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Optimization and Validation of Analytical Conditions for Cholesterol and Cholesterol Oxides Extraction in Chicken Meat Using Response Surface Methodology

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The analytical conditions for the extraction of cholesterol and cholesterol oxides in chicken meat were optimized by means of response surface methodology. The separation and identification were performed by normal phase HPLC using UV and refractive index (RI) detectors, and the confirmation of the 11 cholesterol oxides identities in the samples was verified by HPLC-APCI-MS. The developed methodology showed good analytical performance, presenting recovery levels from 84 to 103% and detection limits varying from 0.01 to 0.06 μg/g for UV detection and from 1.98 to 2.12 μg/g for RI detection. The present study demonstrated the presence of 22*R*-hydroxycholesterol, 24*S*-hydroxycholesterol, and 22*S*-hydroxycholesterol for the first time in chicken meat.

KEYWORDS: Cholesterol; cholesterol oxides; chicken; experimental design; HPLC-APCI-MS

INTRODUCTION

Food of animal origin containing a significant cholesterol amount is susceptible to supplying dietary cholesterol oxides (COP). Although there are no established safety levels for COP ingestion, these compounds can be absorbed from the diet in a proportion of 6−90%, constituting a risk for human health due to chronic exposure (*1*). The toxicological relevance of the COP results from their potentially atherogenic, cytotoxic, mutagenic, and possibly carcinogenic effects (*2–6*). Moreover, COP can influence enzymatic activity in cholesterol metabolism and the modulation of cell membrane stability and function, and COP also have been implicated in some chronic and degenerative diseases (*5, 6*).

Cholesterol oxidation can be initiated by exposure to light, oxygen, radiation, transition metals, and unsaturated fatty acids, by a mechanism of chain reactions of free radicals in a similar way of lipid oxidation (*7, 8*). The cholesterol oxides of biological interest most commonly reported in foodstuffs are those derived from the B-ring of the main chain, such as 5,6α-epoxycholesterol, 5,6β-epoxycholesterol, cholestanetriol, 7-ketocholesterol, 7β-hydroxycholesterol, 7α-hydroxycholesterol, and, in lower concentrations, derivatives of the side chain, such as 20α-hydroxycholesterol and 25-hydroxycholesterol (*2, 9*).

Chicken meat is particularly susceptible to lipid oxidation as it has a high proportion of unsaturated fatty acids, and the formation of COP in processed chicken appears to vary depending on the part of the chicken and cooking method used (*10–17*). The levels of COP reported in fresh or freshly processed chicken are generally low or undetectable (*12, 15–18*). On the other hand, cooking methods can increase significantly cholesterol oxidation up to 1.5% (COP/cholesterol) in fried chicken patties (*10*), raising from 4 to 10 times the total COP content (*10–17*).

To obtain reliable results with good recovery levels and also to avoid artifact formation due to cholesterol instability under the analytical conditions, it is recommended that mild analytical conditions be used with a limited number of steps in the procedure. In fact, the cold saponification of milk powders, followed by the extraction of cholesterol and COP using an organic solvent, showed higher accuracy and repeatability than methods involving previous extraction of the lipids (*19*); this method is also suitable for egg (*20*) and fish (*21*) analysis.

The aims of this work were to (i) optimize saponification and extraction conditions of cholesterol and COP by means of the experimental design methodology, (ii) validate a methodology for simultaneous determination of 11 COP and cholesterol in chicken meat, and (iii) apply the method to the determination of the cholesterol and COP contents in chicken breast subjected to different cooking processes.

MATERIALS AND METHODS

Chemicals and Reagents. Cholesterol and cholesterol oxide standards 20α-hydroxycholesterol (20α-OH), 22*S*-hydroxycholesterol (22S-OH), 25-hydroxycholesterol (25-OH), 5,6α-epoxycholesterol (α-EP), 5,6β-epoxycholesterol (β-EP), and 7-ketocholesterol (7-keto) were purchased from Sigma (Milford, MA); 22*R*-hydroxycholesterol (22R-OH), 24*S*-hydroxycholesterol (24S-OH), 25*R*-hydroxycholesterol (25R-OH), 7β-hydroxycholesterol (7β-OH), and 7α-hydroxycholesterol (7α-OH) were acquired from Steraloids (Newport, RI). The purities of the standards varied from 95 to 98%.

Chicken Samples. For the optimization of the saponification and extraction conditions, 3 kg of chicken breast meat without skin and

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Table 1. Levels of Independent Variables of Factorial Screening Design 2^{7-3}

variable	unit	−1	0	+1
w	g	1.0	1.5	2.0
KOH	%	10	15	20
t	h	20	22	24
H_2O	mL	10	20	30
n		3	4	5
c		2	3	4
s	mL	10	15	20

bones was minced using a food processor; 60 ± 0.5 g was used for each patty. The patties were grilled at 170 °C for 4 min on each side, until the core temperature reached 70 °C. For the application of the methodology, 4 kg of chicken breast without bone was divided into four portions of 1 kg. One portion was analyzed raw (R), and the other portions were subjected to three cooking procedures during 20 min: one portion was roasted at 220 °C (RO), one portion was deep fried in soybean oil at 180 °C (DF), and the other was boiled in water (BO). In addition, two whole ready-to-eat condimented chickens cooked in an electric rotatory oven were acquired, and the breasts (ER) were used in the analysis. All of the apparent connective and adipose tissues were removed after thermal treatments, and the samples were ground before analysis. The samples were purchased at a local market in Campinas, São Paulo, Brazil.

Experimental Design. The saponification and extraction procedures based on the method developed by Mazalli et al. (*20*) were optimized by means of response surface methodology (*22*). First, two factorial screening designs 2^{7-3} (plus three central points) were carried out to verify the simultaneous effects of seven independent variables (**Table 1**) on the cholesterol and COP extraction efficiency and also to choose the best solvent for the extraction of unsaponifiable matter between hexane and diethyl ether. The independent variables were the weight of the chicken sample (*w*), the concentration of the ethanolic KOH solution (KOH), the saponification time (*t*), the volume of water added for partition (H_2O), the number of extractions (*n*), the number of washes for cleanup (*c*) of the unsaponifiable matter, and the solvent volume used to wash the anhydrous sodium sulfate in the filter paper (*s*). After that, a central composite design 2^4 with eight axial points and four central points was developed using the statistically significant variables ($p < 0.10$) plus the weight of chicken sample. Cholesterol and 25-OH contents were set as the response variables.

Method Validation. The analytical method was validated for linearity, recovery, repeatability, and limits of detection. Linearity was observed through correlation coefficients (r^2) of the calibration curves constructed with seven points of standard solutions, with concentrations ranging from 0.2 to 6 mg/mL for cholesterol and from 0.5 to 100 μg/mL for the cholesterol oxides. The recovery analyses for cholesterol and cholesterol oxides were performed separately, and both were carried out in two levels with 10 replicates for each level. Repeatability was evaluated using the relative standard deviations (RSD). Detection and quantification limits were calculated according to IUPAC recommendations (*23*).

HPLC Analysis. A Shimadzu (Kyoto, Japan) liquid chromatograph was used equipped with UV (SPD-10 AVVP) and RI (RID-10 A) detectors. The analytical column was a Nova Pak CN HP (Waters, Milford, MA), 300 × 3.9 mm i.d. × 4 μm; injection loop of 20 μL; oven temperature was 32 °C. The mobile phase was *n*-hexane/2 propanol (97:3) at a flow rate of 1 mL/min (*21*). The epimeric 5,6-epoxides were quantified using the RI detector. Cholesterol and the other COP were quantified using the UV detector at 210 nm. Identification of cholesterol and COP was made by comparison of retention times of peaks in samples with those of reference standards and spiking. Quantification was done by external calibration. Characteristic chromatograms of the cholesterol and COP standards and spiked chicken (there was no sample containing all of the COP at the same time) are shown in **Figures 1** and **2**.

HPLC-APCI-MS Analysis. To confirm the identity of the COP, the chicken samples were injected into a Shimadzu HPLC equipped with quaternary pumps (LC-20AD) and a degasser unit (DGU-20A5) connected in series to a photodiode array detector (PDA) (SPD-M20A) and to a mass spectrometer (MS) from Bruker Daltonics (Esquire 4000 model, Bremen, Germany) with an atmospheric pressure chemical ionization source (APCI) and an ion-trap analyzer. The HPLC conditions were the same as described above. The MS parameters were set as follows: positive mode; source temperature, 400 °C; corona, 4000 nA; dry gas (N_2) 300 °C, 5 L/min flow, and 65 psi nebulizer; scan range from *m*/*z* 80 to 450. The MS spectra of the COP from the chicken samples were compared to the MS spectra of the COP standards at the correspondent retention time. The 25R-OH MS spectrum presented only the fragment with 385 u corresponding to elimination of 1 water molecule, and 7-keto presented the protonated molecule ($[M + H]^+$) at *m*/*z* 401 and the fragment corresponding to the loss of 1 water molecule (*m*/*z* 383). On the other hand, the MS spectra of both epoxides and the other hydroxycholesterols did not show the protonated molecule, and only the fragments at *m*/*z* 385 and 367, corresponding to the loss of 1 and 2 water molecules, were respectively detected. The MS spectra are in Figure 1 of the Supporting Information.

Moisture Content. Moisture content was determined according to AOAC method 950.46 (*24*).

Total Lipid Content. Total lipid content was determined according to the method of Folch et al. (*25*).

Statistical Analysis. The software Statistica for Windows 5.5 (StatSoft Inc., Tulsa, OK) was used to perform the regression analysis and variance analysis (ANOVA) and to obtain the response surfaces in the experimental designs. COP contents were compared by Tuckey test (confidence level of 95%). The second-order polynomial equation expressed below was used to correlate the cholesterol content to the variables (*w*, KOH, H_2O, and *n*) and was adjusted to the experimental results (confidence level of 95%).

$$y = b_0 + \sum_{i=1}^{k} b_i x_i + \sum_{i=1}^{k} b_i x_i^2 + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} b_{ij} x_i x_j \quad (i = 1\text{–}3,\ j = 1\text{–}3) \tag{1}$$

RESULTS AND DISCUSSION

Optimization of Saponification and Extraction Conditions. Two factorial screening designs 2^{7-3} (data not shown) were used to select the significantly different variables ($p < 0.10$) and to choose the best extraction solvent for the unsaponifiable matter. Hexane was chosen as the best extraction solvent due to fewer interference peaks and better repeatability of the central points. The variables *w*, *t*, *c*, and *s* did not present significantly differences ($p > 0.10$) for both cholesterol and 25-OH, so, to optimize the analytical conditions, the variables *t*, *c*, and *s* were fixed at 20 h, 2, and 10 mL, respectively, according to the tendency pointed out by the effects on the screening design. Moreover, *w* was included in the dependent variables of the new central composite design due to the negative signal of its effect, meaning that higher amounts of sample were related to lower contents of cholesterol and 25-OH, probably because of the interfering substances that were extracted together with the compounds of interest.

The optimization of the method was finally carried out using a central composite design 2^4 with eight axial points and four central points; the variables and the levels are presented in **Table 2**.

The variance analysis for the central composite design showed that the mathematical model, taking into consideration all of the variables and interactions among them, had a good correlation coefficient for cholesterol and 25-OH ($r^2 = 82$ and 86%, respectively; $p < 0.05$), demonstrating that the model was predictable and valid. The regression coefficients of the mathematical model are shown in **Table 3**.

The interactions between KOH and H_2O and between KOH and *n* were significant ($p < 0.05$) and negative for cholesterol.

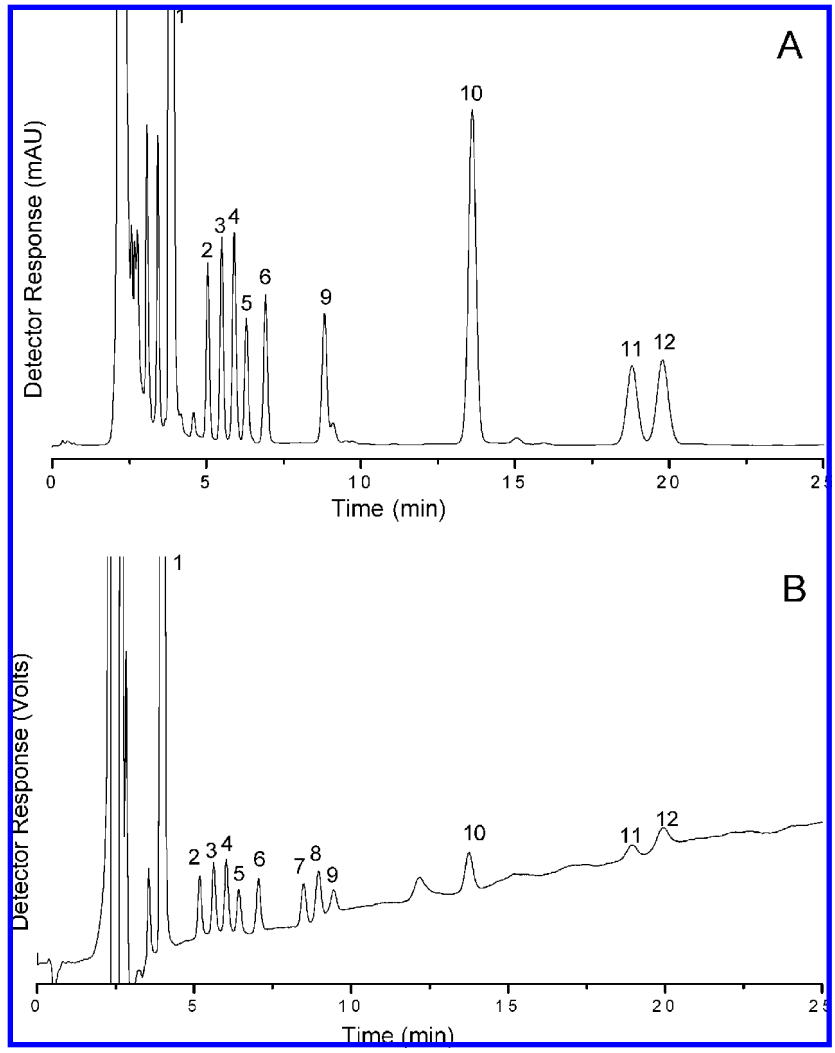

Figure 1. Chromatograms of cholesterol (peak 1) and cholesterol oxides standards: 20α-OH (2), 22R-OH (3), 24S-OH (4), 22S-OH (5), 25-OH (6), α-EP (7), β-EP (8), 25R-OH (9), 7-keto (10), 7β-OH (11), and 7α-OH (12) obtained by (**A**) UV and (**B**) RI detector.

An analysis of the response surfaces for cholesterol (available in Figure 2 of the Supporting Information) clearly shows that the levels set at the central point are optimum for cholesterol extraction. No interactions between the evaluated factors were significant ($p > 0.05$) for 25-OH. The significant ($p < 0.05$) factors for 25-OH extraction were KOH, H_2O, and n, and they all positively influence 25-OH extraction.

The optimum level for each variable was defined from the analysis of the response surfaces obtained from the mathematical models for cholesterol and 25-OH and corresponded to the levels applied to the central points. The methodology was established as follows: direct cold saponification was performed using 1 g of ground sample and 10 mL of a 20% potassium hydroxide solution in 90% aqueous ethanol, in the dark, during 20 h in a shaker at 118 rpm. After the addition of 15 mL of water, the unsaponifiable matter was extracted four times with 10 mL of hexane. The hexane layers were then washed once with 5 mL of 0.5 N KOH aqueous solution and twice with distilled water. The water phase was discarded, and the hexane phase was filtered through anhydrous sodium sulfate and washed using 10 mL of hexane. The filtrate was dried under nitrogen flush, redissolved in 1 mL of mobile phase, filtered through a 0.45 μm Millipore membrane, and injected into the HPLC. The whole procedure was performed under nitrogen atmosphere.

To validate the experimental design, the procedure described above was carried out four times using the same sample, and the relative standard deviations were 1 and 6% for cholesterol and 25-OH, respectively, demonstrating good repeatability.

Validation of the Analytical Methodology. The calibration curves of cholesterol and COP were linear with correlation coefficients between 0.997 and 0.999 (available in Table 1 of the Supporting Information). Similar results were previously obtained using a CN analytical column (*20, 21*). Recovery tests were conducted at two levels of standard addition, 50 and 100 μg of cholesterol/g of chicken, and 25 and 50 μg of COP/g of chicken. The method showed good recovery levels for all compounds (**Table 4**). Cholesterol presented recovery from 96 to 101%, whereas the COP presented recoveries from 84 to 103%. Detection limits were 0.06 μg/g for cholesterol, from 0.01 to 0.04 μg/g for COP detected by UV, and from 1.98 to 2.12 μg/g for COP detected by RI (**Table 5**), expressing adequate sensitivity of the method used for cholesterol and COP analysis. As expected, the UV detector was more sensitive than the RI detector, showing sensitivity about 100 times higher than RI detection. **Table 5** presents the limits of quantification defined as 5 times the limit of detection. No increase in any of the cholesterol oxide peaks was observed when only cholesterol was added, showing no artifact formation.

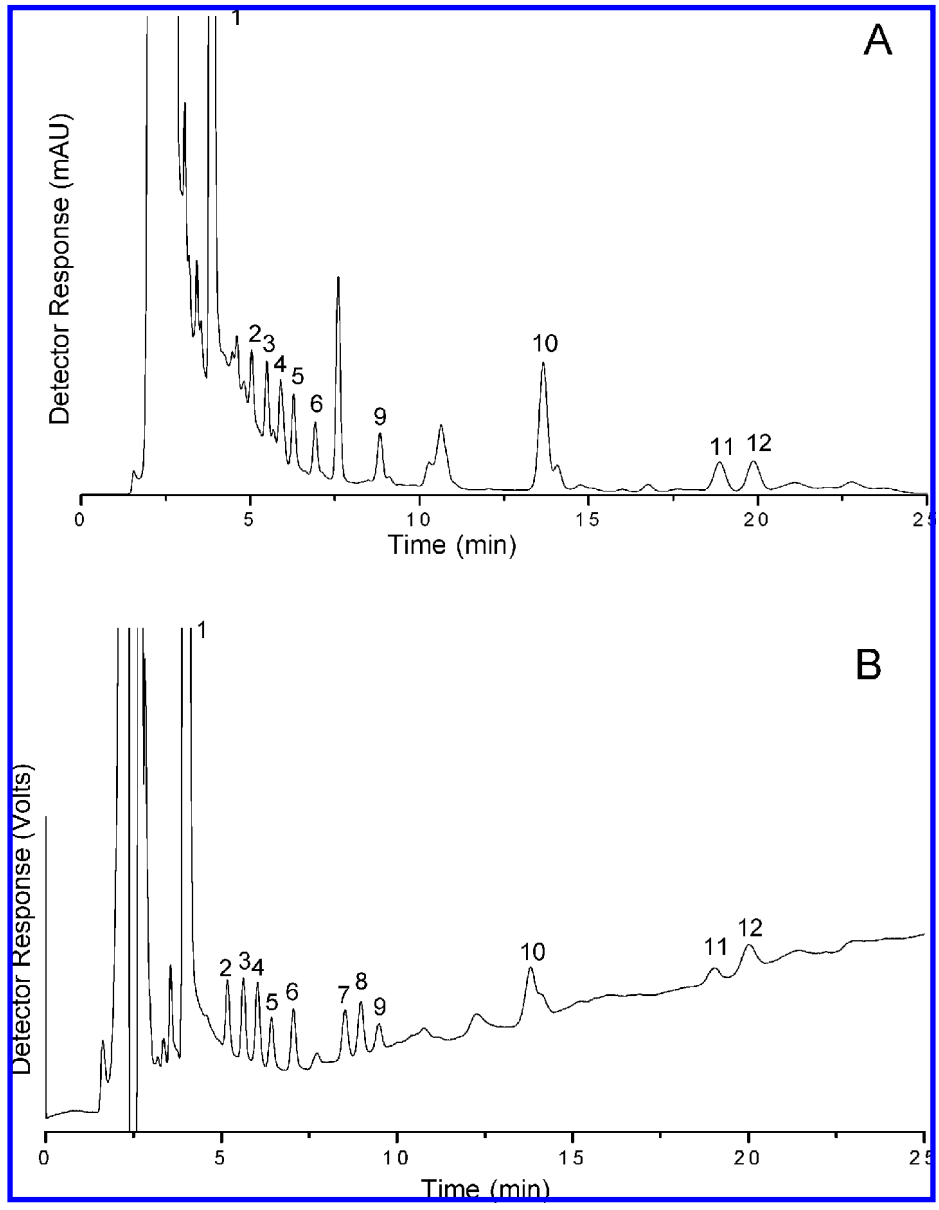

Figure 2. Chromatograms of cholesterol (peak 1) and cholesterol oxides in spiked chicken meat: 20α-OH (2), 22R-OH (3), 24S-OH (4), 22S-OH (5), 25-OH (6), α-EP (7), β-EP (8), 25R-OH (9), 7-keto (10), 7β-OH (11), and 7α-OH (12) obtained by (**A**) UV and (**B**) RI detector.

Table 2. Levels of Independent Variables Used in Central Rotational Design 2^4

variable	unit	−2	−1	0	+1	+2
w	g	0.5	0.75	1.00	1.25	1.50
KOH	%	10	15	20	25	30
H_2O	mL	5	10	15	20	25
n		2	3	4	5	6

Table 3. Regression Coefficients for the Predictive Mathematical Model of Cholesterol and 25-OH Contents[a]

	cholesterol	25-OH
mean	81.07*	25.74*
w (L)	0.01	0.37
w (Q)	−1.99*	−0.25
KOH (L)	0.25	3.35*
KOH (Q)	−4.32*	−1.81
H_2O (L)	1.34*	4.25*
H_2O (Q)	−2.52*	−1.76
n (L)	−0.21	6.52*
n (Q)	−2.12*	0.71
w × KOH	0.39	0.86
w × H_2O	−0.36	−0.45
w × *n*	0.37	−1.10
KOH × H_2O	−3.47*	0.50
KOH × *n*	−1.56*	0.40
H_2O × *n*	−0.32	0.68

[a] *, statistically significant ($p < 0.05$). L, linear; Q, quadratic.

Incidence of COP and Alterations in Cholesterol Content in Chicken Breast Cooked under Different Conditions. The chicken breasts were submitted to different cooking processes and analyzed for moisture, fat, cholesterol, and COP contents (**Table 6**). The presence of cholesterol oxides was confirmed by mass spectrometry.

An increase in the cholesterol and fat contents (**Table 6**) was verified in all cooked chicken regardless of the used method, and this increase could be explained by the loss of water during the cooking procedure. The cholesterol contents in the analyzed raw and cooked samples are similar to that reported in the literature (*10, 13, 15*); however, lower cholesterol values, from 21.7 to 55.7 mg/100 g, were found in raw chicken meat (*17*).

Table 4. Recovery of Cholesterol and Cholesterol Oxides in Grilled Chicken Breast[a]

compound[b]	level of addition (μg)	recovery (%)	RSD (%)
cholesterol	50	101	4
	100	96	4
20α-OH	25	89	4
	50	84	4
22R-OH	25	93	4
	50	90	2
24S-OH	25	87	4
	50	87	5
22S-OH	25	101	2
	50	97	5
25-OH	25	98	2
	50	91	4
α-EP	25	96	4
	50	100	2
β-EP	25	96	3
	50	98	4
25R-OH	25	99	4
	50	103	2
7-keto	25	84	3
	50	98	3
7β-OH	25	92	3
	50	102	2
7α-OH	25	87	1
	50	100	1

[a] Means and relative standard deviations (RSD) of 10 analyses. [b] Compounds are presented according to elution order.

Table 5. Detection and Quantification Limits for Cholesterol and Cholesterol Oxides

compound[a]	detector	DL[b] (μg/g)	QL[c] (μg/g)
cholesterol	UV	0.06	0.32
20α-OH	UV	0.03	0.16
22R-OH	UV	0.03	0.14
24S-OH	UV	0.03	0.13
22S-OH	UV	0.04	0.20
25-OH	UV	0.03	0.17
α-EP	RI	2.12	10.62
β-EP	RI	1.98	9.88
25R-OH	UV	0.03	0.15
7-keto	UV	0.01	0.04
7β-OH	UV	0.02	0.09
7α-OH	UV	0.02	0.11

[a] Compounds are listed according to elution order. [b] Detection limit. [c] Quantification limit, QL = 5 × DL.

This is the first time that the COP 22R-OH, 24S-OH, and 22S-OH were detected in chicken, although they have been previously verified in fish (*21, 26–28*). The oxides 22R-OH, 24S-OH, and 22S-OH were found in R. All of them are derived from the lateral chain oxidation of the cholesterol and apparently formed enzymatically, probably by animal metabolism (*6*). From these, 22R-OH and 24S-OH were also present in all of the cooked samples. 25-OH and β-EP were found just in BO, which was processed using the lowest temperature (90 °C). 22R-OH was the most abundant COP in all of the chicken samples, corresponding to 53−87% of total COP.

The total COP content observed in this study was higher than other values reported in the literature for raw chicken (0.1−1.5 μg/g) and for thermally processed chicken (maximum of 5 μg/g) (*10–17*). Besides, disregarding the COP 22R-OH, 24S-OH, and 22S-OH, the total COP content for R and DF are in agreement with the literature values for chicken meat, although the total COP for RO, BO, and ER are still higher. A large number of different COP were analyzed in this research, so it was expected that a higher total COP would be found; these new COP (22R-OH, 24S-OH, and 22S-OH) could be also present in the samples reported in the literature, but they probably were not detected. The oxides 20α-OH and α-EP, commonly present in foods, were not detected in any of the samples; also 25R-OH was not detected. Baggio and Bragagnolo (*18*) analyzed seven types of chicken products and did not detect COP in any of the samples, probably due to the use of the antioxidant sodium erythorbate in the product formulations.

Considering the extent of cholesterol oxidation, the COP/cholesterol ratio presented values from 1.7 to 5.2%, which were similar or slightly higher than the values shown by some authors (*17*). On the other hand, Maraschiello et al. (*10*) found lower values for total COP/cholesterol ratio, varying from 0.003 to 0.10% for raw chicken and from 0.11 to 0.59% for cooked chicken. BO showed the greatest number of COP, the highest total COP content, and consequently the highest COP/cholesterol ratio. By contrast, DF chicken presented the lowest total COP content among the processed samples and also the lowest COP/cholesterol ratio among all analyzed samples. The high temperature of the deep-frying process could cause thermodegradation of COP, possibly producing new compounds other than oxides, by combination with other molecules such as Maillard reaction products and proteins (*29*); moreover, a small amount of COP could have migrated from the meat to the frying oil (*17*).

In summary, the experimental design proved to be a useful tool for method development and optimization employing a small number of experiments. Optimization of the saponification

Table 6. Cholesterol Oxides (Micrograms per Gram), Cholesterol (Milligrams per 100 g), Moisture (Grams per 100 g), and Fat (Grams per 100 g) Contents and Cholesterol Oxidation (Percent) in Chicken Breast[a]

	raw (R)		roasted (RO)		deep fried (DF)		boiled in water (BO)		roasted in electric oven (ER)	
22R-OH	13.5 c	(5.1)	12.5 c	(0.3)	14.6 c	(3.9)	22.3 a	(4.3)	19.3 b	(1.4)
24S-OH	1.5 b	(0.7)	0.8 d	(1.3)	1.7 a	(0.6)	1.5 b	(0.7)	0.6 c	(1.8)
22S-OH	2.3 b	(1.0)	0.5 d	(0.2)	nd a		nd a		0.9 d	(5.8)
25-OH	nd a		nd a		nd a		4.4 b	(3.9)	nd a	
β-EP	nd		nd		nd		<9.8		nd	
7-keto	nd a		0.4 d	(0.5)	0.5 c	(2.2)	1.0 b	(2.0)	0.4 d	(7.1)
7β-OH	nd a		3.4 c	(6.2)	nd a		4.0 b	(4.0)	3.7 bc	(2.2)
7α-OH	nd a		9.5 c	(0.8)	nd a		6.4 d	(5.0)	11.2 b	(1.9)
total COP	17.3 e	(2.6)	27.1 c	(1.0)	16.8 d	(3.2)	39.6 a	(2.2)	36.1 b	(1.6)
cholesterol	54.7 d	(1.3)	81.2 b	(1.1)	98.5 a	(0.4)	78.34 c	(0.2)	88.4 b	(0.1)
moisture	76.3 a	(0.4)	61.8 c	(0.5)	54.9 d	(0.9)	68.4 b	(0.2)	67.4 b	(0.6)
fat	1.1 d	(3.5)	2.2 b	(3.9)	5.7 a	(2.9)	1.6 c	(2.2)	2.8 b	(1.2)
cholesterol oxidation[b]	3.2 c	(3.8)	3.3 c	(2.1)	1.7 d	(3.6)	5.2 a	(2.0)	4.1 b	(1.5)

[a] Means and relative standard deviations (RSD, parentheses) of duplicate determinations of the same sample. [b] Cholesterol oxidation as total COP × 100/cholesterol content. Different letters in the same line mean that there are statistically significant differences ($p < 0.05$) among the samples. nd, not detected; for detection and quantification limits, see **Table 5**. The oxides 20α-OH, 25R-OH, and α-EP were not detected.

and extraction procedures resulted in a novel reliable method for the simultantaneous detection of cholesterol and COP in chicken meat with no artifact formation. The presence of a large number of COP in chicken breast could be easily confirmed by using HPLC-APCI-MS, leading to total COP contents higher than those previously found in the literature. The COP intake provided by meat can be underestimated, and more studies on this subject are necessary to estimate the real COP intake, as well as to investigate the toxicological role of these new COP found in human health.

Supporting Information Available: MS spectra of cholesterol and cholesterol oxides, response surfaces of the predictive model, and calibration curves and correlation coefficients for cholesterol and cholesterol oxides. This material is available free of charge via the Internet at http://pubs.acs.org.

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