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Gas chromatographic separation and mass spectrometric identification of mixtures of oxyphytosterol and oxycholesterol derivatives Application to a phytosterol-enriched food

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Abstract

Pure individual phytosterols were prepared using reversed-phase HPLC in order to obtain the oxidized compounds of sitosterol, campesterol, stigmasterol and brassicasterol. 7-Hydroxy-, 7-keto-, 5,6-epoxy-, 4β-hydroxy-, 4-ene-6-hydroxy-, 6-keto- and 5α,6β-dihydroxyphytosterols were obtained as well as analogous compounds of cholesterol. The gas chromatographic properties as well as the electronic impact mass spectra of these compounds (as trimethylsilyl ether derivatives) were studied. These data were used to identify oxyphytosterols in a spread enriched in phytosterols: the oxyphytosterols represented no more than 68 μg/g of spread (about 0.08% of phytosterols were oxidised).

Keywords: Food analysis; Oxyphytosterols; Oxycholesterols; Oxysterols; Sterols

1. Introduction

Very numerous studies were carried out during last years on oxidized derivatives of cholesterol (oxycholesterols, often called "oxysterols"). Some of these compounds are synthesised in vivo from cholesterol. But they can also be formed in food during technological procedures and storage. These compounds were described as key intermediates in the metabolism of cholesterol. Some studies also described their potential cytotoxicity, mutagenicity and their possible implication in atherosclerosis. All these effects of oxycholesterols are well known and were already reviewed [1–3]. However, other sterols than cholesterol occurred in food. The plant sterols (phytosterols) present similar amounts than cholesterol in our diet [4]. There is now an important renewed interest for phytosterols in human diet due to the beneficial effects of these compounds on cholesterol metabolism [5–8]. This will probably lead to an increase of their human consumption. However, these phytosterols can undergo oxidation as well as cholesterol [9–12]. But the data in this field are scarce. It is known that these oxyphytosterols may be present at low levels in some foods [12–17]. It was also demonstrated that few amounts of these compounds were absorbed by the intestine in the rat [18]. Recently, some oxyphytosterols (essentially oxysitosterols) were identified in plasmas of human healthy volunteers [19]. In these last studies, an overlapping of some compounds formed from cholesterol, campesterol or sitosterol was observed in gas chromatography. For example, with the conditions used, 27-hydroxycholesterol, α-epoxysitosterol and campestanetriol were observed in the same chromatographic peak, and 7β-hydroxysitosterol was not resolved from 25-hydroxycholesterol [19]. This led us to undertake the study of the chromatographic properties of the oxyphytosterols and to compare them to those of the known oxycholesterols. An important contribution in this field was brought out recently concerning the side-chain oxidized derivatives of stigmasterol [20], sitosterol and campesterol [21]. The present study concerns the phytosterols oxidized on the rings. These compounds were studied using the gas chromatography of the trimethylsilyl ether (TMSE) derivatives of the oxysterols. This method is indeed used very often for the identification and the quantification of the oxysterols [22–27]. In a first time, phytosterols, which are only available in blends, were individually isolated using

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reversed-phase high-performance liquid chromatography (RP-HPLC). All the sterols were then submitted to heating in order to generate the oxysterols. After purification, these compounds were analysed by gas–liquid chromatography (GLC) and gas–liquid chromatography coupled to mass spectrometry (GLC–MS). Finally, the data obtained were applied to the detection of the oxyphytosterols present in a commercial spread enriched with phytosterol esters.

2. Experimental

The compounds examined in this study are described in Fig. 1 and Table 1 (chemical structures and abbreviations used).

2.1. Materials and reagents

Stigmasterol, cholesterol and 5α-cholestane were obtained from Sigma (L'Isle d'Abeau Chesnes, France). The blend of phytosterols was from ICN (Orsay, France). The standard oxycholesterols 7β-hydroxycholesterol, 25-hydroxycholesterol, α-epoxycholesterol, β-epoxycholesterol, cholestanetriol, 6-ketocholestanol and 7-ketocholesterol were from Sigma. 7α-Hydroxycholesterol, 4β-hydroxycholesterol and 27-hydroxycholesterol were obtained from Steraloids (Newport, RI, USA). Cholest-4-ene-3β,6β-diol was obtained by sodium borohydride reduction of 6β-hydroxycholest-4-ene-3-one (Steraloids). 24-Hydroxycholesterol was synthesised according to Lund et al. [28]. The epoxy-, 7-keto- and dihydroxy-derivatives of sitosterol and campes-

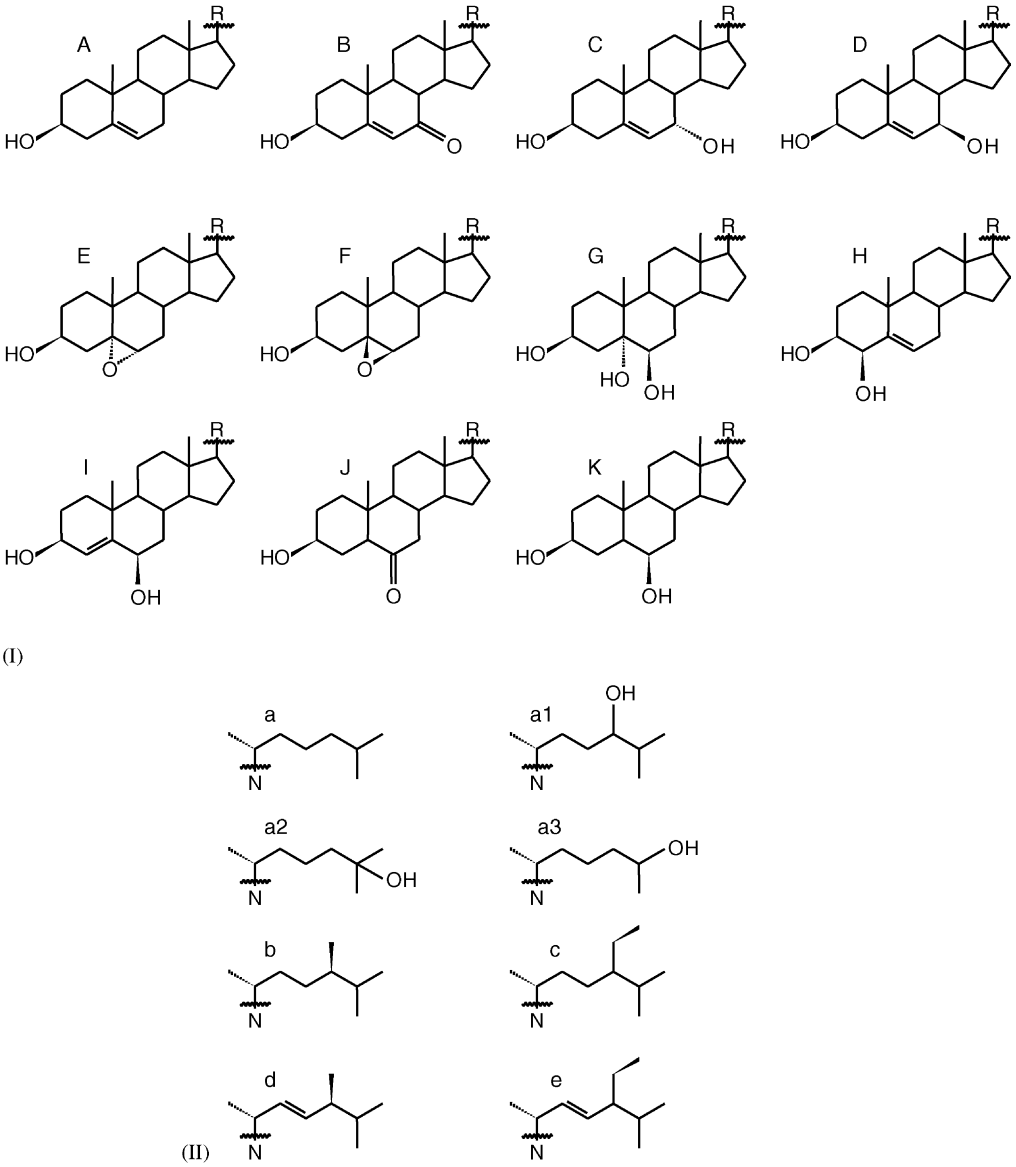

Fig. 1. Structure of compounds examined. (I) Nucleus (N) of the compounds, without the lateral chains (R). (II) Lateral chains (R) linked to the nucleus (N).

Table 1
Nomenclature and abbreviation of compounds examined (as in Fig. 1)

	Nucleus (N)	Lateral chain (R)	Name	Abbreviation
1	A	a	Cholest-5-en-3β-ol	Cholesterol
2	A	b	(24*R*)-Methylcholest-5-en-3β-ol	Campesterol
3	A	c	(24*R*)-Ethylcholest-5-en-3β-ol	Sitosterol
4	A	d	(24*S*)-Methylcholest-5,22-dien-3β-ol	Brassicasterol
5	A	e	(24*S*)-Ethylcholest-5,22-dien-3β-ol	Stigmasterol
6	B	a	Cholest-5-en-3β-ol-7-one	7-Ketocholesterol
7	B	b	(24*R*)-Methylcholest-5-en-3β-ol-7-one	7-Ketocampesterol
8	B	c	(24*R*)-Ethylcholest-5-en-3β-ol-7-one	7-Ketositosterol
9	B	d	(24*S*)-Methylcholest-5,22-dien-3β-ol-7-one	7-Ketobrassicasterol
10	B	e	(24*S*)-Ethylcholest-5,22-dien-3β-ol-7-one	7-Ketostigmasterol
11	C	a	Cholest-5-ene-3β,7α-diol	7α-Hydroxycholesterol
12	C	b	(24*R*)-Methylcholest-5-ene-3β,7α-diol	7α-Hydroxycampesterol
13	C	c	(24*R*)-Ethylcholest-5-ene-3β,7α-diol	7α-Hydroxysitosterol
14	C	d	(24*S*)-Methylcholest-5,22-diene-3β,7α-diol	7α-Hydroxybrassicasterol
15	C	e	(24*S*)-Ethylcholest-5,22-diene-3β,7α-diol	7α-Hydroxystigmasterol
16	D	a	Cholest-5-ene-3β,7β-diol	7β-Hydroxycholesterol
17	D	b	(24*R*)-Methylcholest-5-ene-3β,7β-diol	7β-Hydroxycampesterol
18	D	c	(24*R*)-Ethylcholest-5-ene-3β,7β-diol	7β-Hydroxysitosterol
19	D	d	(24*S*)-Methylcholest-5,22-diene-3β,7β-diol	7β-Hydroxybrassicasterol
20	D	e	(24*S*)-Ethylcholest-5,22-diene-3β,7β-diol	7β-Hydroxystigmasterol
21	E	a	5α,6α-Epoxycholestan-3β-ol	α-Epoxycholesterol
22	E	b	(24*R*)-5α,6α-Epoxy-24-methylcholestan-3β-ol	α-Epoxycampesterol
23	E	c	(24*R*)-5α,6α-Epoxy-24-ethylcholestan-3β-ol	α-Epoxysitosterol
24	E	d	(24*S*)-5α,6α-Epoxy-24-methylcholest-22-en-3β-ol	α-Epoxybrassicasterol
25	E	e	(24*S*)-5α,6α-Epoxy-24-ethylcholest-22-en-3β-ol	α-Epoxystigmasterol
26	F	a	5β,6β-Epoxycholestan-3β-ol	β-Epoxycholesterol
27	F	b	(24*R*)-5β,6β-Epoxy-24-methylcholestan-3β-ol	β-Epoxycampesterol
28	F	c	(24*R*)-5β,6β-Epoxy-24-ethylcholestan-3β-ol	β-Epoxysitosterol
29	F	d	(24*S*)-5β,6β-Epoxy-24-methylcholest-22-en-3β-ol	β-Epoxybrassicasterol
30	F	e	(24*S*)-5β,6β-Epoxy-24-ethylcholest-22-en-3β-ol	β-Epoxystigmasterol
31	G	a	Cholestane-3β,5α,6β-triol	Cholestanetriol
32	G	b	(24*R*)-Methylcholestane-3β,5α,6β-triol	Campestanetriol
33	G	c	(24*R*)-Ethylcholestane-3β,5α,6β-triol	Sitostanetriol
34	G	d	(24*S*)-Methylcholest-22-ene-3β,5α,6β-triol	Brassicastentriol
35	G	e	(24*S*)-Ethylcholest-22-ene-3β,5α,6β-triol	Stigmastentriol
36	H	a	Cholest-5-ene-3β,4β-diol	4β-Hydroxycholesterol
37	H	b	(24*R*)-Methylcholest-5-ene-3β,4β-diol	4β-Hydroxycampesterol
38	H	c	(24*R*)-Ethylcholest-5-ene-3β,4β-diol	4β-Hydroxysitosterol
39	H	d	(24*S*)-Methylcholest-5,22-diene-3β,4β-diol	4β-Hydroxybrassicasterol
40	H	e	(24*S*)-Ethylcholest-5,22-diene-3β,4β-diol	4β-Hydroxystigmasterol
41	I	a	Cholest-4-ene-3β,6β-diol	6β-Hydroxycholesterol
42	I	b	(24*R*)-Methylcholest-4-ene-3β,6β-diol	6β-Hydroxycampesterol
43	I	c	(24*R*)-Ethylcholest-4-ene-3β,6β-diol	6β-Hydroxysitosterol
44	I	d	(24*S*)-Methylcholest-4,22-diene-3β,6β-diol	6β-Hydroxybrassicasterol
45	I	e	(24*S*)-Ethylcholest-4,22-diene-3β,6β-diol	6β-Hydroxystigmasterol
46	J	a	Cholestan-3β-ol-6-one	6-Ketocholestanol
47	J	b	(24*R*)-Methylcholestan-3β-ol-6-one	6-Ketocampestanol
48	J	c	(24*R*)-Ethylcholestan-3β-ol-6-one	6-Ketositostanol
49	A	a1	Cholest-5-ene-3β,24-diol	24-Hydroxycholesterol
50	A	a2	Cholest-5-ene-3β,25-diol	25-Hydroxycholesterol
51	A	a3	Cholest-5-ene-3β,27-diol	27-Hydroxycholesterol
52	K	b	(24*R*)-Methylcholestane-3β,6β-diol	6β-Hydroxycampestanol
53	K	c	(24*R*)-Ethylcholestane-3β,6β-diol	6β-Hydroxysitostanol

terol were prepared in a previous experiment [18]. The 6β-hydroxycampestanol and 6-β-hydroxysitostanol were recently isolated from oil samples [17]. Some other syntheses were effected and are described later in the text. Pyridine was from Sigma. It was dehydrated and maintained on 4 Å molecular sieves. Butylhydroxytoluene (BHT) and *tert*-butyl methyl ether (TBME) were obtained from Merck (Darmstadt, Germany). The other solvents were from SDS (Peypin, France) and were distilled before use. Bistrimethylsilyltrifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) was from Pierce (Rockford, IL, USA).

2.2. Preparation of isolated pure phytosterols

The blend of phytosterols contained 51.3% sitosterol, 28.5% campesterol, 10% stigmasterol, 6.5% brassicasterol and some minor sterols. It was used to prepare purified sitosterol, campesterol and brassicasterol, by semi-preparative RP-HPLC, using slight modifications of the method of Holen [29] with methanol as solvent and refractometric detection. RP-HPLC was effected using a Waters Spherisorb S5 ODS2 250 mm × 10 mm column placed in a HPLC system including a Thermoquest P1000XR pump, a Thermoquest SCM 1000 vacuum membrane degasser, an Igloo-CIL column conditioner, an Interchim Modulocart precolumn and a Shimadzu RID-10A refractometer. The column was operated at 30 °C. The methanol flow was set at 3 ml/min. Three main peaks were observed. The first peak (V_R = 55.8 ml) contained essentially brassicasterol. The second (V_R = 63.6 ml) contained stigmasterol, campesterol and a little part of sitosterol. The third (V_R = 70.8 ml) was pure sitosterol. The Peaks 1 and 3 were individually collected. The second peak was collected in three parts containing, respectively, stigmasterol + campesterol, campesterol alone, and campesterol with a small amount of sitosterol. Each time, 3.4 mg of phytosterol blend were injected in 200 μl ethyl acetate. This separation was effected 51 times and the corresponding fractions gathered. The fractions containing stigmasterol + campesterol and campesterol + sitosterol were run again in order to increase the recovering of campesterol. The chemical structures of these compounds were checked by GLC–MS as described later. At last, we obtained 79 mg of sitosterol (purity 98.6%), 12.3 mg of campesterol (purity 98.6%) and 12.7 mg of brassicasterol (purity 88%).

2.3. Preparation of oxyphytosterols and oxycholesterols

All the prepared campesterol and brassicasterol were used for the preparation of oxysterols. About 20 mg each of sitosterol, stigmasterol and cholesterol were also used for the preparation. The sterols were diluted in acetone in glass tubes, and the solvent was evaporated under nitrogen in order to obtain a thin layer of sterol. The tubes were then placed in an oven and heated at 135 °C, with air circulation, during 24 h. After cooling, the compounds were dissolved in hexane–TBME (90:10) and purified by solid-phase extraction on silica cartridges (LC-Si, 3 ml, 500 mg, Supelco, L'Isle d'Abeau Chesnes, France) using successively 35 ml of hexane–TBME (90:10) and 15 ml of hexane–TBME (80:20). As recommended by Lai et al. [25], a vacuum manifold (Supelco) was used to ensure a regular solvent flow rate of 0.6 ml/min through the cartridge. Theses fractions essentially contained non-oxidized sterols. The sterol oxides were then obtained using 10 ml of acetone. The non-oxidized sterols fractions were submitted again to heating in the same conditions, in order to increase the amounts of recovered sterol oxides. After gathering of the equivalent oxysterol fractions, they were purified by thin-layer chromatography (TLC) on 20 cm × 20 cm silica 60 plates (Merck). The migration was effected two times using hexane–TBME–ethyl acetate (33:33:33). Compounds with R_f between 0.16 and 0.70 were scraped and extracted using acetone. This allowed to keep only 7-hydroxy, 7-keto, 5,6-epoxy, 4β-hydroxy, 4-ene-6-hydroxy, and 6-keto derivatives, as described later. Using this method, the yield of oxidation comprised between 5 and 10%. However, this method did not allow to prepare the 5α,6β-dihydroxyderivatives ("triols"), which are also very important compounds. It is the reason why these compounds were synthesised and added separately to each series of sterol oxides. They were synthesised as described by Li et al. [30], with the exception of brassicastentriol. For this compound, the two epoxy-brassicasterols were first synthesised according to Nourooz-Zadeh and Appelqvist [13], and the brassicastentriol was then obtained using the method described by Dzeletovic et al. [31] for cholestanetriol.

2.4. GLC and GLC–MS of sterol oxides

Before analysis, the samples were transformed in TMSE derivatives as follows: after evaporation of the solvent under nitrogen, the samples were redissolved in 200 μl freshly dehydrated pyridine and 200 μl of BSTFA containing 1% of TMCS was added. The TMSE derivatives were obtained by heating 30 min at 55 °C. The reagents were then evaporated under nitrogen and the residue dissolved in hexane for gas chromatographic analysis.

The analyses were effected using a 5890 Series II Hewlett-Packard gas chromatograph (Palo Alto, CA, USA), equipped with a needle-falling injector (temperature 290 °C) and a flame ionisation detector operated at 300 °C. Two columns were used: a 0.25 μm film thickness, 30 m × 0.25 mm i.d. DB5-MS fused silica capillary column (J&W Scientific, Folsom, CA, USA) and a 0.25 μm film thickness, 30 m × 0.25 mm i.d. DB1-MS fused silica capillary column (J&W Scientific). Helium was the carrier gas. After 1 min at 50 °C, the oven temperature was raised

from 50 to 275 °C at 20 °C/min, then at 1 °C/min until 290 °C. The completion of the analyses was effected at 290 °C. The chromatographic data processing was effected using the Diamir software (JMBS Developments, Fontaine, France).

Gas–liquid chromatography coupled to electronic impact mass spectrometry was effected using a 6890 Hewlett-Packard gas chromatograph coupled to a HP 5973 mass spectrometer (Agilent Technologies, Palo Alto, Ca). The injection was made in splitless mode. The same columns operated in identical temperature conditions as for GLC were used. The transfer line was maintained at 300 °C. The mass spectrometer was operated in the electronic impact mode, with an ionisation energy of 70 eV. Using this method, the detection limit was comprised between 0.4 and 0.9 ng (checked using isolated oxyphytosterols synthesised for a preceding study [18]).

2.5. Analysis of oxyphytosterols in a spread enriched in phytosterol esters

A spread enriched in phytosterol esters was purchased at a local store. It contained 8% of phytosterols (sitosterol 46.9%, campesterol 25.4%, stigmasterol 18% and brassicasterol 3.5%). About 700 mg of spread were used for lipid extraction by a method derived from this of Folch et al. [32]: the extraction solvent was dichloromethane–methanol (2:1), with 0.05% of BHT. Five percent of the lipids were used to quantify the phytosterols, after hot saponification, formation of TMSE and GLC as already described. The main part of the lipids were saponified during 16 h, at room temperature, under argon, in the dark, with 1 M methanolic potassium hydroxide. Unsaponifiable compounds were extracted with dichloromethane. After evaporation of the solvent under nitrogen, they were dissolved in 500 μl of hexane–TBME (90:10) and purified on a silica cartridge (LC-Si, 3 ml, 500 mg; Supelco) using the method already described for the purification of oxyphytosterol series. A known amount of 5α-cholestane was added as internal standard. The compounds were then transformed in TMSE derivatives and analysed by GLC and GLC–MS as already described.

The checking of the eventual formation of artifacts during the analysis was effected as follows: as cholesterol is very low in the analysed spread (near to the limit of detection), the adding of purified cholesterol in an amount equivalent to that of sitosterol was effected to the food sample. The purification of cholesterol was made using two successive silica cartridges and the solvents already described. An amount of 6.53 mg of purified cholesterol were added to a sample of spread containing the same amount of sitosterol. The analysis was effected as previously, in order to check if artifact oxycholesterols could be observed using mass spectrometry, at the end of the analysis.

3. Results and discussion

3.1. Preparation of individual phytosterols

Our first objective was to obtain individual phytosterols with the best possible purity, in order to prepare unambiguous phytosterol oxides. This was reached for campesterol and sitosterol (98.6% purity each). However, brassicasterol was only 88% pure. It was previously observed that the avenasterols and other minor sterols had retention time close to that of brassicasterol using RP-HPLC [33]. Considering the difficulty for the preparation of important amount of brassicasterol, which is only a minor sterol, the purity obtained for brassicasterol was considered as sufficient.

3.2. Preparation of oxyphytosterols

The better way to prepare single oxyphytosterols is the synthesis, for example using the methods described by Li et al. [30]. However, considering the great number of compounds to prepare, we decided to use a method adapted from Osada et al. [34] allowing the formation of several oxysterols in one step. Using this method, some unknown compounds were also formed. It was the reason why we used a purification step by TLC, which allowed to exclude the main part of these unknown compounds. This step also removed the residual non-oxidized phytosterols as well as the side chain oxidized derivatives. We obtained five samples containing, respectively, the oxides of cholesterol, campesterol, sitosterol, stigmasterol and brassicasterol. The sterol oxides obtained from cholesterol and sitosterol are represented in Fig. 2. The other fractions were similar, with intermediate retention times.

3.3. Identification of oxysterols

3.3.1. Identification of oxycholesterols

The identification of the common oxycholesterols was easy, due to the availability of standards, and to numerous data concerning the mass spectra of their TMSE derivatives [23,35,36]. Thus, the structure of 7α-hydroxycholesterol, 7β-hydroxycholesterol, β-epoxycholesterol, α-epoxycholesterol, cholestanetriol and 7-ketocholesterol (as TMSE derivatives) were confirmed. The presence of some other less usual oxycholesterols was also proved using the comparison of their retention times and mass spectra with those of commercial standards; their mass spectra were also equivalent to the published data: the MS data of 6-ketocholestanol, cholest-4-ene-3β,6β-diol and of 4β-hydroxycholesterol were close to those already published [36,37].

3.3.2. Identification of common oxyphytosterols

These identifications of oxycholesterols were useful for the identifications of analogous derivatives of phytosterols.

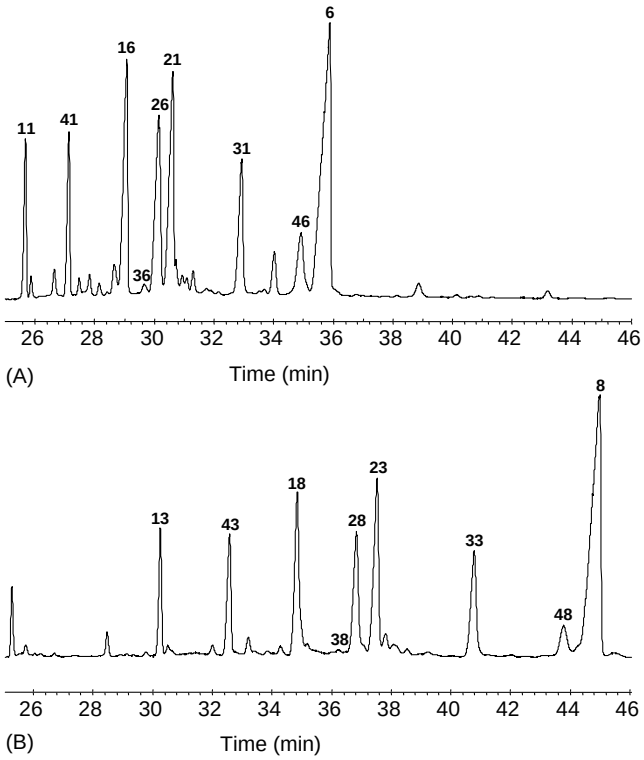

Fig. 2. Gas chromatograms of sterol oxides TMSE derivatives on a column DB5 (chromatographic conditions described in Section 2). The cholesterol oxides are represented at the upper place (A) and the sitosterol oxides at the lower place (B). The numbers represent the compounds described in Table 1.

As seen in Fig. 2, the GLC separation of oxysitosterols is similar to what was observed for oxycholesterols, but with increased retention times. The same was observed for all series of oxyphytosterols. Moreover, in mass spectrometry, the ions comprising the lateral chain presented a mass increase of 14 U for campesterol-, 28 U for sitosterol-, 12 U for brassicasterol- and 26 U for stigmasterol-derivatives, as expected. At last, the mass spectra of the main oxyphytosterols, as TMSE derivatives, were already published. The TMSE spectra of the main oxysitosterols and oxycampesterols are well known, essentially due to the studies of Aringer and Nordström [36], Aringer and Eneroth [38], Dutta and Appelqvist [15] and Dutta [39]. Our laboratory presented also the mass spectra of some synthesised oxysitosterols and oxycampesterols [18]. The oxystigmasterols were studied only recently [16,39–41]. Concerning the brassicasterol oxides, there was an unique study presenting the mass spectra of the 7α- and 7β-hydroxyderivatives [41]. However, Dutta reviewed recently the analysis of the phytosterol oxides and published the mass spectra of the TMSE derivatives of 24 oxyphytosterols, including 7-ketobrassicasterol, α-epoxybrassicasterol, β-epoxybrassicasterol and brassicastentriol, which were not yet known [39]. Our results were in good agreement with the data presented in these different studies, with some slight differences due to various mass spectrometry conditions or to background noise of the columns. All these data permitted an easy identification of all these compounds.

3.3.3. *Tentative identification of other oxyphytosterols*

As observed in Fig. 2, our method of preparation also allowed the formation from cholesterol of noticeable amounts of cholest-4-ene-3β,6β-diol and 6-ketocholestanol, and of a little amount of 4β-hydroxycholesterol. In the four phytosterol series, we observed analogous compounds, presenting ions increased, respectively, from 14 U for campesterol-, 28 U for sitosterol-, 12 U for brassicasterol- and 26 U-for stigmasterol-derivatives. These compounds may be the corresponding analogous oxides for the phytosterols. For example, Fig. 3 presents the comparison between the TMSE derivatives of cholest-4-ene-3β,6β-diol and the compound supposed to be the (24*R*)-ethylcholest-4-en-3β,6β-diol. As expected, the sitosterol derivative spectrum presented 28 U higher ions than this of 6β-hydroxycholesterol. The molecular ion at 574 corresponded to 546. The ions at 559, 545, 484, 469, 431, 417, 394, 379, were analogous, respectively to 531 (*M*–CH_3), 517, 456 (*M*–hydroxytrimethylsilyl fragment (TMSOH)), 441 (*M*–TMSOH–CH_3), 403, 389, 366 (*M*–2TMSOH), 351 (*M*–2TMSOH–CH_3). The ion at 403 was considered by Brooks et al. as characteristic of the 4-ene-6β-hydroxy structure [42], and it was important to note that the analogous 431 ion was present in sitosterol derivative. Some other ions were identical (143, 194 and 283) and represented fragments without the lateral chain. The respective abundances of all these ions were very close from one compound to the other. All these data confirmed that there was a great probability that this compound was (24*R*)-ethylcholest-4-ene-3β,6β-diol.

This kind of comparison was effected for all the 4-ene-6β-hydroxysterols, the 6-ketostanols and the 4β-hydroxysterols (Table 2). All the 4-ene-6β-hydroxysterols had the same kind of fragmentation. The analogous ions issued from each phytosterol ions presented also similar abundances, with some very small differences for the two oxyphytosterols issued from sterols with a second ethylenic bond in positions 22–23 (higher abundances for molecular ions (*M* − 29) and (*M* − 90) ions . . .). Concerning the 6-ketosterols, only three major ions (*M*; *M* − 15; *M* − 29) were observed for 6-ketocholesterol, and hypothetical 6-ketocampesterol and 6-ketositosterol. The other ions including the lateral chain were very low (*M* − 71; *M*–TMSOH; *M*–TMSOH–CH_3; *M* − 161; *M* − 149), as well as the other characteristic ions (305; 211; 159; 107). Two peaks corresponding to the analogous compounds for brassicasterol and stigmasterol were also observed at the respective expected retention times. However, their mass spectrometric fragmentations were more complex, and these compounds were not included in this study. On the contrary, all the phytosterols presented a derivative with the characteristics (retention time, fragmentation) similar to those of 4β-hydroxycholesterol. Two series of TMSOH loss were easily observed among the MS fragments [(*M* − 90); (*M* −

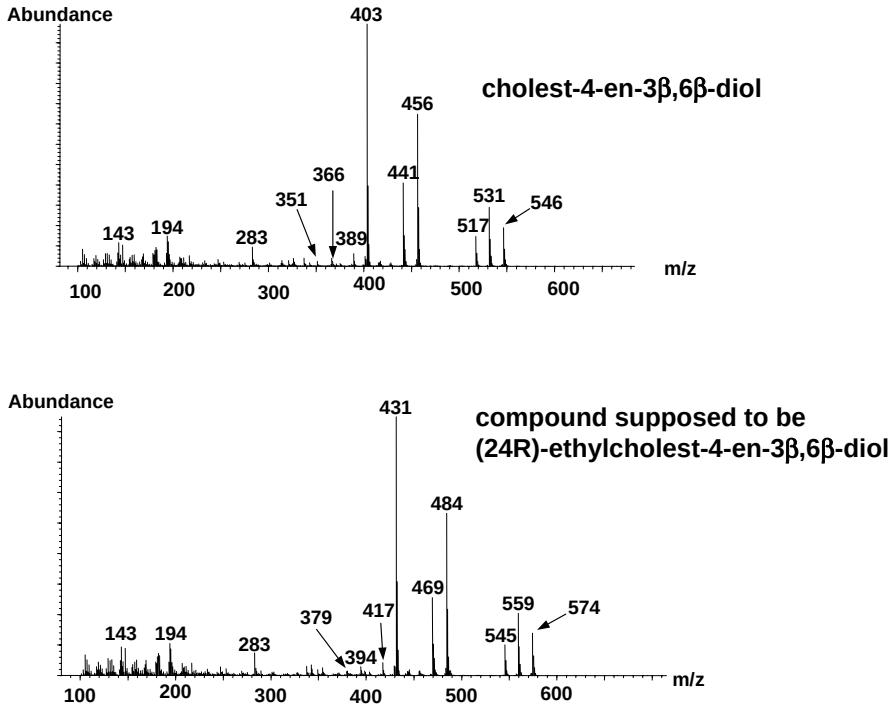

Fig. 3. Electron impact mass spectra of cholest-4-ene-3β,6β-diol (Compound 41) and of the compound supposed to be (24*R*)-ethylcholest-4-ene-3β,6β-diol (Compound 43).

180); ($M - 15$); ($M - 90 - 15$); ($M - 180 - 15$)]. The presence of an ethylenic bond in Δ5 was also evidenced by the fragments 129; ($M - 129$); ($M - 129 - 90$) [35]. As observed by Brooks et al. the fragments 129 and ($M - 129$) presented an equivalent intensity [42]. The unique MS fragmentation difference between these compounds was that the higher ion was ($M - 180$) for the derivatives of cholesterol, campesterol and sitosterol and was ($M - 90$) for the derivatives of brassicasterol and stigmasterol: the presence of an additional ethylenic bond at Δ22 modified probably the fragmentation.

However, the structure of these oxyphytosterols are yet only hypothetical. They were included in this study for informative purpose. But, it is now necessary to separately synthesise these compounds and to check their structure, for example using NMR.

3.4. Gas chromatographic separations of oxycholesterols and oxyphytosterols

Some common oxidised derivatives of cholesterol (24-hydroxycholesterol, 25-hydroxycholesterol, 27-hydroxycholesterol) were added to the study for informative purpose. We also added the 6β-hydroxycampestanol and 6β-hydroxysitostanol, which were recently identified in refined oils [17]. All the compounds indicated in Table 1 were submitted to GLC and GLC–MS on two columns (DB1 and DB5) in the conditions described in Section 2. Their retention times relative to 5α-cholestane were reported, respectively, in Table 3 for the DB-1 column and Table 4 for the DB-5 column.

On these tables, it can be seen that some compounds have equivalent or very close retention times and that some chromatographic separations are not possible using these conditions. However, in several cases, slight modifications of the temperature program allowed better separations of compounds with close retention times. These improvements were not included in the text, because when some separations were better, some other compounds were now gathered in the same peak. We think that the data presented here could be useful to readers as a first approach. They have then to search the best chromatographic conditions to separate their own compounds. It is also possible to use the two columns, DB5 and DB1. Some compounds which are not separated on the first column present often a single peak on the second column. However, we have to keep in mind that the retention data presented here are only for information and that the separations could be different depending of the columns. Moreover, the same column with an identical temperature program could present slight different separations some months later!

Very recently, two studies concerning the identification of oxyphytosterols formed from stigmasterol [20], campesterol and sitosterol [21] were published. These studies essentially concerned the side-chain oxidized compounds, but some other new compounds were also detected as the 4-en-6-ol-3-one derivatives. We wonder why these compounds were not detected in the present study. The probable

Table 2
Mass spectra (*m/z*) of some uncommon oxycholesterols and their possible analogous oxyphytosterols (as TMSE derivatives)[a]

Structure	M^+	Major significant ions									
Cholest-4-ene-3β,6β-diol	546 (16)	531 (24)	517 (12)	456 (63)	441 (34)	403 (100)	389 (5)	366 (3)	351 (2)	283 (8)	194 (12)	143 (10)
(24*R*)-Methylcholest-4-ene-3β,6β-diol[b]	560 (17)	545 (25)	531 (12)	470 (63)	455 (32)	417 (100)	403 (5)	380 (3)	365 (1)	283 (9)	194 (14)	143 (11)
(24*R*)-Ethylcholest-4-ene-3β,6β-diol[b]	574 (16)	559 (24)	545 (11)	484 (61)	469 (30)	431 (100)	417 (5)	394 (3)	379 (1)	283 (9)	194 (14)	143 (12)
(24*S*)-Methylcholest-4,22-diene-3β,6β-diol[b]	558 (21)	543 (26)	529 (15)	468 (65)	453 (32)	415 (100)	401 (3)	378 (3)	363 (2)	283 (11)	194 (13)	143 (14)
(24*S*)-Ethylcholest-4,22-diene-3β,6β-diol[b]	572 (22)	557 (27)	543 (14)	482 (68)	467 (30)	429 (100)	415 (3)	392 (2)	377 (1)	283 (10)	194 (10)	143 (11)
6-Ketocholestanol	474 (18)	459 (58)	445 (100)	403 (3)	384 (3)	369 (2)	345 (2)	325 (2)	305 (3)	211 (4)	159 (8)	107 (6)
6-Ketocampestanol[b]	488 (18)	473 (57)	459 (100)	417 (1)	398 (4)	383 (2)	359 (2)	339 (2)	305 (3)	211 (4)	159 (8)	107 (6)
6-Ketositostanol[b]	502 (19)	487 (58)	473 (100)	431 (2)	412 (3)	397 (2)	373 (2)	353 (1)	305 (3)	211 (4)	159 (7)	107 (5)
4-β-Hydroxycholesterol	546 (26)	531 (15)	456 (77)	441 (25)	430 (10)	417 (38)	366 (100)	351 (8)	327 (39)	253 (18)	147 (57)	129 (37)
4-β-Hydroxycampesterol[b]	560 (29)	545 (13)	470 (85)	455 (26)	444 (11)	431 (45)	380 (100)	365 (7)	341 (41)	253 (17)	147 (52)	129 (35)
4-β-Hydroxysitosterol[b]	574 (23)	559 (15)	484 (79)	469 (25)	458 (11)	445 (41)	394 (100)	379 (9)	355 (31)	253 (20)	147 (48)	129 (32)
4-β-Hydroxybrassicasterol[b]	558 (25)	543 (12)	468 (100)	453 (18)	442 (11)	429 (44)	378 (73)	363 (3)	339 (19)	253 (47)	147 (79)	129 (48)
4-β-Hydroxystigmasterol[b]	572 (34)	557 (13)	482 (100)	467 (20)	456 (10)	443 (44)	392 (78)	377 (2)	353 (13)	253 (44)	147 (57)	129 (40)

[a] Relative abundances are reported into brackets.
[b] Hypothesis.

reason is that we purified our blends by TLC, as described before. The TLC data published by Johnsson et al. [20,21] led us to think that the side-chain oxidized compounds were removed during this step. So, the present study and those of Dutta's team are very complementary and will help to the identification of oxyphytosterols.

3.5. Identification of oxyphytosterols in a commercial spread enriched in phytosterols

All these data were then used for a tentative identification of the oxyphytosterols present in a commercial spread enriched in phytosterol esters. It can be seen in Figs. 4 and 5 that many compounds described in Table 1 can be detected in the food sample, even if some peaks are not yet identified. The main compounds were issued from sitosterol, as expected, but analogous compounds coming from campesterol and stigmasterol were also identified. Some compounds derived from brassicasterol were also present. The 7-keto, 7α-hydroxy, 7β-hydroxy, β-epoxy, α-epoxy, 5α,6β-dihydroxy ("triols") and 6β-hydroxy-4-ene derivatives of sitosterol, campesterol and stigmasterol were all detected, as well as 6-ketositostanol and 6-ketocampestanol. Among the common oxidized derivatives of brassicasterol, only the 7-ketobrassicasterol was identified with certitude. In a recent study concerning oxyphytosterols in refined rapeseed oils [17], the 7-ketobrassicasterol was observed with a rather high level. In the same study, a compound tentatively identified as 6β-hydroxybrassicastanol was present, as well as the analogues 6β-hydroxycampestanol and 6β-hydroxysitostanol. These three compounds were also identified in the present study, as major compounds (a, 52, 53). The analogue compound issued from stigmasterol was then searched in the spread. A compound presenting the required fragmentation for 6β-hydroxystigmastanol was detected on the two columns (e). Compared to 6β-hydroxybrassicastanol, the fragmentation of this compound comprised some identical ions (255, 345, 372, 373) for fragments without the lateral chain and some 14 U higher ions (574, 559, 531, 484, 441) for fragments including the lateral chain. The spread also contained two compounds (c and g) which were tentatively identified as 4-campesten-6β-ol-3-one and 4-sitosten-6β-ol-3-one, using the data recently published by Johnsson and Dutta [21]. A very similar compound, presenting 2 u lower ions compared to 4-sitosten-6β-ol-3-one (498, 483, 442) was also detected (d). It could be 4-stigmasten-6β-ol-3-one. Two other peaks (b and f) presented similar ions than 6β-hydroxycampestanol and 6β-hydroxysitostanol, respectively, with a slight higher rate for the ion (*M*–TMSOH); they could be geometrical isomers of these compounds [36]. At last, two compounds presenting only 131 as unique principal ion were detected (h and i). This kind of fragmentation is considered as characteristic of 25-hydroxycholesterol [35] and was recently also found for 25-hydroxycampesterol, 25-hydroxysitosterol [21] and 25-hydroxystigmasterol [20]. However, in our anal-

Table 3
Retention times of the studied compounds (as TMSE derivatives), relative to the 5α-cholestane, on a DB1 column

	Number in Table 1	Cholesterol derivatives	Campesterol derivatives	Sitosterol derivatives	Brassicasterol derivatives	Stigmasterol derivatives
7α-Hydroxycholesterol	11	1.26				
Cholesterol	1	1.28				
7α-Hydroxybrassicasterol	14				1.29	
6β-Hydroxycholesterol	41	1.34				
Brassicasterol	4				1.34	
6β-Hydroxybrassicasterol	44				1.38	
7α-Hydroxycampesterol	12		1.38			
7α-Hydroxystigmasterol	15					1.41
Campesterol	2		1.42			
7β-Hydroxycholesterol	16	1.43				
β-Epoxycholesterol	26	1.44				
7β-Hydroxybrassicasterol	19				1.44	
Stigmasterol	5					1.46
α-Epoxycholesterol	21	1.47				
β-Epoxybrassicasterol	29				1.48	
6β-Hydroxycampesterol	42		1.48			
4β-Hydroxycholesterol	36	1.49				
7α-Hydroxysitosterol	13			1.49		
α-Epoxybrassicasterol	24				1.50	
4β-Hydroxybrassicasterol	39				1.52	
6β-Hydroxystigmasterol	45					1.53
7β-Hydroxycampesterol	17		1.56			
Sitosterol	3			1.56		
Cholestanetriol	31	1.58				
7β-Hydroxystigmasterol	20					1.59
β-Epoxycampesterol	27		1.60			
6β-Hydroxysitosterol	43			1.62		
α-Epoxycampesterol	22		1.63			
6β-Hydroxycampestanol	52		1.65			
6-Ketocholestanol	46	1.66				
4β-Hydroxycampesterol	37		1.66			
β-Epoxystigmasterol	30					1.66
24-Hydroxycholesterol	49	1.67				
α-Epoxystigmasterol	25					1.68
7-Ketocholesterol	6	1.69				
25-Hydroxycholesterol	50	1.70				
Brassicastentriol	34				1.71	
4β-Hydroxystigmasterol	40					1.71
7β-Hydroxysitosterol	18			1.71		
7-Ketobrassicasterol	9				1.75	
β-Epoxysitosterol	28			1.76		
6β-Hydroxysitostanol	53			1.77		
α-Epoxysitosterol	23			1.79		
Campestanetriol	32		1.81			
27-Hydroxycholesterol	51	1.82				
4β-Hydroxysitosterol	38			1.82		
6-Ketocampestanol	47		1.86			
Stigmastentriol	35					1.87
7-Ketocampesterol	7		1.90			
7-Ketostigmasterol	5					1.98
Sitostanetriol	33			2.00		
6-Ketositostanol	48			2.08		
7-Ketositosterol	8			2.12		

Table 4
Retention times of the studied compounds (as TMSE derivatives), relative to the 5α-cholestane, on a DB5 column

	Number in Table 1	Cholesterol derivatives	Campesterol derivatives	Sitosterol derivatives	Brassicasterol derivatives	Stigmasterol derivatives
7α-Hydroxycholesterol	11	1.20				
7α-Hydroxybrassicasterol	14				1.24	
Cholesterol	1	1.26				
6β-Hydroxycholesterol	41	1.28				
Brassicasterol	4				1.32	
7α-Hydroxycampesterol	12		1.32			
6β-Hydroxybrassicasterol	44				1.33	
7α-Hydroxystigmasterol	15					1.36
7β-Hydroxycholesterol	16	1.37				
4β-Hydroxycholesterol	36	1.39				
Campesterol	2		1.40			
β-Epoxycholesterol	26	1.41				
7β-Hydroxybrassicasterol	19				1.41	
6β-Hydroxycampesterol	42		1.41			
7α-Hydroxysitosterol	13			1.42		
α-Epoxycholesterol	21	1.44				
4β-Hydroxybrassicasterol	39				1.45	
Stigmasterol	5					1.45
6β-Hydroxystigmasterol	45					1.46
β-Epoxybrassicasterol	29				1.47	
α-Epoxybrassicasterol	24				1.49	
7β-Hydroxycampesterol	17	1.51				
7β-Hydroxystigmasterol	20					1.54
Sitosterol	3			1.54		
Cholestanetriol	31	1.55				
4β-Hydroxycampesterol	37		1.56			
6β-Hydroxysitosterol	43			1.56		
6β-Hydroxycampestanol	42		1.57			
β-Epoxycampesterol	27		1.58			
24-Hydroxycholesterol	49	1.59				
Brassicastentriol	34				1.61	
α-Epoxycampesterol	22		1.61			
4β-Hydroxystigmasterol	40					1.62
6-Ketocholestanol	46	1.63				
25-Hydroxycholesterol	50	1.63				
7β-Hydroxysitosterol	18			1.63		
β-Epoxystigmasterol	30					1.64
7-Ketocholesterol	6	1.67				
α-Epoxystigmasterol	25					1.67
4β-Hydroxysitosterol	38			1.71		
β-Epoxysitosterol	28			1.72		
6β-Hydroxysitostanol	53			1.72		
Campestanetriol	32		1.74			
7-Ketobrassicasterol	9				1.75	
α-Epoxysitosterol	23			1.75		
27-Hydroxycholesterol	51	1.76				
Stigmastentriol	35					1.80
6-Ketocampestanol	47		1.86			
7-Ketocampesterol	7		1.90			
Sitostanetriol	33			1.91		
7-Ketostigmasterol	10					1.98
6-Ketositostanol	48			2.05		
7-Ketositosterol	8			2.08		

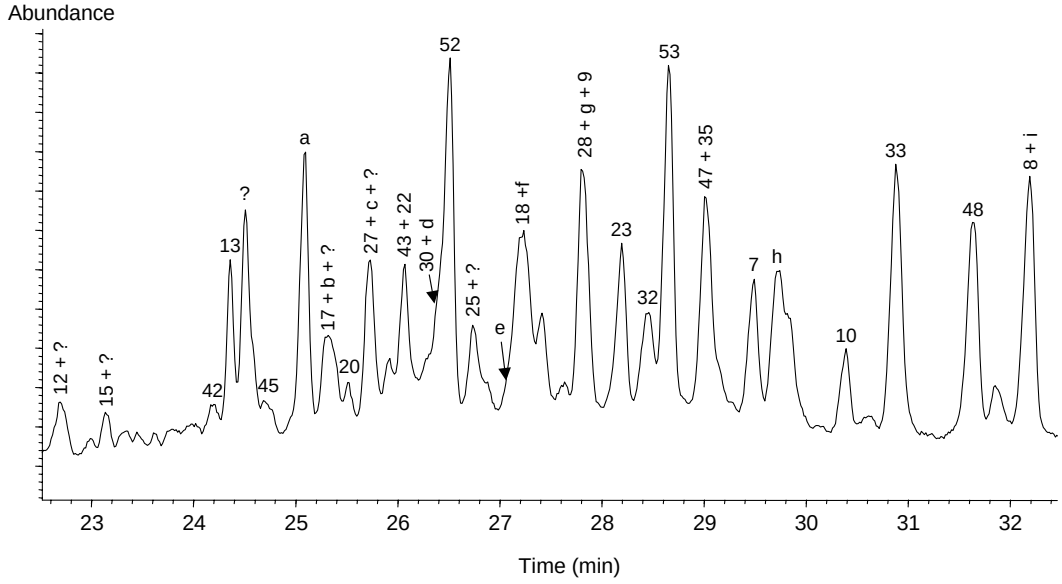

Fig. 4. Total ion current of GLC–MS of the TMSE derivatives on a DB 1 column of the oxyphytosterols of a commercial spread enriched in phytosterols (chromatographic conditions described in Section 2). The numbers represent the compounds described in Table 1. (a) Tentatively identified in a precedent study as 6β-hydroxybrassicastanol [17], (b) this compound may be another 6-hydroxycampestanol [36], (c) 4-campesten-6β-ol-3-one [21], (d) tentatively identified as 4-stigmasten-6β-ol-3-one (see text), (e) tentatively identified as 6β-hydroxystigmastanol (see text), (f) this compound may be another 6-hydroxysitostanol [36], (g) 4-sitosten-6β-ol-3-one [21], (h) and (i) these compounds could have an hydroxylation in position 25 (see text).

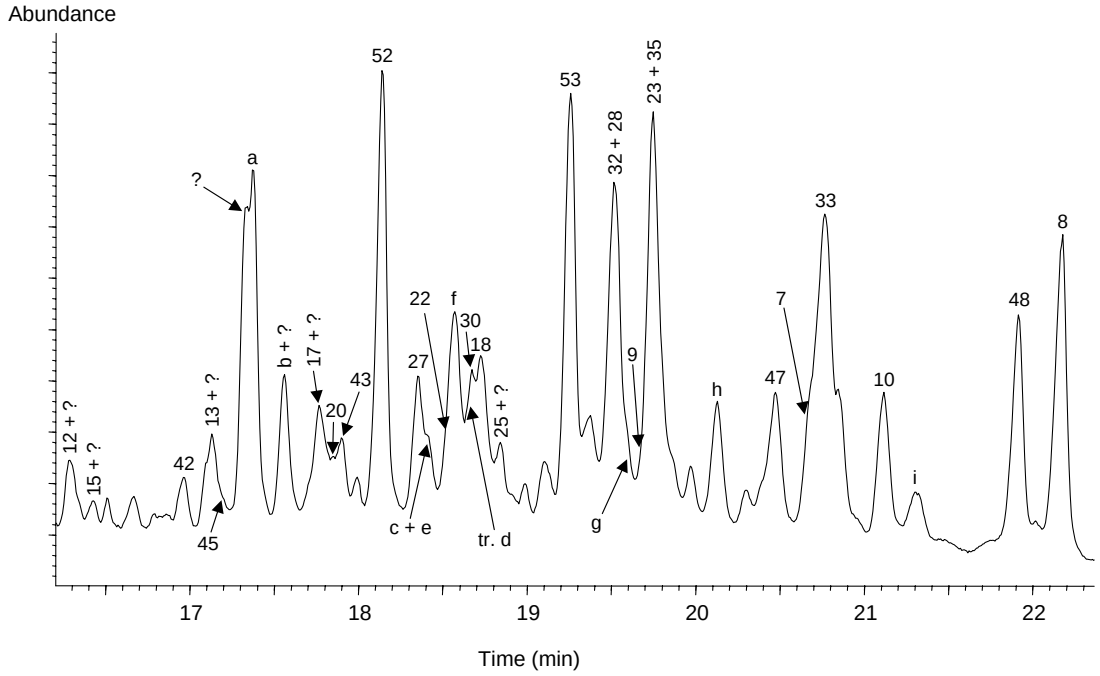

Fig. 5. Total ion current of GLC–MS of the TMSE derivatives on a DB 5 column of the oxyphytosterols of a commercial spread enriched in phytosterols (chromatographic conditions described in Section 2). The numbers and the notes are the same as in Fig. 4.

yses, the little characteristic ions were difficult to see and did not allow to identify these compounds with certitude.

The checking of the absence of formation of artifacts during analyses was effected by adding purified cholesterol to the samples. In these cases, oxycholesterols were not observed. This fact led us to think that the observed oxyphytosterols are not artifacts.

The precise individual quantification of all the oxyphytosterols detected in spread was not possible. It would have been necessary to prepare all the individual compounds in order to calculate the response coefficients and the calibration curves using selective ion monitoring mass spectrometry. This will be the aim of future studies. In these cases the use of 3β,22-dihydroxy-20-homo-5-pregnene, that we recently

synthesised as new internal standard [43], will probably be useful. Nevertheless, a rough estimate of the total oxyphytosterols was effected using mean response coefficients or those of analogue cholesterol oxides when known. With this calculation, the oxyphytosterols represented no more than 68 μg/g of spread. Only about 0.08% of phytosterols were oxidised. The spread had then been good processed. Moreover, a large part of the oxyphytosterols were probably already present in the oils used to prepare the spread: the 6β-hydroxy-derivatives of campestanol, sitostanol and brassicastanol, as well the 7-keto-brassicasterol were recently identified as the main oxyphytosterols of a refined rapeseed oil [17]. However, if the daily human ration is 20–25 g of spread, as recommended, 1.3–1.7 mg of oxyphytosterols are ingested. This amount is not so far of the quantities of oxycholesterols (3–4 mg per day) that were found in daily diets in Netherlands and New Zealand [44,45]. Furthermore, some oxyphytosterols were recently detected in human plasma [19]. This means that new studies on oxyphytosterols are necessary and we hope that the present work will bring some help in this way.

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