection limit (LOD) calculated as the amount of product giving a signal equal to five times background noise.

Sterols	Proposed method (%, w/w)	Official EU method (%, w/w)	LOD (mg/kg)	Repeatability	
				RSD (area)	RSD (tr)
Cholesterol	0.2	0.1	3.16	13.4	0.2
Cholestanol (I.S.)	–	–	3.05	9.1	0.1
24-Methylcholesterol	0.11	0.15	3.63	3.7	0.1
Campesterol	6	6.4	3.99	6.5	0.1
Campestanol	0.2	0.1	3.62	16.1	0.2
Stigmasterol	4.0	4.5	3.72	5.3	0.1
Δ7-Campesterol	0.8	1.6	5.67	13.1	0.1
Δ5,23-Stigmastadienol	1.1	0.2	3.80	8.5	0.2
Clerosterol	0.8	0.9	5.45	5.1	0.2
β-Sitosterol	71.5	68.9	8.82	4.9	0.2
Sitostanol	1.0	0.5	1.69	11.9	0.2
Δ5-Avenasterol	4.4	4.3	3.27	7.5	0.2
Δ5,24-Stigmastadienol	1.4	1.2	5.27	10.0	0.2
Δ7-Stigmastenol	7.3	8.5	4.57	5.1	0.2
Δ7-Avenasterol	1.2	2.9	4.32	6.2	0.1

selected 10 μL as the best reagent volume. In the chromatogram obtained (Fig. 2b), no signals of the non-derivatized compounds, side products or excess of derivatization reagent are observed and a satisfactory separation of the silylated sterols were obtained. The oven temperature was optimized for the transfer step by taking into account the conditions required by both solvent elimination and the on-line derivatization reaction. Three derivatization temperatures were tested (70 °C, 125 °C and 200 °C) and 125 °C was selected as the best to obtain an efficient and fast derivatization. Once the derivatization reaction had finished the temperature was quickly increased to 200 °C to remove volatile impurities from the derivatization reaction. A period of 1 min after the introduction of the derivatization reagent was sufficient for the derivatization reaction to take place.

Fig. 2 shows the chromatograms obtained in the absence (Fig. 2a) and presence (Fig. 2b) of the derivatization reaction. A clearer chromatogram can be observed in Fig. 2b where non-derivatives sterols are not detected. As can be seen from Fig. 2b the LC pre-separation step provides satisfactory clean-up and no interfering compounds from the oil matrix are present in the GC chromatogram. The use of a derivatization reaction that generates more volatile and thermally stable compounds ensures good separation and reliable quantization. Comparing the chromatograms shown in Fig. 2, campestanol peak can be clearly seen in Fig. 2a but not in Fig. 2b, indicating that the on-line derivatization process avoids the reduction of campesterol that occurs when sterols are analyzed by on-line NPLC–GC without derivatization. The quantitative data given in Table 2

indicate a 0.2% (w/w) for campestanol. The data obtained for each sterol are in agreement with those obtained using the EU Official method. Table 3 gives the values obtained for the other two samples (samples B and C) which also fit the values obtained by the EU Official method. We conclude that the results obtained with the proposed procedure are comparable with those obtained with the Official EU method.

The repeatability and limit of detection of the method were studied. The results obtained are shown in Table 2. The relative standard deviation (RSD) for the retention time was 0.1 or 0.2%, indicating no variation in the retention time. The RSDs of the absolute peak areas are lower than 14% except for campestanol, which is a very small peak and could present problems of integration. The good repeatability, lower than 14%, observed indicates the absence of interactions between the derivatized compounds and the adsorbent, unlike those found by Pérez Pavón et al. [19], who indicated that in the case of the Tenax TA liner unrepeatability was exceptionally high, and was also observed that on performing successive injections with the same Tenax TA liner, apart from the signals of the derivatized compounds the chromatograms also exhibited signals corresponding to the non-derivatized compounds, with varying intensities.

The detection limits were calculated as the amount of product giving a signal equal to 5 times the background noise. The values obtained are given in Table 2. Bearing in mind the usual concentration of sterols in edible oils, the detection limits can be considered good.

Table 3
Values of total sterols (%, w/w) obtained with the proposed method versus the Official EU method. Samples studied were two certified reference material, B and C.

Sterols	Sample B		Sample C	
	Proposed method (%, w/w)	Official EU method (%, w/w)	Proposed method (%, w/w)	Official EU method (%, w/w)
Cholesterol	0.24	0.13	0.06	0.12
Cholestanol (I.S.)	–	–	–	–
24-Methylcholesterol	0.16	0.20	0.17	0.1
Campesterol	3.54	3.40	3.14	3.10
Campestanol	0.08	0.10	0.18	0.15
Stigmasterol	0.65	0.71	0.77	1.10
Δ7-Campesterol	n.d.	0.09	–	0.09
Δ5,23-Stigmastadienol	n.d.	0.03	0.45	0.80
Clerosterol	0.97	1.00	0.48	1.20
β-Sitosterol	86.50	84.90	89.92	87.27
Sitostanol	0.82	0.60	1.95	1.80
Δ5-Avenasterol	6.18	7.50	1.72	1.52
Δ5,24-Stigmastadienol	0.34	0.50	0.97	1.74
Δ7-Stigmastenol	0.24	0.50	0.13	0.47
Δ7-Avenasterol	0.25	0.40	0.07	0.12

4. Conclusion

On-line derivatization combined with on-line NPLC–GC using the TOTAD interface was used for the analysis of total sterols in edible oils. An additional LC injection valve allows the automated introduction of the derivatizing reagent. The analytes were derivatized on-line at the adsorbent sites inside the liner of the TOTAD interface, before the GC analysis and after the LC pre-separation step. The method required only saponification and extraction of the unsaponifiables as sample pre-treatment, and eliminated the laborious step of thin layer chromatography and off-line derivatization needed in the EU Official method. The total time for the analysis was about 60 min, which is much lower than that needed by the Official EU method. The result obtained for each sterol is in good agreement with those obtained using the Official EU method.

Acknowledgment

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