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A new SPE/GC-fid method for the determination of cholesterol oxidation products. Application to subcutaneous fat from Iberian dry-cured ham

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ABSTRACT

A new method for the isolation and analysis of cholesterol oxidation products (COPs) using solid phase extraction (SPE) and silica columns was developed using gas chromatography-flame ion detection (GC-FID). The method comprises of saponification and liquid–liquid extraction of the unsaponifiable fraction prior to the isolation and derivatization of the COPs to trimethylsilyl ethers. The COPs used in this study are cholestane-5α-6α-epoxide, cholestane-3β-5α-6β-triol, 25-hydroxycholesterol and 5-cholesten-3β-ol-7-one. In order to identify the COPs fraction a GC-ion-trap-mass spectrometry experiment were conducted using authentic standards to verify the presence of the COPs. The method was effective at rapidly separating the COPs (25 min run). Calibration curves were linear with the LODs and LOQs bellow 0.03 and 0.07 mg kg^{-1} for all cases, respectively. This methodology gave a total recovery for every compound that was used in the study. Betulin was used as an internal standard to monitor the recovery. The method was validated with a standard mixture of COPs. The method has been applied to characterize the COP fraction of subcutaneous fat from Iberian dry-cured ham. Cholestane-5α-6α-epoxide, cholestane-3β-5α-6β-triol, 25-hydroxycholesterol and 5-cholesten-3β-ol-7-one have been identified for the first time in these samples.

1. Introduction

The oxidation of cholesterol occurs as part of the lipid peroxidation process in membranes. Several oxidation products that are characteristic of free-radical mechanisms are formed and some can serve as indices of the nature and extent of cholesterol oxidation and lipid peroxidation [1]. Some cholesterol oxidation products (COPs) have been shown to have a variety of potentially cytotoxic and mutagenic effects [1] and for such reason the study of these compounds is of great interest to the food industry. Cholesterol-containing foods, when consumed fresh, contain low levels of COP and the levels go up during processing, storage and cooking, So, the COPs are related to processing temperature, heating time, storage conditions, level of activator present, packaging and most of the COPs found in foods have been subjected to processing conditions or exposure to heat [2]. Emanuel et al. [3] concluded in their study that the human subjects had the capacity to absorb COPs from food sources. These researchers reported that the brief residence time of COPS observed in both chylomicrons and plasma suggested rapid transfer of COPS among lipoprotein fractions and/or clearance from plasma.

Analysis of COPs generally consists of four major steps that include extraction of lipids from foods, saponification of extracted lipids, and subsequent enrichment of COPs and quantification by gas chromatography (GC) or by high performance liquid chromatography (HPLC). An alternative to saponification of extracted lipids is transesterification of total lipids and subsequent enrichment of COPs [4]. Both hot [5] and cold saponifications [6], using either ethanolic or methanolic potassium hydroxide (KOH), have been used in the analysis of COPs.

Solid phase extraction (SPE), thin layer chromatography (TLC) or the combination of the two methods has been used to enrich COPs. García Regueiro and Maraschiello [7] developed a method to enrich COPs through combining SPE and TLC. Both silica-phase [7] and amino-phase [4] columns have been used to separate COPs when SPE is used.

Prior to GC and GC–MS analyses, samples are derivatised to trimethylsilyl (TMS)-ethers using BSTFA [8], Tri-Sil reagent (Pierce Chemical Co., Rockford, IL) [9] and the mix of hexamethyldisilazane:trimethylchlorosilane:anhydrous pyridine (3:1:9, v/v/v) [10].

Different capillary columns have been used for the separation, determination and quantification of COPs using the GC. Garcia

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Regueiro and Maraschiello used a fused-silica open tubular (FSOT) capillary column (30 m × 0.25 mm I.D., 0.25 μm) coated with 5% phenylmethylsilicone [7]. Columns coated with 100% dimethylpolysiloxane, such as DB-1, have also been used [8,11]. Grandgirard et al. [12] used DB5-MS and DB1-MS (30 m × 0.25 mm i.d., 0.25 μm), to analyze COPs and obtained similar results. Azadmard-Damirchi and Dutta [9] used a combined column of DB5-MS (10 m × 0.18 mm, 0.18 μm) and DB17 (10 m × 0.18 mm, 0.18 μm), which were joined together with a universal press-fit connector.

Although GC has been the most used technique for the analysis of COPs, some HPLC methods have been reported in literature. Raith et al. [13] developed a new method using reversed-phase LC/atmospheric pressure chemical ionization mass spectrometry for the successful determination of cholesterol and COPs in processed foods such as pork, beef, chicken and eggs. Kermasha et al. [14] did a comparative HPLC analysis of these compounds using both diode-array ultraviolet and laser light-scattering (LLSD) detection, concluding that the use of LLSD in the HPLC of cholesterol and COPs could provide a reliable tool for the determination of these compounds, since the use of this allowed the detection of cholestanetriol and α- and β-epoxides.

For the quantitation of COPs, different internal standards have been added; 5α-colestane [9,12], betulin [8], 19-hydroxycholesterol [11] and betamethasone-17, 19-dipropionate [13] being between them.

The aim of this work is to develop an analytical method for isolation, resolution and quantification of cholesterol oxidation products, which shows a total recovery, a good linear correlation for low levels of concentrations and an adequate resolution for a short runtime. This method has been applied to characterize the cholesterol oxidation products from Iberian dry-cured ham subcutaneous fat.

2. Experimental

2.1. Reagents and standards

LiChrosolv grade n-hexane and diethyl ether were procured from Merck (Darmstadt, Germany). Potassium hydroxide 85% pellets and PA-ACS grade sodium sulfate anhydrous were supplied by Panreac (Barcelona, Spain). Chloroform, methanol, acetone and pyridine anhydrous for analysis grade were provided by Merck (Darmstadt, Germany). Chlorotrimethylsilane and hexamethyldisilazane were supplied by Fluka (Seelze, Germany) and betulin (Sigma Chemical Co., St. Louis, MO, USA) was used as an internal standard. All other materials were analytical grade.

Cholesterol and the standards used for the identification of oxy-cholesterols (COPs): cholestane-5α-6α-epoxide, cholestane-3β-5α-6β-triol, 25-hydroxycholesterol and 5-cholesten-3β-ol-7-one were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Samples and sample treatment

Samples of subcutaneous fat from Iberian dry-cured ham were used. The lipids were obtained from 1.5 g of each sample by extraction with chloroform-methanol (2:1 v/v) according to the procedure described by Folch et al. [15]. The samples were cut up into small pieces and homogenized prior to extraction with 3 × 10 mL of solvent using an agitator. The solution was filtered and evaporated to dryness in a rotary evaporator at 30 °C under reduced pressure.

The unsaponifiable fraction was extracted as described below. Fat (0.5 g) was placed in a glass Pyrex tube where 0.5 mL of betulin solution (0.15 mg mL^{-1} of chloroform) was previously added and evaporated under nitrogen. The mixture was saponified with 5 mL of ethanolic potassium hydroxide (2 M) that contained 20% (v/v) water in an oven with air circulation and heated at 85 °C for 45 min. After cooling at room temperature, 10 mL distilled water was added and the solution was transferred to a 50-mL decanting funnel prior to extraction. Then, the mixture was extracted with three 10 mL portions of diethyl ether. The extracts were combined in another funnel and were washed several times with 10-mL portions of distilled water, until the wash was at neutral pH. The solution of diethyl ether and COPs was dried over anhydrous sodium sulfate and evaporated to dryness in a rotary evaporator at 30 °C under reduced pressure.

2.3. COPs purification

The COPs fraction was separated from unsaponifiable matter by means of Solid Phase Extraction (SPE). The extract was dissolved in 0.5 mL n-hexane:diethyl ether (75:25 v/v) and transferred into a silica gel bonded column, Supelclean LC-Si, 3 mL volume; 500 mg sorbent (Supelco Bellefonte, PA, USA) which was previously conditioned with 10 mL n-hexane. The column was washed with 4 mL n-hexane:diethyl ether (75:25 v/v), to remove the compounds with minor polarity, and then, the COPs were eluted with 3 mL n-hexane:diethyl ether (60:40 v/v) and 4 mL of acetone: methanol (60:40 v/v). Both recovered fractions were combined and evaporated to dryness in a rotary evaporator at 30 °C under reduced pressure.

Trimethylsilyl ether derivatives were prepared by adding 0.2 mL of Pyridine: Chlorotrimethylsilane: hexamethyldisilazane (9:3:1, v/v/v) at room temperature for 20 min [16].

Standards of cholesterol, cholestane-5α-6α-epoxide, cholestane-3β-5α-6β-triol, 25-hydroxycholesterol and 5-cholesten-3β-ol-7-one were derivatized and chromatographed in a similar manner.

2.4. Instrumentation

The COPs were analyzed by injecting 1 μl of the derivatized solution into an Agilent (Palo Alto, CA, USA) 7890A gas chromatograph that was equipped with a G4513A split/splitless injector and a flame ionization detector. Separation of the COPs was accomplished using a capillary DB5-MS column (30 m × 0.25 mm i.d., 0.25 μm film thickness, J&W Scientific, Albany, NY, USA) using hydrogen (Airgas, Columbus, MS) as the carrier gas at 10 psi constant head pressure. The programming sequence for the GC oven temperature was as follows: an initial temperature of 90 °C held for 0.5 min and increased to 290 °C at a rate of 30.0 °C min^{-1}. Once the column reached 290 °C, the temperature was increased to 300 °C at a rate of 1.0 °C min^{-1} and held for a total running time of 25 min. The injector was operated in the splitless mode at 350 °C with a purge flow to split vent of 15 ml min^{-1} at 0.5 min. The detector temperature was 360 °C, and air and hydrogen had flow rates of 300 and 30 mL min^{-1} to the detector respectively, which had an auxiliary flow of 30 mL min^{-1} of nitrogen.

In order to identify the COPs, an experiment was conducted using a Varian-CP3800 gas chromatograph coupled to a Saturn 2000 ion trap mass spectrometer (Varian, Palo Alto, CA, USA). The GC was equipped with a CP8400 autosampler. Separation was accomplished on a DB-5 MS capillary column (30 m long × 0.25 mm i.d, 0.25 μm film thickness; J&W Scientific, Folsom, CA) with a flow rate of 1.0 mL min^{-1} using ultra high purity helium (Airgas, Columbus, MS) as the carrier gas. The injector temperature was 350 °C. Splitless injection mode was used with a ratio of 1:10 at 0.5 min and injection volume was 1 μL. The programming sequence for the GC oven temperature was the same as for GC-FID. Once the column reached 290 °C, the

temperature was increased to 300 °C at a rate of 1.0 °C min^{-1} and for 7.833 min for a total running time of 25 min. The MS conditions were as follows: transfer line maintained at 290 °C; electronic ionization mode at an ionization voltage of 70 eV; scan rate of 1.0 s $scan^{-1}$; m/z range of 25–550 amu and manifold temperature of 180 °C. The dwell time and inter-channel delay was 0.08 s and 0.02 s, and the emission current was 10 μA. Varian Mass Spectrometry Workstation version 6.30 software was used for data acquisition and processing of the results.

2.5. Quantitative and recovery analysis

The peak area was used as analytical signal. The quantification of individual COPs was carried out using calibration curves. Betulin was used as internal standard to monitor the recovery.

Aliquots of a blank subcutaneous fat sample of Iberian pig, free of COPs, were spiked at ten different concentration levels (between 0.001 and 1.00 mg mL^{-1}) to obtain the matrix matched calibration curves, which have been used for COPs quantification.

In order to study the recovery and reproducibility (R) of the present method, a complementary experiment was conducted. Recovery data were calculated by comparing the results of COPs determination added to a blank subcutaneous fat matrix with those obtained from the GC direct analysis of COP standards that were analyzed in the same way. Eight replicates were utilized in each case. For the determination of repeatability, the replicates were analyzed on different days in the same laboratory.

The limit of detection (LOD) of the method was reported as the signal to noise ratio of 3 for blank samples. The limit of quantification (LOQ) was reported as the signal to noise ratio of 10 [17]. Eight independent determinations of COPs were made using subcutaneous fat from Iberian pig blank samples.

3. Results and discussion

3.1. Identification of components

A gas chromatogram of COP standard solutions is listed in Fig. 1A, and the relative retention times (T_{RR}) of betulin are shown in Table 1. Fig. 1B shows the gas chromatogram profiles of the COP fractions purified by the SPE Si columns from the subcutaneous fat of Iberian dry-cured ham samples. These figures indicate that there was good separation between the different compounds using this method. The compounds elute between 16 and 20 min. This method is more rapid than others published using a GC-FID [18]. The present method is also more rapid than the HPLC methods [13,14]. In the method described by Raith et al. [13], COPs elute in 22 min using reversed-phase LC/atmospheric pressure chemical ionization mass spectrometry, but it takes 18–20 h to extract the compounds (with n-hexane/isopropanol, 3:2) and approximately 27 min to isolate them using HPLC-ELSD (normal phase column). Other compounds, such as phospholipids [19], are more easily analyzed using HPLC methods, but it seems that GC methods are better for the analysis of COPs.

Four compounds have been identified in the COP fraction of the subcutaneous fat from Iberian dry-cured ham. Identification of the peaks was carried out using a GC ion-trap-MS and comparing the spectra to those from the NIST (National Institute of Standards and Technology) and Wiley libraries and verified by standards (as TMSE derivatives). Fig. 2A corresponds to the GC-ion trap-MS analysis of the COPs standards and Fig. 2B corresponds to the GC-ion trap-MS analysis of the COP fractions obtained from the subcutaneous fat of the Iberian dry-cured ham using the proposed method. Table 1 shows the retention times, the base peak and the molecular ion for their trimethylsilyl ethers.

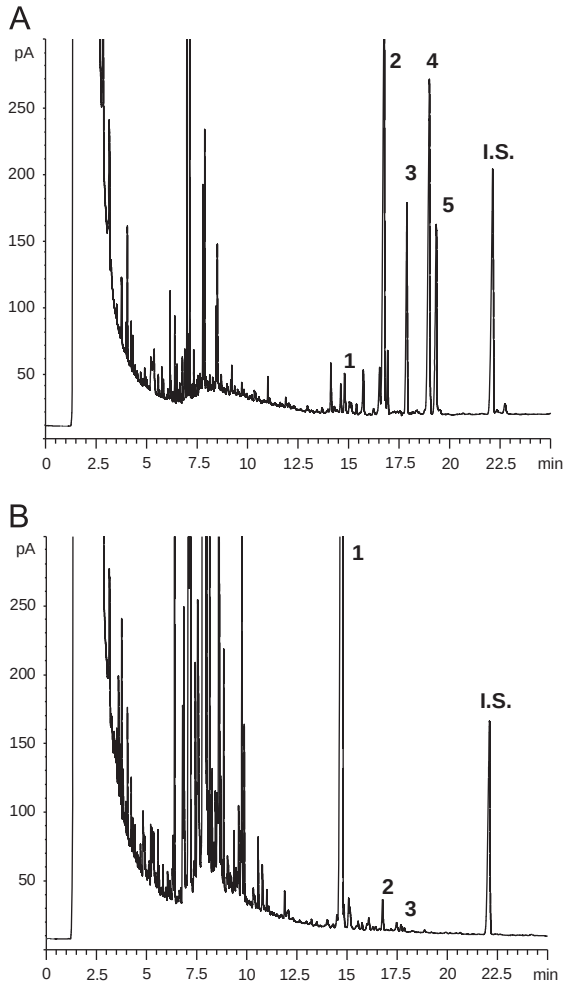

Fig. 1. GC-FID chromatograms of COPs that were obtained from A: standard solutions; B: the subcutaneous fat of Iberian dry-cured ham (See Table 1 for peak assignments).

Table 1
COPs identified in the subcutaneous fat of Iberian dry-cured ham (see Fig. 1).

Peak	Compound	T_{RR}	Base peak	M^+
1	Cholesterol	0.663	369	458
2	Cholestane-5a-6a-epoxide	0.752	367	474
3	Cholestane-3b-5a-6b-triol	0.809	456	636
4	25-Hydroxycholesterol	0.856	131	546
5	5-Cholesten-3b-ol-7-one	0.875	473	472
S.I.	Betulin	1.000	497	586

T_{RR}: relative retention time; M^+: molecular ion.

3.2. Validation of the method

For each COP, a calibration curve was developed to validate the method and the corresponding range of linearity was determined. Each curve was prepared and injected in triplicate. The different calculated equations (Area= Slope × [mg kg^{-1}]+Intercept) are presented in Table 2 and it can be observed that there is a good linear relationship ($R^2 > 0.98$) between concentration and peak area in all cases. The respective peak areas fitted a linear model within the following ranges. Linearity was observed for cholestane-5α-6α-epoxide from 0.021 to 0.400 mg kg^{-1}, for cholestane-3β-5α-6β-triol from 0.015 to 0.371 mg kg^{-1}, for 25-hydroxycholesterol from 0.033 to 0.375 mg kg^{-1} and for 5-cholesten-3β-ol-7-one from 0.051 to 0.380 mg kg^{-1}. The method

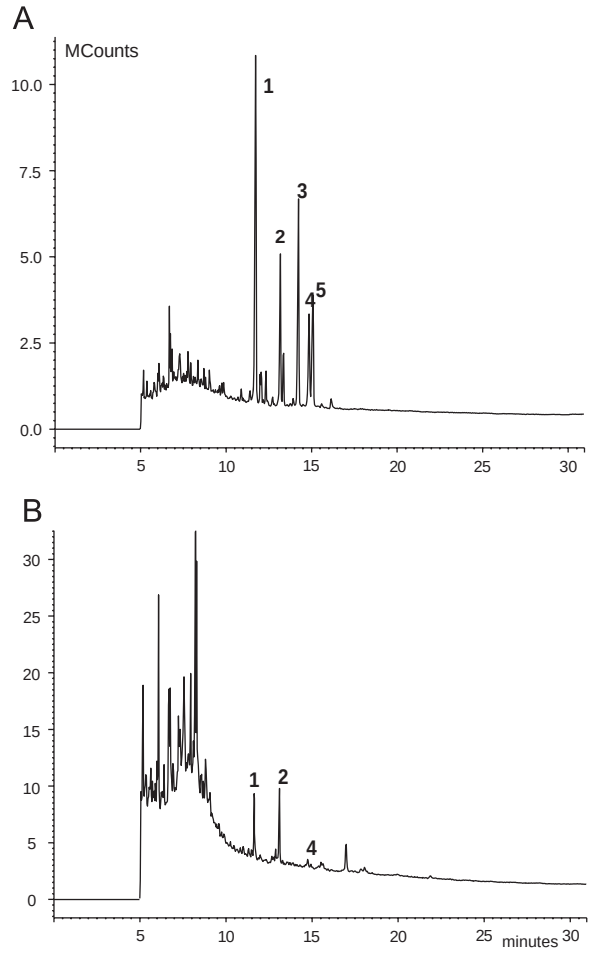

Fig. 2. GC-ion trap-MS chromatograms of COPs that were isolated in full scan mode. For peak assignments see Table 1. A: standard solutions; B: the subcutaneous fat of Iberian dry-cured ham.

Table 2
Simple linear regression coefficients and correlations (R^2) for the linear equation Area = Slope × [mg kg^{-1}] + Intercept corresponding to calibration curves for each cholesterol oxidation products and the internal standard (Betulin).

COP	Slope	Intercept	R^2
Cholestane-5α-6α-epoxide	8279.640	−20.946	0.995
Cholestane-3β-5α-6β-triol	4347.750	−7.657	0.998
25-Hydroxycholesterol	7044.561	−25.924	0.986
5-Cholesten-3β-ol-7-one	4469.081	−42.288	0.975
Betulin	6280.509	−10.553	0.999

shows an excellent linear correlation at a much low concentration range of 10^3 times lower than those indicated in some works [13,14], which makes this analysis much more sensitive than those methods.

The trueness was assessed based on recovery assays. In order to evaluate the recovery of the proposed SPE-GC method, mean peak area values of different COPs obtained by direct injection and by isolation from a mixture of subcutaneous fat of Iberian dry-cured ham free of COPs with 0.400, 0.371, 0.375, 0.380 and 0.375 mg kg^{-1} of Cholestane-5α-6α-epoxide, Cholestane-3β-5α-6β-triol, 25-hydroxycholesterol, 5-cholesten-3β-ol-7-one and betulin respectively. Eight replicates were performed and their corresponding recoveries are reported in Table 3. It can be seen that in all cases the recoveries lie within the range of acceptable values based on the analyte concentration [8,9,18] and accordingly, trueness is significant. This new SPE method presents a total recovery of all COPs in contradiction to others methods described in literature [8,9,18]. The analytical technique is repeatable and the recovery is complete. In addition, it has been verified that none of these compounds elute out when n-hexane:diethyl ether (75:25 v/v) was used to wash the sample. This demonstrates that the separation obtained with SPE silica columns is adequate. The recovery using the aminopropyl solid phase extraction columns is much lower (between 87.5% and 95.0%) [8] than in the proposed method. The same can be observed in the method used by Cardenia et al. [20] where the NH_2-SPE was used and the recovery for some COPs is much less than 80% (triol recovery was just 72%). The recovery is also much more lower (78.7–87.6%) in the method developed by Clariana et al. [18]. In addition, their method requires the use of a higher quantity of solvent to wash and elute the COPs and two SPE separation steps with both silica and aminopropylsilica SPE columns. The method of Azadmard-Damirchi and Dutta [9] also involves single step using silica SPE columns, but recovery is still lower (87.5–95.8%) than the proposed method and required higher quantities of solvents. Therefore, the new proposed method shows better recovery and requires a smaller amount of solvent when compared to other methods reported in the literature. In addition, this method also shows a better recovery than the preparative normal-phase LC isolation method (around 90%) [13].

The repeatability of this method that was developed was evaluated using the subcutaneous fat of Iberian dry-cured ham samples as described in Sections 2.4 and 3.2 and a new SPE column for each replicate. The COPs were being characterized and quantified in subcutaneous fat of Iberian dry-cured ham for the first time (Table 4). With the exception of CH-triol, the relative standard deviations are better than the reference value derived from the Horwitz equation [21] (R.S.D$_H$ = 16.4%). Therefore, the results for different COPs indicate good repeatability for the assay. The LOD and LOQ obtained are also shown in Table 4. The LOD for the different compounds were between 0.007 and 0.032 mg kg^{-1}, and the LOQ ranged from 0.015 and 0.067 mg kg^{-1}. The lowest LOD and LOQs were for cholestane-3β-5α-6β-triol (excluding betulin) and the highest LOD and LOQs were for 5-cholesten-3β-ol-7-one. The LODs for this method are much lower than LODs that have been reported in other methods [18], and is approximately 35 times less in the case of cholestane-3β-5α-6β-triol. The LOQ cannot be compared to other methods because no data could be located.

Similar concentrations were obtained for cholestane-3β-5α-6β-triol, 25-hydroxycholesterol and cholesten-3β-ol-7-one in the subcutaneous fat of Iberian dry-cured ham. The concentration of cholestane-5α-6α-epoxide was approximately half the concentration of the other cholesterol oxidation products and was similar to the concentration of 5-cholesten-3β-ol-7-one that was reported by Clariana et al. [18] for subcutaneous fat from Iberian dry-cured shoulders. Higher concentrations of cholestane-3β-5α-6β-triol and 25-hydroxycholesterol and a lower concentration of cholestane-5α-6α-epoxide were also reported in this study. Petrón et al. [10] didn't study the presence of cholestane-5α-6α-epoxide in intramuscular fat from Iberian dry-cured ham and only found traces of the other three COPs.

4. Conclusions

The new SPE/GC-FID method that has been developed allows for the rapid, sensitive and highly reproducible separation and quantification of the cholesterol oxidation products, which have been identified and quantified for the first time in the subcutaneous fat from Iberian dry-cured hams. A linear response has been achieved for a wide range of concentrations with very low LODs. The new SPE methodology that has been developed permits the

Table 3
Evaluation of the recovery of cholesterol oxidation products using SPE-GC. GC peak areas of the cholesterol oxidation products and the internal standard (Betulin) were obtained by direct injection and by isolation from a mixture with Iberian pig subcutaneous fat.

($n=8$)	(SPE-GC analysis)					(Direct analysis)				
	CH-epoxide	CH-triol	Hydroxy-CH	CH-one	I.S.	CH-epoxide	CH-triol	Hydroxy-CH	CH-one	I.S.
Mean	2222	812	4048	941	1552	1766	733	1434	819	1299
S.D.	209	196	323	80	125	255	48	205	70	148
R.S.D. (%)	9.43	24.13	7.97	8.50	8.07	14.47	6.54	14.30	8.53	11.42
Recovery (%)	126	111	158	115	119					

Table 4
Repeatability, LOD and LOQ data for the cholesterol oxidation products and the internal standard (Betulin) using SPE-GC to quantify these compounds in Iberian pig subcutaneous fat samples.

($n=8$)	(SPE-GC analysis)				
	CH-epoxide	CH-triol	Hydroxy-CH	Ch-one	I.S.
Mean	0.251	0.585	0.526	0.536	0.621
S.D.	0.024	0.141	0.042	0.051	0.050
R.S.D. (%)	9.43	24.13	7.97	9.51	8.07
LOD (mg kg^{-1})	0.013	0.009	0.021	0.032	0.007
LOQ (mg kg^{-1})	0.028	0.019	0.046	0.067	0.015

isolation of cholesterol oxidation products from other lipids with a shorter analysis time, high recovery and the use of less solvent than other reported methods.

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