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Procedure for the determination of eight cholesterol oxides in poultry meat using on-column and solvent venting capillary gas chromatography.

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Abstract

A procedure for the determination of eight relevant cholesterol oxides in poultry meat has been developed. The method consists of the enrichment of cholesterol oxides by means of the combined use of solid-phase fractionation and thin-layer chromatography. Florisil and silica columns of 10 g permitted the handling of the total cholesterol oxides content included in the lipid bulk obtained after the Folch's extraction of 20 g of muscle meat. The determination of cholesterol oxides under their trimethylsilyl derivatives was performed by using capillary gas chromatography. The use of a fused-silica open tubular capillary column 30 m×0.25 mm I.D. coated with 5% phenylmethylsilicone and with a film width of 0.25 μm permitted the separation of all the species. Two modes of injection (on-column and solvent venting) were evaluated and compared for the analysis of cholesterol oxides. On-column capillary gas chromatography (cGC) gave better absolute areas relative standard deviation (R.S.D.) values: 3% to 6% vs. 5% to 7% for solvent venting cGC. Regression analysis for each cholesterol oxide was performed for the two modes of injection. The possibility of large volume injection (10 μl) by using the solvent venting mode was also evaluated in order to increase the sensitivity of the detection of cholesterol oxides. R.S.D. values for absolute areas ranging from 6% to 14% were obtained. The validation of the method was carried out within the range of 0.1–1 ppm. Absolute and relative recovery values ranging from 80% to 100% were obtained. Statistical analysis revealed that the method was reproducible. cGC–mass spectrometry was also used to confirm the peaks detected by cGC: the total ion chromatogram mode was used for the analysis of samples containing concentrations down to 0.1 ppm of cholesterol oxides. The analysis of fresh and cooked chicken meat revealed the presence of cholesterol oxides proceeding from the autoxidation of the cholesterol B-ring. Finally, saponification was found to be not as accurate as the described procedure for cholesterol oxides analysis.

Keywords: Food analysis; Injection methods; Cholesterol oxides

1. Introduction

Atherosclerosis is the thickening of the inner wall of the artery that results from an injury to the endothelial cells and subsequent accumulation of lipid-enriched macrophages (foam cells), adherence of platelets and proliferation of muscle cells. All these processes can lead to the occlusion of the artery and produce tissue death by ischemia. In 1984, the implication of oxidized LDL (low-density lipoprotein, major cholesterol carrying lipoprotein) in the acceleration of the atherogenic process was

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demonstrated [1]. It has been emphasized that the toxicity of oxidized lipoprotein can be attributed to their cholesterol oxides content [2]. The oxidation of cholesterol and lipids modifies the lipoprotein and causes its increased absorption by macrophages leading to an enhanced production of foam cells, one of the hallmarks of atherogenesis. Emmanuel et al. [3] showed a rapid transfer of dietary administrated oxysterols (30–90 ppm) from the plasma to the lipoprotein fraction of human subjects. Brooks et al. detected amounts of α-epoxycholesterol, 7-keto-cholesterol and 26-hydroxycholesterol in human plasma [4]. It has also been demonstrated that exogenous 25-hydroxycholesterol, 7α-hydroxy-cholesterol and 20α-hydroxycholesterol preferentially distributed into LDL and HDL (high-density lipoprotein) of human serum [5]. Cholesterol oxides can also produce vascular injury which triggers off the atherogenic process [6]. Cholestan-3β,5α,6β-triol and 25-hydroxycholesterol induced lesion development when respectively fed to rabbits and Carneau pigeons [7–9]. Enhanced in vitro platelet adhesion onto endothelial cells was also observed after incubation with 7-ketocholesterol, 25-hydroxycholesterol and cholestanetriol while pure cholesterol had no effect [10]. Besides their primary role in atherosclerosis, some oxysterols have been shown to be also implicated in carcinogenesis [11].

Products of cholesterol oxidation occur in the diet or can be produced in vivo in the human body [2,3]. The determination of cholesterol oxides in foods involves procedures which consist first of all in extracting the lipid bulk. The following steps involved different techniques like thin-layer chromatography (TLC), high-performance liquid chromatography and solid-phase extraction (SPE) in order to purify cholesterol oxides [12–19]. Recently, there is a growing application of SPE for the determination of oxysterols especially in dry-egg powder [20–23]. Detection and quantitation of cholesterol oxides was generally performed by capillary gas chromatography (cGC) and sometimes confirmed by cGC–mass spectrometry (MS) [14,20,24,25]. Cold saponification has also been used as an alternative to the hot alkali procedure for the detection of cholesterol oxides [14,24–26].

As meat is one of the major constituents of the human diet and a cholesterol-rich food, many researchers applied themselves to the development of analytical procedures which would render possible the quantitation of its oxysterol content [16,24,27–29]. Due to their very low concentrations in fresh meat, cholesterol oxides cannot be detected and quantified accurately. The determination of the cholesterol oxides content of fresh meat is of relevant importance since its knowledge would permit us to quantify accurately its increment when meat is submitted to oxidative conditions like cooking, storage and processing wherein cholesterol oxides levels superior to 1 ppm have been estimated [24,25,30,31].

Most of the procedures developed until so far are laborious, time consuming and seem to be unsuitable for a multi-sample analysis. Furthermore, some of them lack sensitivity and/or were designed for the detection of a limited number of oxysterols. The method presented in this paper, has been elaborated for the enrichment of eight relevant oxysterols [32] with a sensitivity within the 0.1–1 ppm range. Validation of the method has been performed within this range of concentrations. The entire procedure was repeated four times, absolute and relative recoveries were calculated, the total assay variation for each cholesterol oxide was evaluated and the reproducibility of the entire procedure was statistically tested. cGC was used for the detection of cholesterol oxides: their chromatographic separation has been optimalized and two methods of sample injection were compared (on-column and solvent venting [33]). The possibility of large volume injection (10 μl) in the solvent venting mode was also assessed. cGC analysis has been complemented by cGC–MS in the TIC (total ion chromatogram) mode. In order to test the sensitivity of the procedure, samples obtained from fresh and cooked chicken meat were analyzed for their cholesterol oxides content. Finally, cold saponification has also been evaluated and compared with the described procedure.

2. Experimental

2.1. Chemicals

Cholesterol, 19-hydroxycholesterol, 7β-hydroxy-cholesterol, α-epoxycholesterol, 20α-hydroxy-cholesterol, cholestanetriol, 7-ketocholesterol and 25-

hydroxycholesterol were purchased from Sigma (Madrid, Spain). β-epoxycholesterol and 7α-hydroxycholesterol were obtained from Research Plus (Bayonne, NJ, USA) and Sylon HTP from Supelco (Bellefonte, PA, USA). SPE columns (Florisil and silica Mega Bond Elut 10 g) were purchased from Varian Associates (CA, USA) and TLC plates (silica gel 60, 20×20 cm, 0.25 mm thickness) from Merck (Darmstadt, Germany). Rhodamin was obtained from Sigma. Chloroform, hexane, and diethyl ether for organic trace analysis were obtained from Merck and 2-propanol from Panreac (Barcelona, Spain). Chicken meat was purchased from a local store.

2.2. Sample pre-treatment

A 20 g weight of chicken muscle meat was subjected to total lipid extraction by the procedure of Folch et al. [34] using 200 ml of $CHCl_3$–MeOH (2:1, v/v). The mixtures were homogenized with a Ultra-Turrax T25 (IKA-Labortechnik, Staufen, Germany) at speed 7 for 45 s. The homogenates were filtered through Whatman No. 1 filter paper. The filters were washed with 50 ml of $CHCl_3$–MeOH (2:1, v/v). A 80 ml volume of distilled water containing 10% of NaCl was added to the filtrates which were left for 12 h and decanted. The lipid extract was evaporated to dryness by means of a Büchi rotavapor (Switzerland) and re-dissolved in 7% 2-propanol in hexane.

2.3. SPE

The lipid extract was first of all applied onto a silica column (10 g). The columns were conditioned with hexane. Neutral lipids were eluted with 25 ml of chloroform–methanol (9:1, v/v). The obtained fraction was evaporated to dryness, re-dissolved in 7% 2-propanol in hexane and applied to a Florisil column (10 g). The major part of triglycerides were eliminated by elution with 250 ml of hexane–diethyl ether (9:1, v/v) (fraction A). A 40 ml volume of diethyl ether–methanol (8:2, v/v) was necessary to elute the cholesterol oxides (fraction B).

2.4. TLC and derivatization

The fraction containing the oxysterols was evaporated to dryness and re-dissolved in 200 μl of 7% 2-propanol in hexane. A 20 μl volume of the extract and a solution containing 20 μg of each cholesterol oxide plus cholesterol were deposited on a silica TLC plate. The plate was developed with hexane–diethyl ether (50:50, v/v) and dried. Only the zone corresponding to the migration of the cholesterol and the oxysterols standards was sprayed with Rhodamin. The oxysterols area was scrapped and recovered by extracting the silica twice with 10 ml of $CHCl_3$–MeOH (9:1, v/v). The extract was evaporated until dryness. Cholesterol oxides trimethylsilyl (TMS) ether derivatives were obtained by reaction with 33% of hexamethyldisilazane (HMDS)+11% trimethylchlorosilane (TMCS) in pyridine (Sylon HTP, Supelco) for 1 h at 60°C. The silylating reagent was evaporated under nitrogen stream and derivatized cholesterol oxides were resuspended in chloroform.

2.5. cGC

Oxysterols were detected and quantified by using a Dani gas chromatograph HR 3800-PTV (Monza, MI, Italy) equipped with a flame ionization detection (FID) system. A fused-silica open tubular (FSOT) capillary column 30 m×0.25 mm I.D. coated with 5% phenylmethylsilicone and with a film width of 0.25 μm (J&W Scientific, Folsom, CA, USA) was used. Helium was the carrier gas and delivered to the column at a head pressure of 1.4 bar. The detector temperature was 340°C. Sample injection was carried out via a programmed-temperature vaporizer (PTV) injector in the solvent venting mode: the inlet was ballistically heated from 50°C to 300°C which was held for 90 s. The split flow was 100 ml/min and the split valve was opened for 6 s and then closed for 60 s. Temperature programming was used: initial column temperature at 220°C programmed at a rate of 5 C°/min to 310°C which was held for 25 min. Volumes of 0.5 μl were injected.

Oxysterols were also analyzed by a 5300-HT Mega-series high-resolution gas chromatograph (Carlo Erba Strumentazione, Rodano, MI, Italy) equipped with an on-column injector and a secondary cooling system. Temperature programming was also used: initial column temperature at 100°C programmed to 220°C at a rate of 10 C°/min. Final

column temperature (320°C) was reached by means of a temperature increase of 5 C°/min and was held for 25 min. The detector temperature was 340°C. Helium was the carrier gas and delivered to the column at a head pressure of 120 kPa. Nitrogen was used as make-up gas. The FSOT capillary column 30 m×0.25 mm I.D. coated with 5% phenylmethylsilicone and with a film width of 0.25 μm (Ultra 2; Hewlett-Packard, USA) was connected to a deactivated retention gap (1 m×0.32 mm, Hewlett-Packard). Volumes of 0.5 μl were injected.

2.6. cGC–MS

Two types of coupled cGC–MS systems were used:

(1) An HP 5890 gas chromatograph equipped with a DB-5 capillary column (20 m×0.18 mm I.D., 0.18 μm film thickness) (J&W Scientific) and coupled to an HP 5970 mass-selective detector (Hewlett-Packard) by direct interface at 280°C was used. In this case, a two-step temperature program was applied: (a) initial column temperature at 80°C, held for 1 min and programmed to 250°C at a rate of 10 C°/min and (b) final column temperature (280°C) reached by means of a temperature increase of 4 C°/min and held for 20 min. The injector temperature was 270°C and the inlet pressure was 0.7 bar.

(2) A Fisons Instruments GC 8000 series equipped with an Ultra 2 capillary column (30 m×0.25 mm I.D., 0.25 μm film thickness) (Hewlett-Packard) and coupled to a mass spectrometer (Fisons Instruments TRIO 2000) by direct interface at 280°C was also used with a two-step temperature program: (a) initial column temperature at 80°C, held for 2 min and programmed to 250°C at a rate of 10 C°/min and (b) final temperature (280°C) reached by means of a temperature increase of 4 C°/min and held for 19 min. The injector temperature was 290°C and the inlet pressure was 120 kPa.

Cholesterol oxide samples were introduced onto the capillary columns by splitless injection (split valve closed for 1 min). The mass spectra of oxysterols were obtained in the electron-impact mode (70 eV) and scanned within the mass range m/z=50–550. Selected ions were used for isolating cholesterol oxide peaks from the TIC obtained by mass scan (50–550 m/z) (see Table 5 for selected ions). Calibration curves were also calculated for each cholesterol oxide using the 19-hydroxycholesterol as internal standard: 1, 2, 4, 10 and 20 ng of each oxysterol were analyzed in the TIC mode.

2.7. Evaluation of the solvent venting and on-column injection for oxysterols cGC analysis

The response linearity was evaluated for cGC: standards solutions (n=4) were analyzed by cGC in the solvent venting and on-column mode. Concentrations of 2.5, 6.25, 12.5 and 25 ng of each oxysterol (including 19-hydroxycholesterol) were analyzed by cGC in the solvent venting mode. In the case of on-column cGC, 6, 10, 20 and 40 ng of each compound were injected. Linear plots of concentration vs. peak areas were calculated. The reproducibility of absolute areas and retention times for each cholesterol oxide were also evaluated by injecting seven times a solution containing the eight cholesterol oxides plus 19-hydroxycholesterol. The response factors were calculated by varying standard oxysterols concentrations vs. a fixed concentration of 19-hydroxycholesterol (internal standard). Oxysterols recovered from spiked meat were also analyzed by the two types of cGC in order to compare chromatogram profiles.

The solvent venting injection of a 10 μl standard solution was performed. The injection was repeated six times and the reproducibility of absolute areas and retention times for each cholesterol oxide was evaluated. The analytical conditions are as described in Section 2.5 with the exception that the split valve was left open for 15 s.

2.8. Validation

A 20 g weight of fresh chicken muscle was spiked with 1 ppm of oxysterols (including the 19-hydroxycholesterol as internal standard). Five samples were analyzed by the SPE-based procedure and the experiment was repeated three times. Absolute and relative recoveries for each cholesterol oxide were evaluated. A 20 g weight of fresh chicken meat was also spiked with 0.1 ppm of oxysterols: three samples were analyzed and relative and absolute recoveries for each oxysterol were calculated. R.S.D. (relative standard deviation) values for each cholesterol oxide

were determined. Four non-spiked samples (controls) were screened for their cholesterol oxides content in order to determine the amount of oxysterols originally presents in the fresh chicken meat. Cooked meat was also analyzed for its oxysterols content.

A regression analysis was performed with four meat samples (20 g) spiked with 0.1, 1, 2 and 3 ppm of cholesterol oxides. The recovered cholesterol oxides were analyzed by cGC in the solvent venting mode.

2.9. Saponification

A 20 g weight of muscle meat spiked with 1 and 5 ppm of 7α-hydroxycholesterol, α-epoxycholesterol, β-epoxycholesterol, 7-ketocholesterol and 19-hydroxycholesterol were extracted as described in Section 2.1. The total fat extract was saponified by 2 *M* KOH in methanol for 22 h at room temperature. When the reaction was completed, 20 ml of distilled water containing 10% of NaCl were added. The unsaponifiable matter was recovered by consecutive washings with 50, 50 and 25 ml of diethyl ether. Finally, the pooled extracts were washed with 20 ml of 2 *M* KOH in water, passed through anhydrous sodium sulfate and evaporated until dryness. The unsaponifiable matter was redissolved in 7% 2-propanol in hexane. Volumes of 10 μl were applied on a TLC plate as described in Section 2.4. Cholesterol oxides were recovered after TLC runs, derivatized and detected by solvent venting cGC.

2.10. Statistical analysis

Means and R.S.D. (or coefficients of variation) were calculated by using the MEANS procedure and differences between means were tested by using the least significant difference test from the SAS statistical package [35].

3. Results and discussion

3.1. cGC separation of cholesterol oxides

Three types of FSOT columns coated with various stationary phases (DB-1, DB-1701, DB-5) have been tested for the resolution of cholesterol oxides. Underivatized cholesterol oxides were first of all analyzed with unsuccessful resolution of the epoxy isomers (α, β) and the 7-epimers (α, β). Moreover, cholesterol oxides (including 19-hydroxycholesterol) were found to be labile compounds and were subject to thermal instability when flash evaporation was used (see solvent venting injection parameters, Section 2). This latter fact has already been reported by some authors [36–38]. As a consequence of this analytical behaviour, we decided to analyze cholesterol oxides under their TMS-ether derivatives form. The full resolution of all species was achieved by using a 30 m×0.25 mm I.D. column coated with 5% phenylmethylsilicone and with a film width of 0.25 μm. The flow and the temperature program were adjusted to permit an optimalized resolution of oxysterols (Fig. 1 and Section 2). Regarding the derivatization process, the reagent formed by HMDS–TMCS–pyridine (3:1:9, v/v/v) was used. The derivatization was effective at 60°C for 1 h or by overnight reaction at room temperature.

3.2. Enrichment of cholesterol oxides by the SPE-based procedure

Cholesterol oxides are included in the lipid bulk obtained by means of the Folch's extraction [34]. Silica-SPE has been used to isolate neutral lipids (oxysterols and triglycerides) from free fatty acids and phospholipids [39]. The neutral lipids fraction obtained from poultry meat can also include compounds belonging to the carotenoid family like β-carotenes and xantophylls. The use of Florisil-SPE permitted the efficient separation of the cholesterol oxides from the triglycerides and the major part of pigments. The presence of carotenoids in samples was monitored by absorbance detection within the 300–500 nm range. Combined SPE extraction was first of all evaluated for each cholesterol oxide standard and ranged from 95 to 100% recovery (data not shown, eluting solvents are described in Section 2).

In fresh meats, cholesterol oxides generally range from 0.1 to 1 ppm. These trace quantities generate problem of cGC detection when FID is used. In order to solve the problem, we decided to use Mega Bond Elut SPE columns which permitted us to load the total fat extract (up to 1 g) obtained from the 20

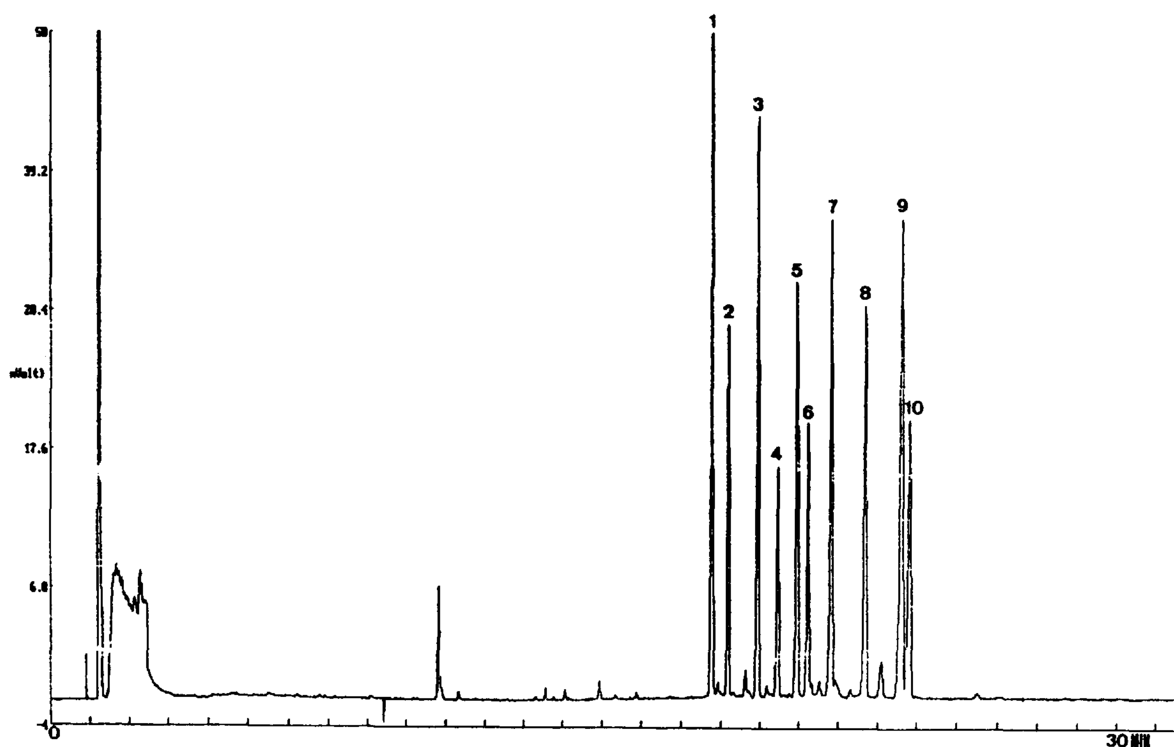

Fig. 1. Capillary gas chromatographic resolution of cholesterol oxides standards. Injection was performed in the solvent venting mode. Analytical conditions are described in Section 2. (1) 7α-Hydroxycholesterol; (2) cholesterol; (3) 19-hydroxycholesterol; (4) 7β-hydroxycholesterol; (5) β-epoxycholesterol; (6) α-epoxycholesterol; (7) 20α-hydroxycholesterol; (8) cholestanetriol; (9) 25-hydroxy-cholesterol; (10) 7-ketocholesterol.

g of muscle meat. By working with the total cholesterol oxides content, it increased the recovered amount of oxysterols and facilitated their analysis by cGC–FID. Hexane–diethyl ether (9:1, v/v) removed the major part of triglycerides with insignificant loss of the less polar cholesterol oxides and 40 to 60% of the cholesterol content was lost. The use of TLC allowed further cleaning of the cholesterol oxides fraction obtained after Florisil fractionation principally by removing the remaining cholesterol which was found to interfere with 7α-hydroxycholesterol and 19-hydroxycholesterol (internal standard) during the cGC analysis. An efficient resolution of cholesterol from its autoxidation products is necessary and this was the reason why we did not opt for preparative TLC. Rhodamin has been used as revelator and avoided harsh treatment such as H_2SO_4 spraying followed by charring on a hot plate [13]. Only the oxysterols standards zone was sprayed with rhodamin in order to eliminate possible interferences during the cGC detection. The use of $CHCl_3$–MeOH (9:1, v/v) improved the recovery of oxysterols from the silica instead of the use of diethyl ether [14].

3.3. Validation

Table 1 presents the absolute and relative recoveries for each cholesterol oxide. Absolute recoveries ranging from 77 to 100% were obtained. The reproducibility of the procedure was satisfactory since the R.S.D. values, evaluated in the two experiments (0.1 and 1 ppm) for each cholesterol oxide, are reflecting the total procedure from the extraction to the TLC step and not only the SPE fractionation [21]. The oxysterol recovery values given by each repetition at the 1 ppm level were compared. No

Table 1
Absolute and relative recoveries for oxysterols

Oxysterols	Absolute recovery						Relative recovery					
	1 ppm[a] (n=15)				0.1 ppm (n=3)		1 ppm[a] (n=15)				0.1 ppm (n=3)	
	Rep. 1	Rep. 2	Rep. 3	R.S.D.	Rep. 4	R.S.D.	Rep. 1	Rep. 2	Rep. 3	R.S.D.	Rep. 4	R.S.D.
7α-Hydroxycholesterol	88.90	91.24	77.03	11.50	91.76	10.09	100.35	95.02	84.23	7.17	90.36	1.91
19-Hydroxycholesterol	89.36	91.30	91.34	7.70	99.97	7.09	100.00	100.00	100.00	0.00	100.00	0.00
7β-Hydroxycholesterol	84.65	96.95	92.32	10.11	93.93	9.03	99.61	106.54	98.90	7.33	101.30	3.39
β-Epoxycholesterol	97.20	97.34	94.79	10.94	83.84	14.98	100.08	100.02	100.08	7.83	100.20	11.19
α-Epoxycholesterol	89.23	89.68	90.22	7.93	94.23	8.75	108.03	96.27	91.89	8.18	105.62	4.23
20α-Hydroxycholesterol	81.73	85.34	94.24	11.18	77.20	11.04	95.22	90.37	101.08	10.34	84.50	1.63
Cholestanetriol	100.32	85.18	88.88	15.76	93.33	6.19	114.23	94.82	94.85	13.00	99.79	3.45
25-Hydroxycholesterol	97.75	85.71	86.60	12.17	84.46	15.56	115.54	94.21	91.48	11.67	94.40	11.60
7-Ketocholesterol	99.81	89.22	93.24	12.09	95.60	13.92	111.91	92.40	100.42	11.00	91.61	12.65

Mean values are presented for each repetition. Repetitions 1 to 3 have been performed with five samples spiked each time with 1 ppm of each oxysterol. Repetition 4 was carried out with three samples spiked with 0.1 ppm of each cholesterol oxide. R.S.D. values for the 0.1 and 1 ppm experiments are listed separately. Results are expressed in %.

[a] No significant differences between columns were detected (p>0.05).

significant differences (p>0.05) were found, indicating a similar efficiency of the procedure for all the repetitions and for each cholesterol oxide. The same conclusions were drawn for the relative recovery values. As a diol derivative of cholesterol, 19-hydroxycholesterol has quite the same analytical behaviour as cholesterol oxides. Its use as an internal standard corrected losses of cholesterol oxides during the entire procedure. Relative recoveries ranging from 84 to 100% were obtained (Table 1). The use of 19-hydroxycholesterol as an internal standard has already been reported in the literature [14,23,24]. Values greater than 100.00% for some oxysterols are explained by the fact that they were either recovered more efficiently than the internal standard (19-hydroxycholesterol) or that some impurity co-eluted with these oxysterols. The use of an internal standard also contributed to the improvement of the R.S.D. values (Table 1). For a correct evaluation of the relative recovery, the calculation of the relative response factor was performed each time before each repetition analysis.

The results of the regression analysis carried out on spiked samples are presented in Table 2. Calibration curves with R^2 values ranging from 0.9778 to 0.9951 were calculated by using the 19-hydroxycholesterol as internal standard. The sample spiked with 1 ppm of cholesterol oxides was manually injected five times and R.S.D. values for absolute areas and retention times were evaluated and listed in Table 2.

3.4. Evaluation of the solvent venting and on-column injection for oxysterols cGC analysis

A regression analysis was performed for on-column and solvent venting cGC. Table 2 presents calibration curves with their corresponding coefficient of determination (R^2) obtained for each cholesterol oxide and 19-hydroxycholesterol. Figs. 2 and 3 respectively represent the analysis of two samples, obtained from 20 g of muscle spiked with 1 ppm of cholesterol oxides, by cGC in the solvent venting and on-column mode. Lower absolute areas R.S.D. values where obtained when on-column cGC was used (Table 2). This could be explained by a possible thermal degradation of cholesterol oxides under the high evaporation temperature [40] used for solvent venting injection. The latter problem is circumvented by on-column injection and secondary cooling which prevents selective vaporization of the sample and by this, contributes to improved R.S.D. values [41,42]. The longer analysis time obtained with on-column cGC (see Section 2.5) was due to on-column injection at 100°C in order to optimalize oxysterol peaks shape while the initial column temperature was 220°C, in the case of the solvent venting cGC. The results indicate that these two

Table 2
Evaluation of the on-column and the solvent venting injections

Oxysterols	On-column injection				Solvent venting injection							
	Pure standards				Pure standards				Spiked samples			
	$t_R^{a,b}$	AA[a,b]	Regression analysis ($n=4$)		$t_R^{a,b}$	AA[a,b]	Regression analysis ($n=4$)		$t_R^{a,c}$	AA[a,c]	Regression analysis ($n=4$)	
			Calibration curves	R^2			Calibration curves	R^2			Calibration curves[d]	R^2
7α-Hydroxycholesterol	0.26	3.95	$y=-0.18+0.28x$	0.9980	0.27	7.34	$y=1.91+1.48x$	0.9937	0.16	8.33	$y=0.06+0.83x$	0.9951
19-Hydroxycholesterol	0.28	4.00	$y=0.11+0.30x$	0.9995	0.27	6.44	$y=1.63+1.13x$	0.9936	0.12	5.19	(internal standard)	
7β-Hydroxycholesterol	0.30	5.20	$y=-1.95+0.55x$	0.9998	0.28	6.60	$y=0.84+0.55x$	0.9930	0.16	6.73	$y=0.09+1.42x$	0.9748
β-Epoxycholesterol	0.31	4.35	$y=0.56+0.037x$	0.9804	0.29	7.76	$y=1.15+1.14x$	0.9897	0.20	14.10	$y=0.08+1.24x$	0.9904
α-Epoxycholesterol	0.33	2.70	$y=-1.41+0.50x$	0.9993	0.30	6.85	$y=0.87+0.67x$	0.9944	0.16	8.33	$y=0.14+1.04x$	0.9778
20α-Hydroxycholesterol	0.34	4.65	$y=-0.60+0.32x$	0.9913	0.31	6.44	$y=1.47+1.20x$	0.9944	0.18	13.48	$y=0.11+0.82x$	0.9820
Cholestanetriol	0.35	4.30	$y=-2.49+0.60x$	0.9971	0.30	6.68	$y=1.45+1.08x$	0.9928	0.15	8.30	$y=0.12+0.99x$	0.9822
25-Hydroxycholesterol	0.34	6.00	$y=-0.83+0.41x$	1.0000	0.35	6.29	$y=2.10+1.57x$	0.9926	0.15	10.38	$y=0.15+0.94x$	0.9857
7-Ketocholesterol	0.36	4.65	$y=-0.43+0.32x$	0.9887	0.32	5.43	$y=1.47+0.90x$	0.9910	0.14	12.03	$y=0.19+1.22x$	0.9814

Reproducibility of retention times and absolute areas was evaluated for pure standards and spiked samples. For both cases, results from the regression analyses are also presented for each cholesterol oxide. Calibration curves were obtained by plotting concentration (y) vs. peak area (x). For the regression analysis carried out on spiked samples, 19-hydroxycholesterol was used as the internal standard. The R.S.D. values for retention times and absolute areas are expressed in %. For analytical conditions, see Section 2.

[a] t_R: retention times, AA: absolute area.

[b] R.S.D. values calculated from seven consecutive manual injections.

[c] R.S.D. values calculated from five consecutive manual injections.

[d] Samples were spiked with 0.1, 1, 2, 3 ppm of cholesterol oxides including 19-hydroxycholesterol.

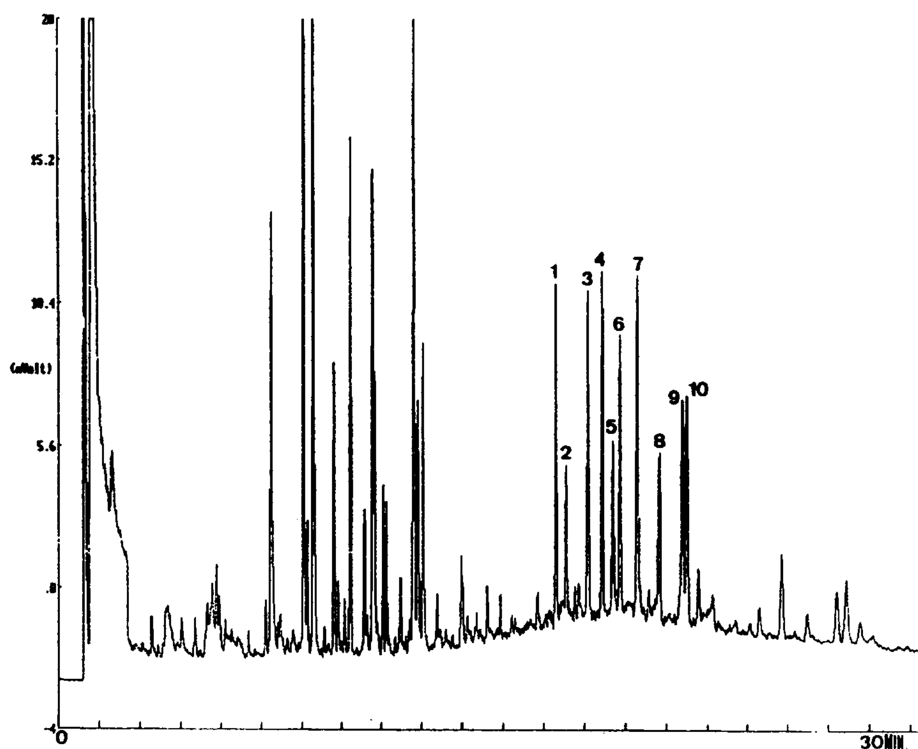

Fig. 2. The presented chromatogram corresponds to cholesterol oxides recovered from a spiked sample by applying the SPE-based procedure. The oxysterols were introduced onto the column by solvent venting injection. Analytical conditions are described in Section 2. (1) 7α-Hydroxycholesterol; (2) cholesterol; (3) 19-hydroxycholesterol; (4) 7β-hydroxycholesterol; (5) β-epoxycholesterol; (6) α-epoxycholesterol; (7) 20α-hydroxycholesterol; (8) cholestanetriol; (9) 25-hydroxycholesterol; (10) 7-ketocholesterol.

modes of injection are suitable for cholesterol oxide cGC analysis.

The solvent venting injection, as already described [33], can be used to inject large sample volumes (e.g. for very dilute samples, 10 μl). Table 3 shows R.S.D. values obtained after six manual consecutive injections of a 10 μl cholesterol oxide standards solution. The obtained R.S.D. values were satisfactory. The introduction of such volumes onto the column requires the split valve to be left open for 15 s instead of 6 s in order to allow the efficient evaporation of the solvent. Furthermore, a constant flow-rate injection is required in order to avoid peak discrimination and to minimize R.S.D. values. This kind of injection would allow a 10-fold increase in the sensibility. On-column injection of volumes up to 2 μl into a glass capillary column of 0.23–0.35 mm I.D. has been described and evaluated [42,43] and could be applied to cholesterol oxides analysis.

3.5. Identification of cholesterol oxides

The identification of cholesterol oxides by cGC can be achieved by using the relative retention times [t_R (solute)/t_R (internal standard), Table 4]. This way of proceeding is appropriate when huge peaks are detected and without the presence of overlapping peaks (Fig. 1). A 1 ng amount is the typical limit of detection for cGC–FID. In our work, cholesterol oxides from samples spiked with 0.1 and 1 ppm can be concentrated up to 20 ng/μl which can be analyzed by cGC–FID (Figs. 2 and 3). Nonetheless, the use of cGC–MS is of great help for confirming cholesterol oxides detected by cGC–FID [23]. Regression analysis was performed for each of the cholesterol oxide standards which were scanned in the TIC mode. Table 5 shows the calibration curves and the corresponding coefficients of determination.

Samples were analyzed by cGC–MS in the TIC

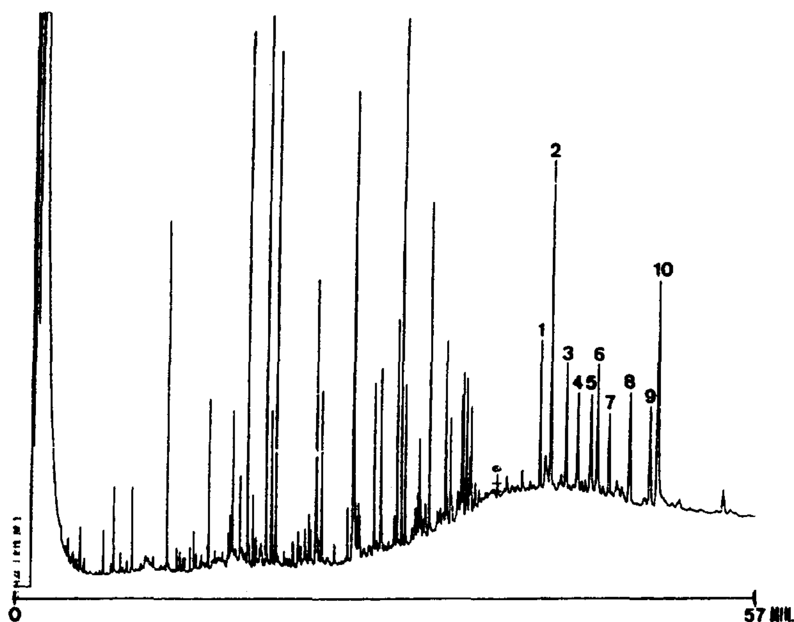

Fig. 3. The presented chromatogram corresponds to cholesterol oxides recovered from a spiked sample by applying the SPE-based procedure. On-column injection was used. Analytical conditions are described in Section 2. (1) 7α-Hydroxycholesterol; (2) cholesterol; (3) 19-hydroxycholesterol; (4) 7β-hydroxycholesterol; (5) β-epoxycholesterol; (6) α-epoxycholesterol; (7) 20α-hydroxycholesterol; (8) cholestanetriol; (9) 25-hydroxycholesterol; (10) 7-ketocholesterol.

mode in order to confirm the peaks detected by cGC. Fig. 4 shows the cGC–MS analysis of a sample spiked with 0.1 ppm of cholesterol oxides. The sample was analyzed in the TIC mode and cholesterol oxides peaks were isolated by selecting characteristic ions (see Table 5). Mass spectra were acquired in order to confirm their identification.

3.6. Analysis of non-spiked meat samples by the SPE-based procedure

Table 6 presents the cholesterol oxides content of four non-spiked samples. The B-ring autoxidation products, i.e., the 7α- and 7β-hydroxycholesterol and the 7-ketocholesterol were detected in most of the

Table 3
Reproducibility of the solvent venting injection

Oxysterols	R.S.D. values for retention times (%)	R.S.D. values for absolute areas (%)
7α-Hydroxycholesterol	1.77	6.43
19-Hydroxycholesterol	1.63	8.07
7β-Hydroxycholesterol	1.63	8.35
β-Epoxycholesterol	1.59	13.56
α-Epoxycholesterol	1.56	8.25
20α-Hydroxycholesterol	1.52	12.60
Cholestanetriol	1.50	10.76
25-Hydroxycholesterol	1.45	6.07
7-Ketocholesterol	1.45	11.12

A 10 μl cholesterol oxide standards solution was injected six times. R.S.D. values for absolute areas and retention times are presented.

Table 4
Relative retention times (RRT) [t_R (solute)/t_R (internal standard)] evaluated for each cholesterol oxide

Oxysterols	RRT (solvent venting)	R.S.D. values (%)	RRT (on-column)	R.S.D. values (%)
7α-Hydroxycholesterol	0.938	0.13	0.954	0.12
7β-Hydroxycholesterol	1.026	0.09	1.020	0.05
β-Epoxycholesterol	1.044	0.17	1.043	0.07
α-Epoxycholesterol	1.058	0.16	1.056	0.11
20α-Hydroxycholesterol	1.089	0.18	1.076	0.14
Cholestanetriol	1.130	0.23	1.111	0.21
25-Hydroxycholesterol	1.174	0.25	1.147	0.29
7-Ketocholesterol	1.181	0.20	1.160	0.25

A mixture of cholesterol oxides was analysed by cGC in the on-column and solvent venting mode. R.S.D. values are presented for both cases. For analytical conditions, see Section 2. 19-Hydroxycholesterol was used as internal standard.

samples. The epoxy-isomers (α, β) were also identified in all the samples with the predominance of the β-form. Cholestanetriol was not detected. Autoxidation products like 20α-hydroxycholesterol and 25-hydroxycholesterol resulting from radical attack on the cholesterol lateral chain were also detected. The level of cholesterol oxides detected by this procedure agreed with those listed in the literature [13,14,24].

The oxysterols content of cooked meat has also been determined by applying the SPE-based procedure. Fig. 5 shows the cGC–FID analysis. 7α-Hydroxycholesterol, 7β-hydroxycholesterol and 7-ketocholesterol were detected and confirmed by cGC–MS. Fig. 6 shows the TIC of a sample obtained from cooked chicken meat. These latter results are presented in order to show that the SPE-based procedure is suitable for the analysis of processed meats and will be the subject of a forthcoming publication.

3.7. Saponification

Fig. 7 shows cholesterol oxides obtained from a 20 g muscle sample spiked with 5 ppm of 7α-hydroxycholesterol, α-epoxycholesterol, β-epoxycholesterol, 7-ketocholesterol and 19-hydroxycholesterol. The four oxysterols were chosen on the basis of the levels detected in non-spiked samples and reported in the literature [12,14,24]. Saponification of the total fat extract (about 1 g) was performed in order to permit the handling of the total cholesterol oxides content. Cholesterol oxides were poorly recovered (50–60%) and greater loss was observed for 7-ketocholesterol. This fact has already been

Table 5
cGC–MS calibration curves obtained by plotting concentration (y) vs. peak area (x) performed for each cholesterol oxides and achieved in the TIC mode

Oxysterols	Calibration curves	R^2	Ions			
7α-Hydroxycholesterol	$0.07+0.31x$	0.9995	*546*	*458*	457	456
7β-Hydroxycholesterol	$-0.04+0.38x$	0.9996	546	458	*457*	456
β-Epoxycholesterol	$-0.10+0.54x$	0.9991	*474*	459	445	*384*
α-Epoxycholesterol	$0.10+0.57x$	0.9931	474	459	384	366
20α-Hydroxycholesterol	$0.07+0.98x$	0.9993	462	*461*	281	*201*
Cholestanetriol	$-0.09+0.35x$	0.9905	546	456	*404*	*403*
25-Hydroxycholesterol	$1.12+0.48x$	0.9155	546	456	327	*131*
7-Ketocholesterol	$0.03+0.51x$	0.9795	514	*472*	*367*	131
Cholesterol	–	–	458	*368*	*353*	329
19-Hydroxycholesterol	–	–	*366*	354	353	352

19-Hydroxycholesterol was used as internal standard. The major abundant ions characterizing TMS-ether cholesterol oxides including cholesterol are also presented. The ions chosen for selected ion monitoring of the samples are printed in italics. For analytical conditions, see Section 2.

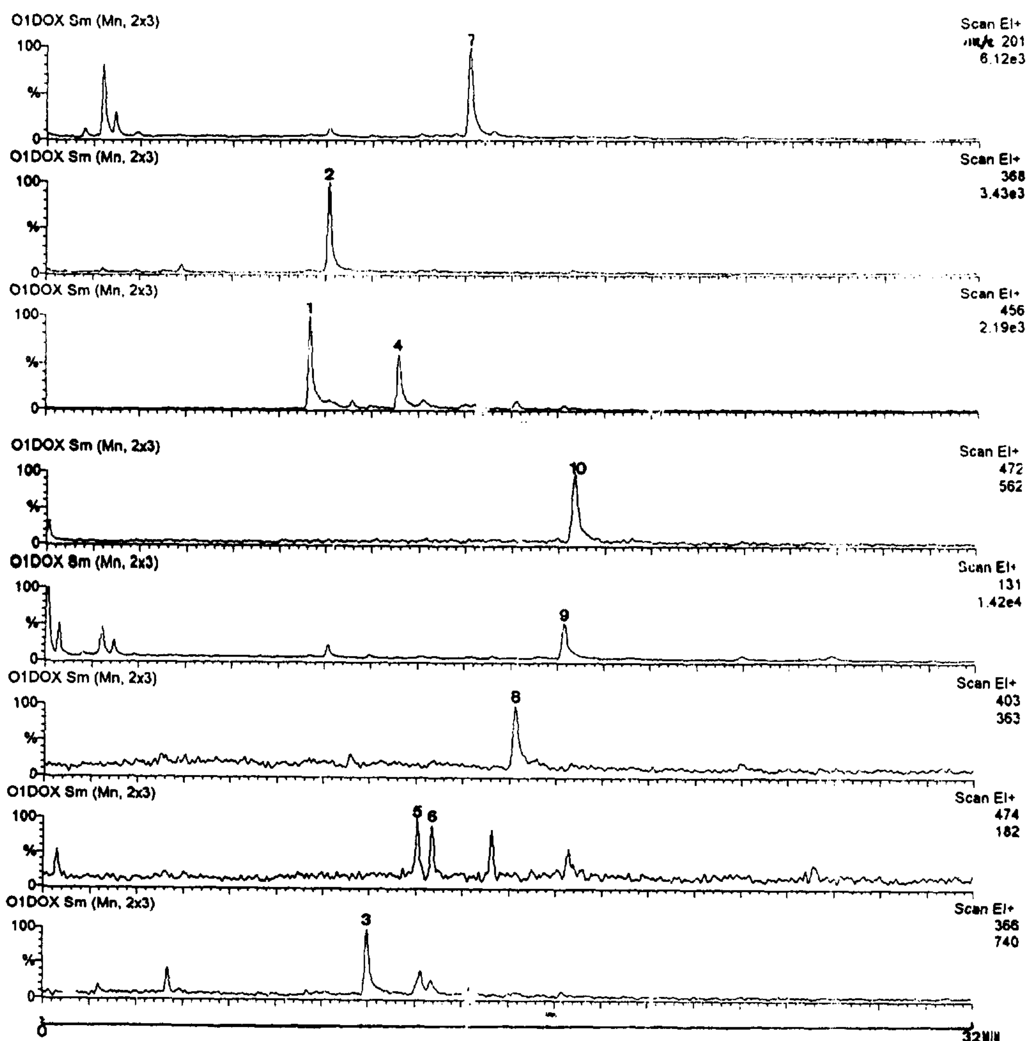

Fig. 4. cGC–MS analysis of a sample spiked with 0.1 ppm of cholesterol oxides. Cholesterol oxides were recovered by applying the SPE-based procedure. Splitless injection was used. Selected ions were used for isolating cholesterol oxide peaks from the TIC obtained by mass scan (50–550 m/z). For selected ions and analytical conditions, see respectively Table 5 and Section 2. (1) 7α-Hydroxycholesterol; (2) cholesterol; (3) 19-hydroxycholesterol; (4) 7β-hydroxycholesterol; (5) β-epoxycholesterol; (6) α-epoxycholesterol; (7) 20α-hydroxycholesterol; (8) cholestanetriol; (9) 25-hydroxycholesterol; (10) 7-ketocholesterol.

underlined by van de Bovenkamp et al. [40]. Moreover, the quantification of the 7α-hydroxycholesterol is complicated by an interfering compound (see Fig. 7, peak 1). For the 1 ppm spiked sample, the accurate quantitation of cholesterol oxides was problematic due to the poor recoveries. The sensibility of the detection could be increased by applying larger volumes on the TLC plate (>20 μl) but we observed an overloading of the silica gel which caused an inefficient resolution of the solutes. The use of preparative TLC to increase the recovered amounts of oxysterols presented the inconvenience of a bad resolution. We think that saponification is not as accurate as the SPE-based procedure for the determination of cholesterol oxides in fresh meat: poor recoveries limit the detection of critical quan-

Table 6
Cholesterol oxides content of fresh chicken meat (non-spiked samples, $n=4$)

Oxysterols	Sample			
(μg/g)	1	2	3	4
7α-Hydroxycholesterol	0.13	0.08	n.d.	0.10
7β-Hydroxycholesterol	0.17	0.12	n.d.	n.d.
β-Epoxycholesterol	0.24	0.41	0.38	0.30
α-Epoxycholesterol	0.15	0.25	0.16	0.06
20α-Hydroxycholesterol	0.11	0.08	n.d.	0.18
Cholestanetriol	n.d.	n.d.	n.d.	n.d.
25-Hydroxycholesterol	0.11	n.d.	n.d.	n.d.
7-Ketocholesterol	0.12	0.14	0.22	0.16

Results are expressed in μg/g.
n.d.: not detected

tities (e.g. 0.1–1 ppm). Finally, the saponification generated artifacts which complicated the quantitation of some cholesterol oxides.

4. Conclusions

The SPE-based procedure has been proved to be statistically reproducible (p>0.05) and provided high absolute recoveries (Table 1). This method permits the handling of the total cholesterol oxide content of the sample and by this, an increase in the sensibility of the detection is gained. The use of 19-hydroxycholesterol as an internal standard is appropriate for the calculation of relative recoveries (Table 1). Solvent venting and on-column cGC analysis have been found to be suitable for the determination of the eight chosen cholesterol oxides. On-column cGC would permit a more accurate analysis with reduced absolute area R.S.D. values. Solvent venting cGC offers the possibility of large volume injections and could improve the detection of low levels of cholesterol oxides. cGC–MS clearly proved to be a necessary tool at the time to confirm a quantitative cGC analysis and could permit the

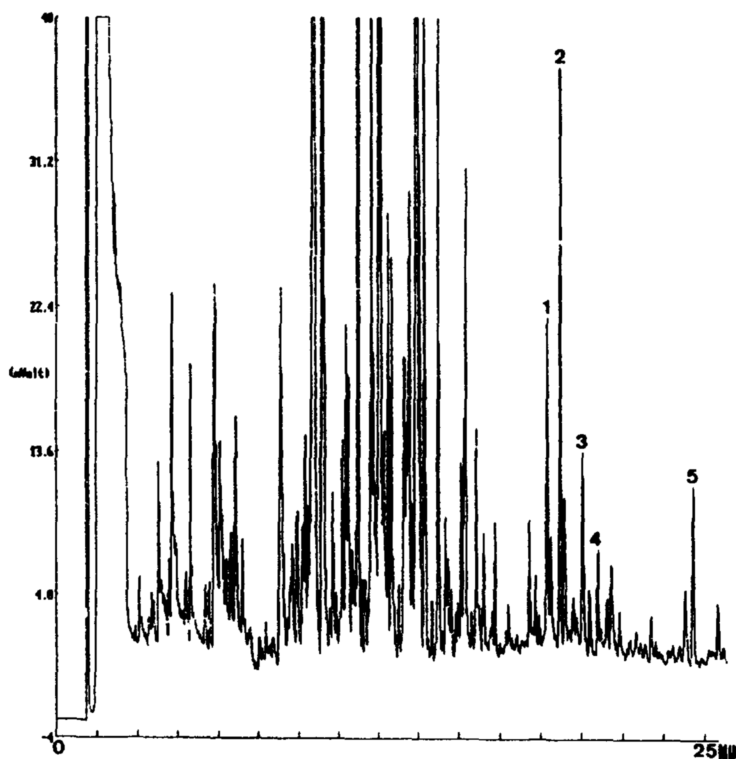

Fig. 5. Detection of cholesterol oxides in cooked meat. Solvent venting was used. Analytical conditions are described in Section 2. (1) 7α-Hydroxycholesterol; (2) cholesterol; (3) 19-hydroxycholesterol; (4) 7β-hydroxycholesterol; (5) 7-ketocholesterol.

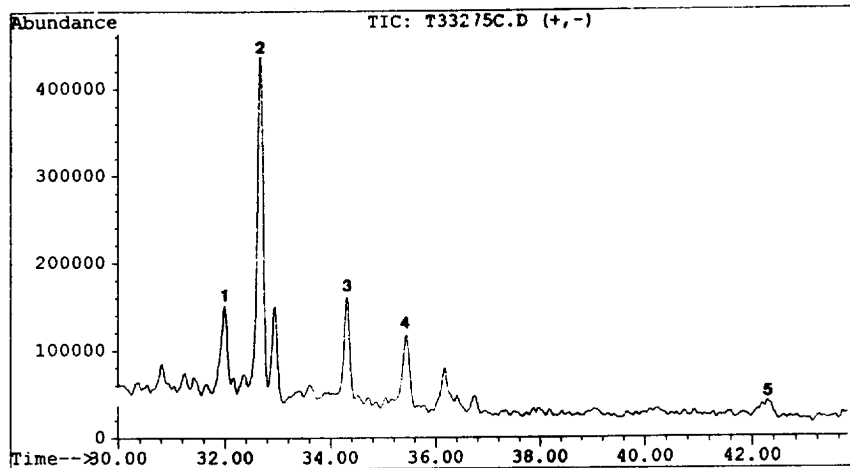

Fig. 6. Cholesterol oxides obtained from cooked meat and analyzed by cGC–MS. The TIC was obtained by mass scan (50–550 m/z). For analytical conditions, see Section 2. (1) 7α-Hydroxycholesterol; (2) cholesterol; (3) 19-hydroxycholesterol; (4) 7β-hydroxycholesterol; (5) 7-ketocholesterol.

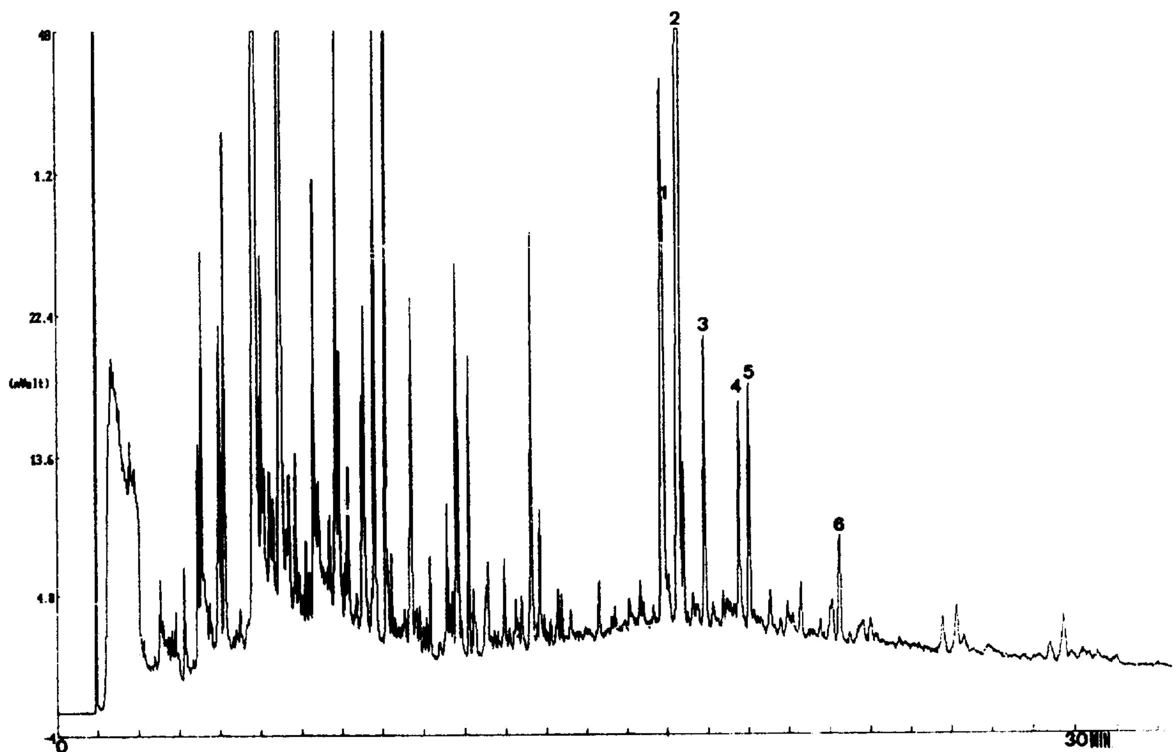

Fig. 7. Cholesterol oxides recovered by saponification followed by TLC and analyzed by cGC in the solvent venting mode. For analytical conditions, see Section 2. (1) 7α-Hydroxycholesterol; (2) cholesterol; (3) 19-hydroxycholesterol; (4) β-epoxycholesterol; (5) α-epoxycholesterol; (6) 7-ketocholesterol.

detection of oxysterol concentrations at the ng/g order. The described procedure is more appropriate for a multi-sample analysis than saponification which was found to be laborious, time-consuming and characterized by poor recoveries. The analysis of four non-spiked samples, obtained from fresh chicken meat, by the SPE-based procedure revealed that the majority of the detected cholesterol oxides resulted from the autoxidation of the cholesterol B-ring. Finally, the SPE-based procedure permitted the detection of cholesterol oxides in cooked meat and from this, it can be concluded that the described method is appropriate for accurate determination of cholesterol oxides in fresh and processed meats.

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