

Development of a Reliable Analytical Protocol for the Isolation of Cholesterol Oxidation Products—A Comparison of Different Lipid Extraction and Saponification Methods

Michalis S. Constantinou · Christiana A. Georgiou ·
Constantina P. Kapnissi-Christodoulou

Received: 28 August 2014 / Accepted: 23 October 2014 / Published online: 5 November 2014
© Springer Science+Business Media New York 2014

Abstract The main objectives of this study were (i) the optimization and the validation of a high performance liquid chromatographic method for the simultaneous determination of seven cholesterol oxidation products (COPs), (ii) the development of a reliable analytical protocol for their selective isolation from food samples, and (iii) the application to several foodstuffs. Baseline separation was achieved using a cyano-bonded high-performance liquid chromatography (HPLC) column and a mobile phase of *n*-hexane/2-propanol/acetone (97:1.5:1.5, v/v). The criteria studied for the choice of the optimum extraction procedure were the selectivity towards COPs and its efficiency in removing matrix interferences. Special attention was given to the stability of 7-keto under saponification conditions. The ideal procedure involved extraction of lipids from the food matrix with chloroform/methanol (2:1, v/v), alkaline hydrolysis with 1 M potassium hydroxide (KOH) solution in 60 % aqueous ethanol and subsequent enrichment of COPs with solid phase extraction using an NH₂ column. COPs presented recoveries from 76 to 103 %.

Keywords Cholesterol oxidation products · Lipid extraction · Sample preparation · Saponification · Solid phase extraction

Introduction

In the context of cholesterol oxidation products (COPs) analysis, sample preparation is considered to be the most important and challenging step for numerous reasons. Except from

exhaustive isolation, the analytical protocol must be capable to provide an interference-free COPs fraction with minimal artifact generation prior to chromatographic determination and quantification. Despite the fact that numerous methods have been proposed, no method has, so far, been established as a routine method for the analysis of COPs in several foodstuffs (Georgiou et al. 2014).

The methods used, over the years, in COPs analysis, follow three possible routes: lipid extraction or direct saponification with subsequent solid-phase extraction (SPE) or saponification of the lipid extract and purification of the unsaponifiables (consisting COPs) followed by SPE (Guardiola et al. 2002; Sieber 2005). It is generally accepted that the two latter combinations have found widespread application in relation to the former. This clearly demonstrates that saponification is the most powerful purification tool in COPs analysis, as it can eliminate the interfering substances to a satisfactory extent, ensuring their selective isolation from the bulk lipids.

Nevertheless, it should be noted that saponification procedure is considered to be the number one cause of artifact formation. More specifically, much of the research regarding the stability of COPs under saponification conditions has focused on the most abundant but vulnerable COP in foods, 7-keto (Busch and King 2009a, b). It is well known that 7-keto is dehydrated to cholesta-3,5-diene-7-one in a strong alkaline solution and at high incubation temperatures (Busch and King 2010; Park et al. 1996). Even though, in current protocols, saponification is entirely conducted in ambient temperature in order to minimize 7-keto degradation; some analysts still support the negligible contribution of extreme alkali conditions to the degradation of 7-keto (Saldanha et al. 2006; Mariutti et al. 2008).

Except from selective isolation and minimal artifact generation, the proposed method should also provide maximum recoveries of the target analytes. Recovery studies are often omitted in several publications, leading to erroneous

M. S. Constantinou · C. A. Georgiou ·
C. P. Kapnissi-Christodoulou (✉)
Department of Chemistry, University of Cyprus, 1678 Nicosia,
Cyprus
e-mail: ckapni1@ucy.ac.cy

quantitative results. Thereby, the goal of this study was to investigate, further, the influence of saponification on 7-keto and determine the most reliable strategy for isolation of COPs, based on time, adequate removal of impurities, recovery, reproducibility, and artifact generation. This objective was achieved by comparing some of the most recent and most used saponification and lipid extraction procedures. To the best of our knowledge, no comparisons have yet been made among the most recent sample preparation methods.

Regarding chromatographic analysis of COPs, GC-MS has become the preferred analytical method since it provides efficient resolution. However, the use of high temperatures and the need for derivatization can contribute to artifact formation and increased time of analysis, respectively (Chen et al. 2012; Vicente et al. 2012). As a consequence, high-performance liquid chromatography (HPLC) is considered as the alternative method to GC. Although it is a faster and a milder technique, it suffers from poor baseline separation of isomeric COPs (Raith et al. 2005). Frequently, quantitative results of the enantiomeric COPs are reported as the sum of the both forms due to inability of separation by HPLC (Georgiou and Kapnissi-Christodoulou 2013).

Despite the fact that both normal- and reversed-phase modes have been used in COPs analysis, the former has demonstrated enantiomeric selectivity (Saldanha et al. 2006; Baggio et al. 2005). Some studies regarding the adverse effects of COPs on human health have demonstrated, unlike 7 α , positive correlation between 7 β and atherogenesis, lung cancer, and apoptosis, thus, indicating that enantiomeric separation is very important for the monitoring of COPs in foodstuffs (Brown and Jessup 1999; Linseisen et al. 2002; Ryan et al. 2005; Otaegui-Arazola et al. 2010). Based on the above, another objective of this study was the development of a stable and a reliable HPLC-UV method for the separation of seven COPs, including two enantiomeric pairs.

Materials and Methods

Materials COPs standards, 20 α -hydroxycholesterol (20 α -OH), 22R-hydroxycholesterol (22R-OH), 25-hydroxycholesterol (25-OH), 7 α -hydroxycholesterol (7 α -OH), and 7 β -hydroxycholesterol (7 β -OH) were purchased from Steraloids (Newport, RI); 22S-hydroxycholesterol (22S-OH) and 7-ketocholesterol (7-keto) were acquired from Sigma (Milford, MA). The HPLC-grade solvents, *n*-hexane, methanol, ethanol, 2-propanol, chloroform, and ethyl acetate were purchased from Merck (Darmstadt, Germany), while acetone was obtained from Sharlau (Sentmenat, Spain). The HPLC solvents were filtered through a 0.45- μ m membrane filter Millipore (Cork, Ireland) under vacuum prior to use. The reagents, potassium hydroxide (KOH), sodium chloride (NaCl), and sodium

sulfate (Na₂SO₄) were purchased from Merck, Sharlau, and Sigma, respectively. A Visiprep SPE Vacuum Manifold equipped with NH₂-SPE columns (3 ml, 500 mg) from Supelco (Bellefonte, PA, USA) was used for COPs purification.

Instruments A Shimadzu (Kyoto, Japan) liquid chromatograph was equipped with a PDA detector (SPD-M20A), a solvent delivery module (LC-20 AD), a degassing system (DGU-20A₅), an autosampler (SIL-20AHT), and a column oven (CTO-10ASVP). A Nova Pak CN HP column (300 \times 3.9 mm I.D., particle size 4 μ m) supplied with a Hypersil BDS CN guard column (7.5 \times 4.6 mm I.D., particle size 5 μ m) was purchased from Waters (Milford, MA, USA), and it was used to separate the examined COPs. For the determination of the COPs, chromatograms were obtained at 206 nm, except from 7-keto, which was quantified at 230 nm due to its maximum absorbance. Identification of COPs was made by comparing the retention times of the reference standards peaks with those of the real samples.

Normal-phase chromatography was selected for the separation of the examined COPs, as they differ from each other in the position of their polar group. According to the literature, cyano column seems to be capable of separating these isomers. Saldanha et al. were able to separate several COPs by using hexane/2-propanol (97:3, v/v) as a mobile phase at a flow rate of 1 ml/min and a temperature of 32 °C (Saldanha et al. 2006). Nevertheless, baseline separation between 7 α -OH and 7 β -OH was not achieved since the *R_s* was less than 1.5. On the contrary, Mariutti et al., two years later, under the same chromatographic conditions, reported baseline separation of COPs including the 7-OH isomers (Mariutti et al. 2008). However, in a more recent study, their coworkers changed the composition of the mobile phase by replacing *n*-hexane with a mixture of hexanes (minimum 63 % of *n*-hexane), indicating probably the instability of the chromatographic method (Noguiera et al. 2010). Thereby, it was considered important to develop a more stable and a more reproducible NP-HPLC method. Hexane was selected as the main solvent of the mobile phase. Taking into account the miscibility tables, hexane was examined in different proportions with 2-propanol, acetone, and ethanol. The concentration of the COPs in the mixture was 0.014 mg/ml; the injection volume was set to 20 μ L and the temperature at 25 °C.

Sample Preparation As mentioned before, extensive sample preparation must be carried out in order to isolate selectively the COPs from the complex lipid matrix. On the other hand, it is generally accepted that any additional operations introduced into the analytical protocol can contribute to a loss of recovery. Therefore, a minimum number of steps must be applied before quantitative determination. In addition, having also in mind that saponification is the most efficient procedure for bulk

lipids elimination, direct saponification followed by SPE was first studied due to simplicity and rapidness.

Direct Saponification Procedures Three slightly modified direct saponification procedures, as well as a fourth one derived from the combination of two, were compared in order to determine the most optimum method, mainly based on recovery. The main differences among the four methods were based on the composition of the saponification solvent *X*, the amount of water *Y* added after saponification, and the extraction solution *Z*.

One gram of sample, spiked with 20 µg of each COP, underwent saponification at room temperature, in a screw cap bottle, covered with aluminum foil, with 10 ml of 3.6 M KOH solution *X* for 18 h. After saponification, a volume of *Y* ml of water was added, and the unsaponifiables were extracted four times with 10 ml of solution *Z* (Mariutti et al.: *X*=ethanol/water (90:10, v/v), *Y*=15 ml, *Z*=hexane; Saldanha et al.: *X*=ethanol/water (60:40, v/v), *Y*=5 ml, *Z*=hexane; Mariutti-Saldanha et al.: *X*=ethanol/water (60:40, v/v), *Y*=15 ml, *Z*=hexane; Ubhayasekera et al.: *X*=7-ml ethanol/water (60:40, v/v) and 3-ml dichloromethane, *Y*=10 ml, *Z*=dichloromethane) (Saldanha et al. 2006; Mariutti et al. 2008; Ubhayasekera et al. 2004). The organic phase was filtrated through a filter paper with 0.5-g anhydrous Na₂SO₄ for moisture removal. After washing the filter with 10 ml of solution *Z*, the organic phase was evaporated in a rotary evaporator at 40 °C.

For further purification of COPs, Rose-Sallin's SPE method was applied (Rose-Sallin et al. 1995). The solid residue was dissolved in hexane/ethylacetate (95:5, v/v) and loaded into an NH₂ column, which was preactivated with 3 ml of hexane. Apolar lipids and cholesterol were eliminated with 6-ml hexane/ethylacetate (95:5, v/v) and 10-ml hexane/ethylacetate (90:10, v/v), respectively. COPs were finally eluted with 10-ml acetone, evaporated under N₂ steam, redissolved in hexane/2-propanol (97:3, v/v), and injected into the HPLC system.

Lipid Extraction Procedures The optimum saponification procedure determined by comparing the four methods mentioned above was also applied on a lipid extract with milder alkaline conditions. In this case, the optimum lipid extraction procedure was determined by comparing three methods: the "Folch," the "Lee," and the "Boselli" methods. The first is the most commonly used method, while the other two are modifications of the first. These three lipid extraction methods, which differ mainly in the purification of lipids from polar contaminants, are described below.

Folch Method: One or 5 g of sample were homogenized with 90 ml of chloroform/methanol (2:1, v/v) for 3 min. The blender was rinsed with an extra 15-ml lipid extraction solvent and filtrated through a filter paper with 0.5-g anhydrous Na₂SO₄.

The filter paper was washed with 15-ml chloroform/methanol (2:1, v/v), and the filtrate volume was readjusted to 120 ml before being transferred to a 500-ml screw cap bottle. Then, a 30-ml NaCl solution (0.88 %, w/w) was added in order to obtain a ratio of 8:4:3 v/v (chloroform/methanol/water). The mixture was stirred and left overnight at 4 °C for phase separation. The lower organic phase was dried with anhydrous Na₂SO₄ and evaporated in a rotary evaporator. A 250-mg lipid extract was subjected to saponification and NH₂-SPE (Folch et al. 1957).

Boselli Method: Five grams of sample were homogenized with 200 ml of chloroform/methanol (1:1, v/v) for 3 min. The blender was transferred to a 250-ml screw cap bottle, and then, it was placed for 20 min in a water bath at a constant temperature of 60°. After further homogenization with 100 ml of chloroform for 3 min, the mixture was filtrated through a filter paper with 0.5-g anhydrous Na₂SO₄. The filtrate was transferred into a 500-ml bottle, where 100 ml of NaCl solution (1 M) was added. The mixture was stirred and left overnight at 4 °C for phase separation. The lower organic phase was dried with anhydrous Na₂SO₄ and evaporated in a rotary evaporator. A 250-mg lipid extract was subjected to saponification and NH₂-SPE (Boselli et al. 2005).

Lee Method: Five grams of sample were homogenized with 90 ml of chloroform/methanol (2:1, v/v) for 3 min. The blender was rinsed with an extra 15-ml lipid extraction solvent and stirred for 30 min. Then, the mixture was filtrated through a filter paper with 0.5-g anhydrous Na₂SO₄ and evaporated in a rotary evaporator. The filter paper was washed with 15-ml chloroform/methanol (2:1, v/v). The resulted oily liquid was dissolved in 10 ml of hexane and filtrated through a membrane filter (0.45 µm), which had been previously activated with methanol. The solution was then transferred into a 25-ml bottle and evaporated under N₂ flow. The resulted solid was subjected to saponification and NH₂-SPE (Lee et al. 2006).

Results and Discussion

Method Development The first objective of this research project was to optimize the chromatographic parameters of a HPLC method that will allow the simultaneous separation of seven COPs. Initially, the mobile phase hexane/2-propanol (97:3, v/v) was examined, which, according to Mariutti et al., provided baseline separation. However, the separation of the two 7-OH isomers could not be achieved under these chromatographic conditions (Rs: 0.8). Neither a decrease in the flow rate from 1.0 to 0.8 ml/min, nor a reduction in temperature, could affect the resolution; thus, the optimum chromatographic conditions were determined by evaluating

several parameters, particularly the composition of the mobile phase, the flow rate, and the column temperature.

Hexane was chosen as the main solvent in the mobile phase, and based on the miscibility tables, 2-propanol, acetone, and ethanol were chosen as additives. Different combinations and percentages of the solvents were examined, and the optimum one, in regard to resolution, proved to be hexane/2-propanol/acetone (97:2:1, v/v). The presence of acetone in the mobile phase reduced the polarity of the mobile phase, which, in turn, increased the resolution of the two isomer 7-OH peaks (R_s : 1.4). Due to the long analysis time (35 min), though, it was considered important to substitute 2-propanol with ethanol. Although the latter decreased the analysis time by 13.5 min, the resolutions of the peaks that correspond to 22S-OH and 25-OH and the two 7-OH isomers decreased to 1.0, in both cases. A decrease in the flow rate from 1 to 0.6 ml/min was able to increase the resolution of the isomers (R_s : 1.7), while the effect on the resolution of the first pair was insignificant. In another attempt to optimize the separation, in regard to resolution and analysis time, the percentage of acetone in the mobile phase and the flow rate were increased to 1.5 % [hexane/2-propanol/acetone (97:1.5:1.5, v/v)] and 1.5 ml/min, respectively. These chromatographic parameters provided a baseline separation of the seven COPs with resolution values of $R_{s3,4}$: 1.8 and $R_{s6,7}$: 1.5 (Fig. 1).

Method Validation The reliability of the optimum chromatographic method was evaluated in terms of linearity, limit of detection (LOD), limit of quantification (LOQ), and precision. The standards were diluted in hexane:2-propanol (96:4, v/v) at a final concentration of 0.1 mg/ml. The calibration curves were constructed by plotting the peak areas of the COPs against their concentrations, which were in the range of 0.5–32 $\mu\text{g/ml}$ for 20 α -OH, 22R-OH, and 25-OH, and 1–32 $\mu\text{g/ml}$ for 7 α -OH, 7 β -OH, 22S-OH, and 7-keto. The quantitation of COPs was performed at 206 nm, except from 7-keto, whose quantitation was performed at 230 nm. All seven analytes demonstrated a good linearity response in the range of concentrations investigated, with correlation coefficients higher than 0.99.

The LODs and LOQs were calculated as three and ten times the standard deviation, respectively, via the slope of the calibration curve. The LOD and LOQ values for the seven COPs were in the range 0.15–0.63 $\mu\text{g/ml}$ and 0.45–1.91 $\mu\text{g/ml}$, respectively.

The precision of the chromatographic method was evaluated by calculating the relative standard deviations (RSDs) of the migration times of all seven analytes and their corresponding peak areas. The intra-day precision was obtained from five replicate analyses performed within the same day, and the inter-day precision was obtained by use of twelve runs (three

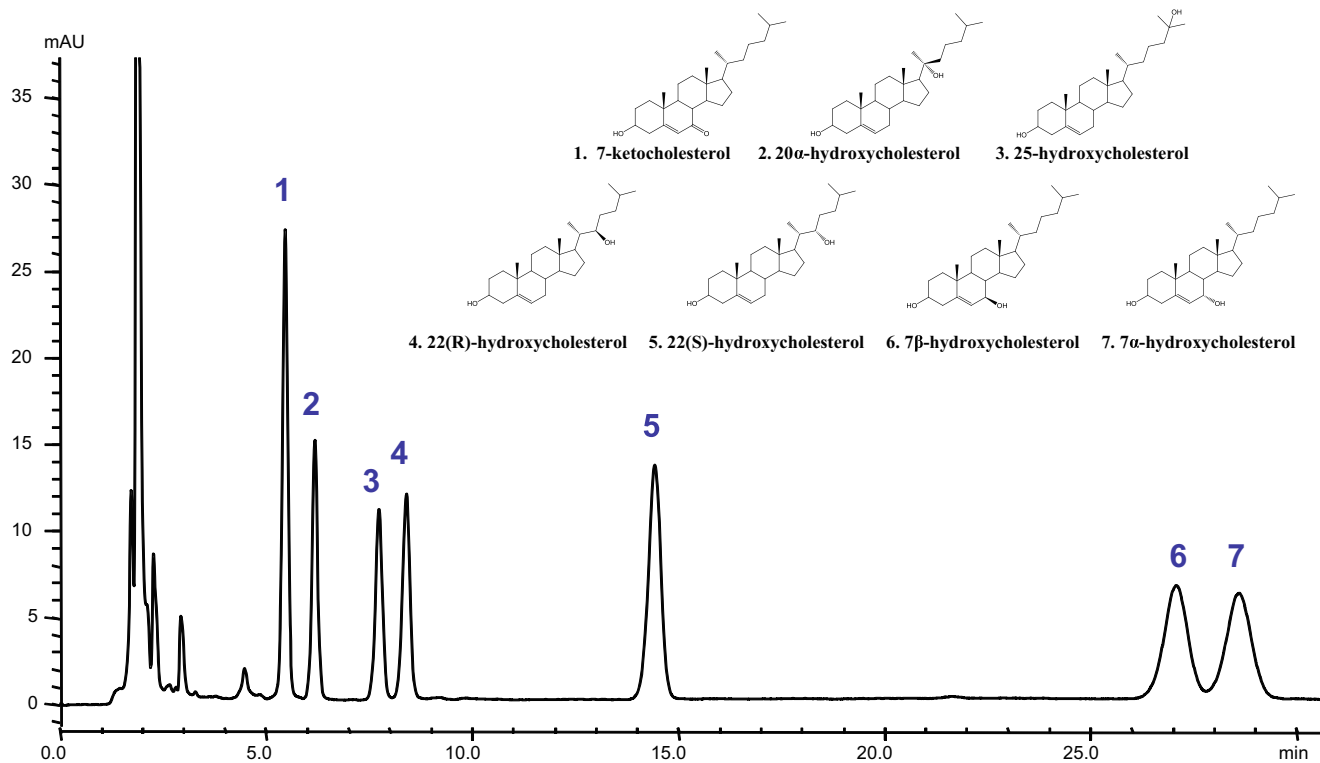


Fig. 1 Optimum chromatographic separation of the examined COPs. Baseline separation was achieved by the use of *n*-hexane:2-propanol:acetone (97:1.5:1.5, v/v) at a flow rate of 1 ml/min and a

temperature of 25 °C. (1) 20 α -OH, (2) 22R-OH, (3) 22S-OH, (4) 25-OH, (5) 7-keto, (6) 7 β -OH, (7) 7 α -OH

replicate analyses performed on four consecutive days). In the first case, the RSD values of the migration times and the peak areas of all seven analytes were below 2 %, indicating very good run-to-run reproducibilities. However, the inter-day precision, in regard to peak area, was not very satisfactory, since the RSD values were varied from 6.90 to 8.55 %. This is probably due to the instability of the analyte-standard solutions. Even though they were stored at 4 °C, evaporation was observed, which, in turn, caused an increase in the peak areas along with time.

Optimization of Sample Preparation Optimization of saponification method. As mentioned earlier, the primary objective of this work is the development of a reliable analytical protocol for the selective isolation of COPs from food samples. Therefore, a comparison among available direct saponification methods was performed. The method of Mariutti, which involves a saponification solution of ethanol/water (90:10, v/v), 15-ml H₂O, and hexane, was first examined. The method was applied in both a spiked and a blank “lountza” sample. Table 1 demonstrates the results from the recovery studies for each COP, in comparison with the relative findings of Mariutti and his coworkers for a 25- μ L spiked sample. As shown in Table 1, the agreement between the reference and the experimental values was excellent for almost all COPs, with the exception of 7-keto, whose recovery value was smaller (52 %) in comparison to the one reported by Mariutti et al. (84 %). Similar results for most of the analytes were obtained, when Saldanha’s method (ethanol/water (60:40, v/v), 5-ml H₂O) was applied. The latter, as well as the coupling of the two methods, “Mariutti-Saldanha” (ethanol/water (60:40, v/v), 15-ml H₂O), demonstrated higher selectivity to 7-keto (recovery values 63 and 67 %, respectively). Another important observation involves the decrease in the recovery values of 7-OH isomers, when the combined method was applied (75 % for 7 α -OH and 75 % for 7 β -OH). The latter observation indicates the influence of the saponification’s solvent composition on recovery.

Both Mariutti and Saldanha methods provided high recovery values for 7-keto, and their findings are in conflict with the majority of the published studies, which support that 7-keto degrades to cholesta-3,5-diene-7-one under strongly alkaline conditions. This created the need to further study the influence of KOH concentration on 7-keto recovery. It is worth here to mention that, in order to exclude the nature of the saponification solvent as the cause for the low recovery of 7-keto, a completely different saponification method, according to Ubhayasekera ($X=7$ -ml ethanol/water (60:40, v/v) and 3-ml dichloromethane, $Y=10$ ml, Z =dichloromethane), was applied. As expected, the recovery of 7-keto was 59 %, which strengthened the suspicion for its degradation.

The study on the degradation of 7-keto in alkaline conditions was performed by using the Saldanha method, because it was considered as the optimum saponification method, according to recovery and interference. In particular, as illustrated in Fig. 2 (i), the Saldanha method provided a free of interference COPs fraction. The unknown substances that eluted at 7 min and 11.5 min did not affect the qualitative and quantitative analysis of COPs. On the contrary, the use of the other two methods provided several impurities, which coeluted with the target analytes, and, in turn, contributed to quantitative errors (Fig. 2 (ii),(iii)). In brief, in the case of the Mariutti method, the impurities contribute to a positive calculation of the recovery of 22S-OH (Fig. 2 (ii)). In addition, the Ubhayasekera method was found to be the most inappropriate for the removal of impurities because, as illustrated from the chromatogram of a blank sample in Fig. 2 (iii), the impurities coeluted with the 7-OH and 22-OH isomers.

A two-fold 7-keto stability experiment was designed in order to elucidate whether the concentration of KOH affects the degradation of 7-keto. In the first part, the stability of 7-keto was studied under the strong alkaline conditions of direct saponification, 3.6 M KOH, versus the incubation time, and particularly for 0, 2, 10, and 18 h. The Saldanha saponification conditions were applied, and the experiments were performed in solution without the presence of a matrix. For comparison

Table 1 Recoveries (record) of COPs after the application of different direct saponification methods and different lipid extraction methods, in combination with the optimum saponification method, in spiked lountza samples

COPs	Mariutti bibliography record	Mariutti experimental record	Saldanha	Mariutti-Saldanha	Lee-Saldanha	Boselli-Saldanha	Folch-Saldanha
20 α -OH	89 %	89 %	85 %	93 %	92 %	78 %	102 %
22R-OH	93 %	92 %	94 %	93 %	101 %	87 %	91 %
22S-OH	101 %	106 %	88 %	105 %	88 %	78 %	103 %
25-OH	98 %	88 %	88 %	94 %	88 %	65 %	98 %
7-keto	84 %	52 %	63 %	67 %	78 %	63 %	76 %
7 β -OH	92 %	91 %	95 %	75 %	93 %	46 %	83 %
7 α -OH	87 %	85 %	95 %	74 %	87 %	52 %	80 %

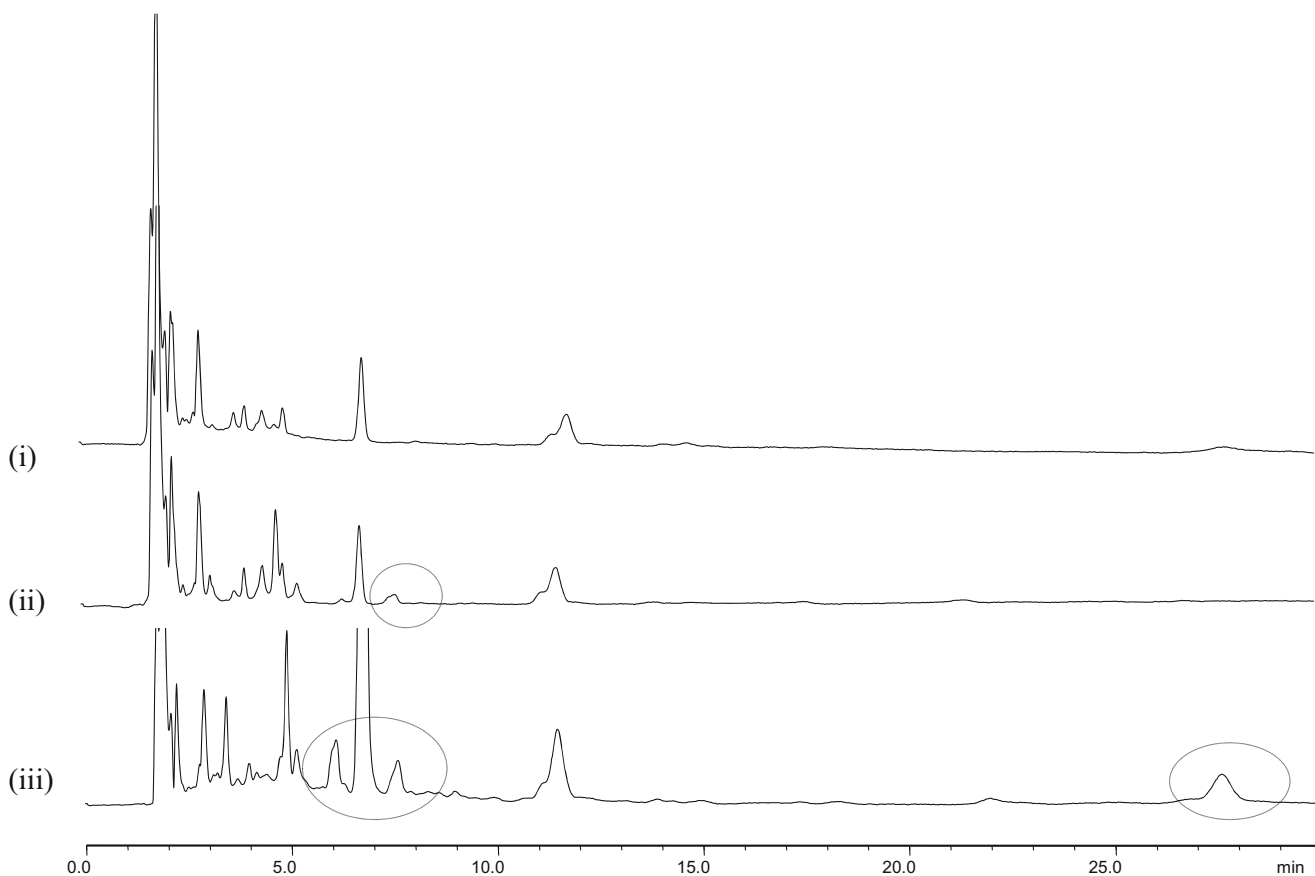


Fig. 2 Chromatograph of blank lountza samples at 206 nm which were treated according to the method of **a** Saldanha, **b** Mariutti, and **c** Ubhayasekera. In circle, the contaminations that led to quantitative errors

purposes, the saponification solution was spiked with 7-keto (20 μg) and 20 α -OH (11.5 μg). As shown in Fig. 3a, when the incubation time increased, the recovery of 7-keto decreased, while the recovery of 20 α -OH stayed unaffected. As mentioned earlier, 7-keto is converted to cholesta-3,5-dien-7-one in an alkaline environment. The latter is a conjugated triene, which absorbs strongly at 270–280 nm.

The fractions were also analyzed by use of a UV–vis system in order to further confirm the formation of cholesta-3,5-dien-7-one. As demonstrated in Fig. 3b, the intensity of the peak at 275 nm increased, while the intensity of the peak at 230 nm decreased, with an increase in the saponification time. This variation clearly demonstrates the conversion of 7-keto to cholesta-3,5-dien-7-one. Therefore, the choice of direct saponification under strong alkaline conditions (3.6 M KOH) was rejected.

The saponification technique though appears to be a necessary step in the sample preparation procedure because it provides clear chromatograms. Therefore, in an attempt to increase the 7-keto recovery, milder saponification conditions were examined, and particularly, 1 M and 2 M KOH. As illustrated in Fig. 3c, the use of a 1 M KOH solution resulted in a significant increase on the recovery rate by 21 and 11 %, in comparison to the amount that was recovered when 3.6 and

2 M KOH solutions were used, respectively. In a similar study performed by Busch and coworkers, it was reported an increase of 29 % (Busch and King 2009a, b). It should also be noted that the recovery of 7-keto, after its solution saponification for 18 h in strong alkaline conditions (3.6 M KOH), was 6 % higher than the recovery obtained when the Saldanha method was used in a spiked lountza sample (Table 1). This suggests that the presence of a matrix may interfere, to some extent, to the recovery of COPs.

Optimization of Lipid Extraction Procedure The optimum saponification conditions determined cannot be applied directly to the sample matrix because the concentration of 1 M KOH cannot dissolve the sample completely. Therefore, it was considered necessary to apply a lipid extraction method before saponification. The optimum method was determined by comparing three different lipid extraction procedures, which were described earlier, and by applying the optimum saponification method along with NH_2 -SPE. The performance of the three lipid extraction methods was evaluated by using the terms of simplicity, rapidness, recovery, and selectivity.

Initially, the method according to Lee and his coworkers was examined because it requires less time than the methods reported by Folch and Boselli. In this case, 1 g of lountza

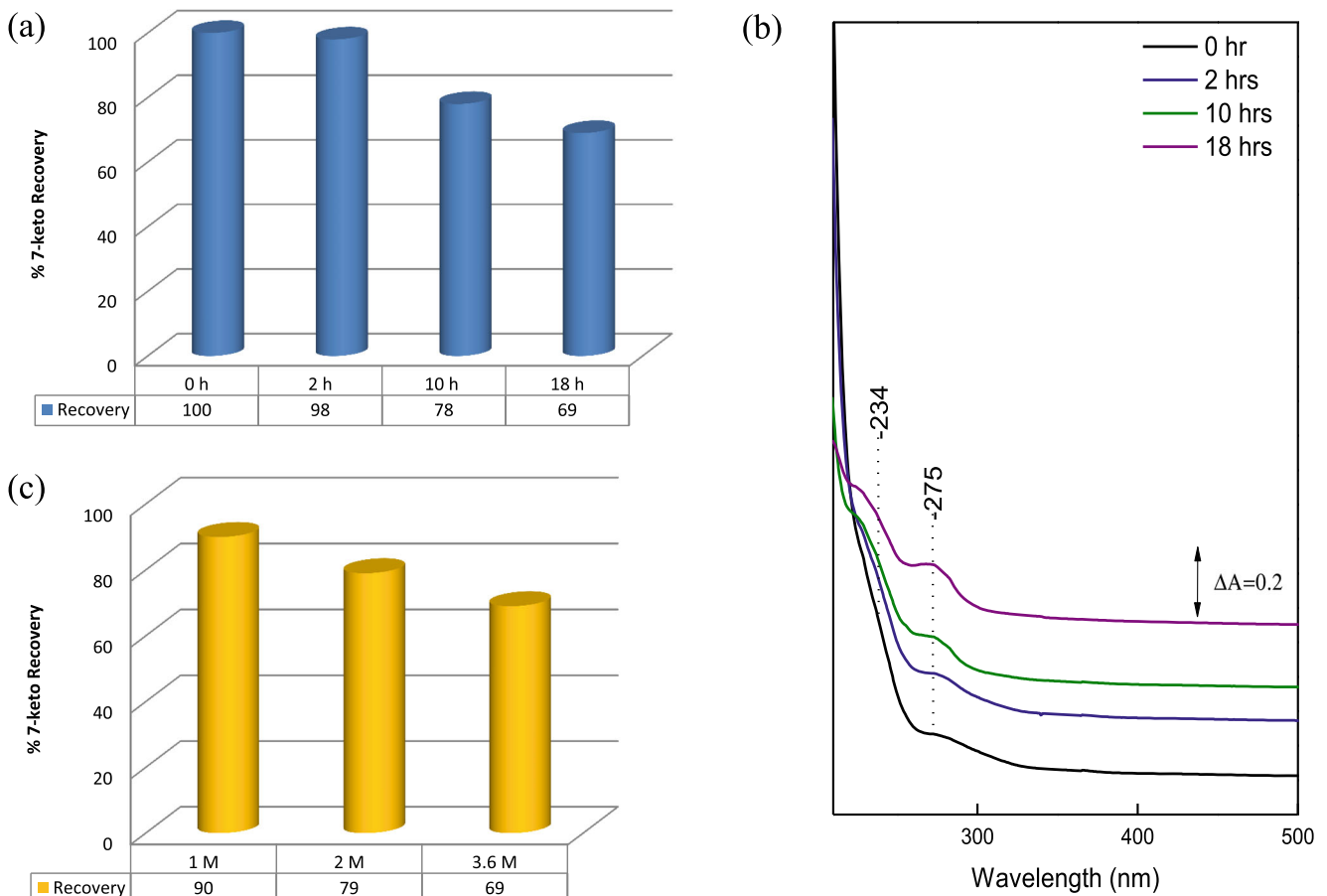


Fig. 3 **a** Effect of incubation time, under saponification with KOH 3.6 M, to the recovery of 7-keto. **b** Effect of KOH concentration to the recovery of 7-keto at 18 h of saponification. **c** UV spectra confirming the

increase of conversion of 7-keto (230 nm) to cholesta-3,5-dien-7-one (275 nm) with the increase of saponification time with KOH 3.6 M

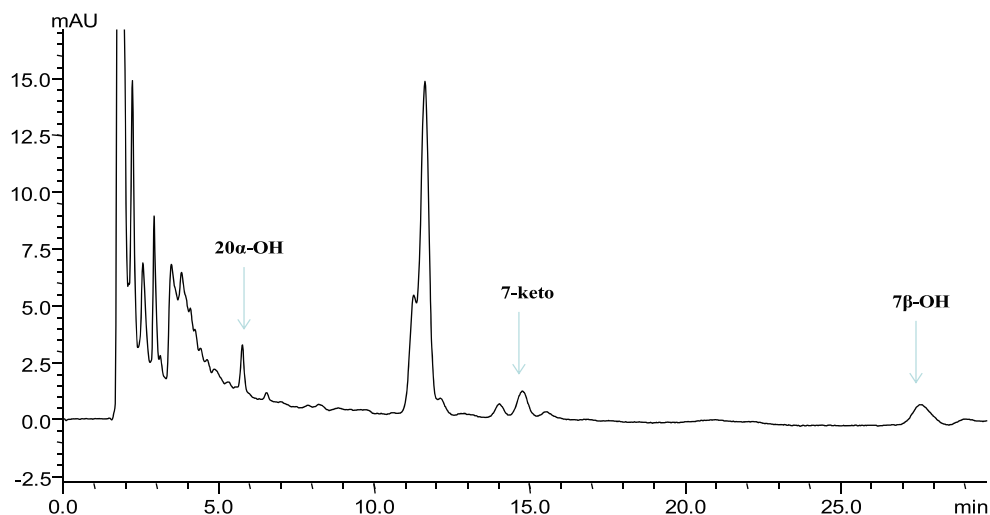
sample was used in order to obtain comparable results with the direct saponification of the sample. The recovery values obtained from the “Lee-Saldanha” method are demonstrated in Table 1. It was observed that the recovery value of 7-keto increased by 15 %, when compared with the value obtained by use of the Saldanha method (Table 1), while the recovery values of 22S-OH, 25-OH, and 7 β -OH remained constant. In an effort to increase the sample mass in order to improve the possibility of detecting more COPs, a 5-g sample was used, and the chromatogram obtained was compared with the one obtained when a 1 g of sample was analyzed (data not shown). Although the latter provided a clear chromatogram, a bigger quantity of sample generated several impurities, which may interfere with the qualitative and quantitative analysis of important COPs. In this case, a peak, which absorbs both at 206 and 230 nm, appears at the elution time of 22R-OH (~15 min). Another major problem of this particular method involves the difficulties in the practical implementation. In particular, the lipid-fraction purification step, which involved the use of a 0.45- μ m membrane filter, could not be performed easily, due to the presence of polar impurities. Therefore, the optimum

saponification method was further applied in combination with other lipid extraction procedures.

According to the Folch method, the ratio of the extraction solvent chloroform/methanol/water must be exactly 8:4:3 so that the most polar lipids (COPs) are prevented from being transported into the aqueous phase during the cleaning step. On the contrary, the Boselli method reports the use of an 8:4:4 ratio. This is probably the reason for the low recovery values obtained by use of the latter method (Table 1). Even though the recoveries of the most polar COPs, 7-keto and 7-OH, were lower with the Folch method than with the Lee method, the first one proved to be more selective. Based on the above, the method “Folch-Saldanha” was chosen as the optimum sample preparation procedure, and it was further applied to different food samples.

Application for Analysis of Food Samples Samples of Cypriot smoked lountza, marinated lountza in red wine, grilled lamb chops, boiled egg, and grilled sea bream fillet were analyzed by using the optimum preparation and chromatographic methods. In the lipid extraction procedure, 5 g of each food

Fig. 4 Application of the optimum sample preparation method in a smoked lountza sample with detection at 206 nm. Chromatographic conditions same as in Fig. 1



sample were used. The lipid content varied from 250 mg, found in lountza and lamb chops, to 1755 mg, found in the boiled egg. The saponification method though was applied, in all cases, to 250 mg of lipids, because this is the maximum lipid mass that can effectively be saponified under mild conditions. The COPs fraction was dissolved in 500- μ L hexane/2-propanol (97:3, *v/v*) solution.

Cypriot-smoked lountza was the first sample analyzed in this study, and the chromatogram obtained clearly demonstrates the peaks that correspond to 20 α -OH, 7-keto, and 7 β -OH (Fig. 4). In addition, the predominant COPs, 7-OH and 7-keto, found in lamb chops, in our study, were similar to the findings reported by Morán et al. (2012). On the contrary, their concentrations in the marinated lountza were significantly lower, probably due to the antioxidant activity of polyphenols, which are found in red wine. Similarly, a study reported by Rodríguez-Carpena et al. demonstrated that the addition of natural antioxidants, during food processing, is an excellent strategy for the inhibition of COPs formation (Rodríguez-Carpena et al. 2012).

Although the optimum preparation method was applied effectively to meat samples, selectivity was less satisfactory when it was applied to fish and egg samples. In both cases,

more impurities were present in the chromatograms (data not shown). However, the presence of these impurities did not inhibit the quantitation of the COPs 7-keto and 7-OH. In addition, the concentrations of the two latter COPs determined in the egg are in agreement with the corresponding concentrations found in a study reported by Mazalli and Bragagnolo (2009). Table 2 demonstrates all the COPs concentrations that were found in the meat, fish, and egg samples, as calculated from the calibration curves.

In this study, an HPLC method was developed for the separation of seven COPs by using a cyano-bonded column and a mobile phase of *n*-hexane/2-propanol/acetone (97:1.5:1.5, *v/v*). The developed method, along with the optimized sample preparation procedure, proved to be effective for the analysis of COPs in different food samples. The sample preparation procedure, in this study, was optimized by comparing different saponification methods in combination with different lipid extraction procedures. The optimum saponification conditions involve the use of ethanol/water (60:40, *v/v*) as the saponification solvent and the addition of 5-ml water after the completion of the process. The application though of direct saponification to the sample confirmed the degradation of 7-keto to cholesta-3,5-diene-7-one. This problem, along with the sample mass limitation, was partially solved by

Table 2 COPs found (μ g/g) in food samples that were analyzed by using the optimum analytical protocol

COPs	Smoked Lountza	Grilled lamb chops	Grilled sea bream	Boiled eggs	Lountza in wine
20 α -OH	0.106	0.147	0.145	–	–
22R-OH	<LOQ	<LOQ	–	–	–
22S-OH	–	–	–	–	–
25-OH	–	–	–	–	–
7-keto	<LOQ	0.133	<LOQ	1.092	<LOQ
7 β -OH	0.204	0.220	0.589	2.535	<LOD
7 α -OH	<LOQ	<LOQ	0.623	<LOQ	–
Total	0.310	0.500	1.357	3.627	–

extracting the lipids with chloroform/methanol (2:1 v/v) before the mild alkaline hydrolysis (1 M KOH). In addition, the application of the optimum sample preparation and chromatographic methods to different food samples confirmed the predominance of B-ring COPs over the side-chain COPs. Finally, the results obtained from the analysis of a sample that was marinated in red wine revealed that antioxidants can significantly inhibit the formation of COPs.

Acknowledgments C.P.K.C. acknowledges the University of Cyprus for the support of this research. The authors would like to thank Ms. Andri Ioannou for her help in the performance of UV experiments and also for helpful discussions regarding the particular studies.

Conflict of Interest Michalis S. Constantinou declares that he has no conflict of interest.

Christiana A. Georgiou declares that she has no conflict of interest.

Constantina P. Kapnissi-Christodoulou declares that she has no conflict of interest.

This article does not contain any studies with human or animal subjects.

References

- Baggio SR, Miguel AMR, Bragagnolo N (2005) Simultaneous determination of cholesterol oxides, cholesterol and fatty acids in processed turkey meat products. *Food Chem* 89:475–484
- Boselli E, Caboni MF, Rodriguez-Estrada MT, Toschi TG, Daniel M, Lercker G (2005) Photooxidation of cholesterol and lipids of turkey meat during storage under commercial retail conditions. *Food Chem* 91:705–713
- Brown AJ, Jessup W (1999) Oxysterols and atherosclerosis. *Atherosclerosis* 142:1–28
- Busch TP, King AJ (2009) Artifact generation and monitoring in analysis of cholesterol oxide products. *Anal Biochem* 388:1–14
- Busch TP, King AJ (2010) Stability of cholesterol, 7-ketocholesterol and β sitosterol during saponification: ramifications for artifact monitoring of sterol oxide products. *J Am Oil Chem Soc* 87:955–962
- Chen YC, Chien JT, Inbaraj BS, Chen BH (2012) Formation and inhibition of cholesterol oxidation products during marinating of pig feet. *J Agric Food Chem* 60:173–179
- Folch J, Lees M, Stanley GHS (1957) A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem* 226:497–509
- Georgiou CA, Kapnissi-Christodoulou CP (2013) Qualitative and quantitative determination of COPs in Cypriot meat samples using HPLC. Determination of the most effective sample preparation procedure. *J Chromatogr Sci* 51:286–291
- Georgiou CA, Constantinou MS, Kapnissi-Christodoulou CP (2014) Sample preparation: a critical step in the analysis of cholesterol oxidation products. *Food Chem* 2013:918–926
- Guardiola F, Dutta P C, Codony R, Savage G P (2002) Cholesterol and phytosterol oxidation products analysis, occurrence, and biological effects. AOCs Press
- Lee HW, Chien JT, Chen BT (2006) Formation of cholesterol oxidation products in marinated foods during heating. *J Agric Food Chem* 54:4873–4879
- Linseisen J, Wolfram G, Miller AB (2002) Plasma 7β -hydroxycholesterol as a possible predictor of lung cancer risk. *Cancer Epidem Biomar* 11:1630–1637
- Mariutti LRB, Nogueira GC, Bragagnolo N (2008) Optimization and validation of analytical conditions for cholesterol oxides extraction in chicken meat using response surface methodology. *J Agric Food Chem* 56:2913–2918
- Mazalli MR, Bragagnolo N (2009) Increase of cholesterol oxidation and decrease of PUFA as a result of thermal processing and storage in eggs enriched with n-3 fatty acids. *J Agric Food Chem* 57:5028–5034
- Morán L, Andrés S, Bodas R, Prieto N, Giráldez FJ (2012) Meat texture and antioxidant status are improved when carnosic acid is included in the diet of fattening lambs. *Meat Sci* 91:430–434
- Nogueira GC, Costa BZ, Crotti AEM, Bragagnolo N (2010) Synthesis of 7-hydroperoxycholesterol and its separation, identification, and quantification in cholesterol heated model systems. *J Agric Food Chem* 58:10226–10230
- Otaegui-Arrazola A, Menendez-Carreño M, Ansorena D, Astiasaran I (2010) Oxysterols: a world to explore. *Food Chem Toxicol* 48:3289–3303
- Park WT, Guardiola F, Park SH, Addis PB (1996) Kinetic evaluation of 3β -hydroxycholest-5-en-7-one (7-ketocholesterol) stability during saponification. *J Am Oil Chem Soc* 73:623–629
- Raith K, Brenner C, Farwanah H, Muller G, Eder K, Neubert RHH (2005) A new LC/APCI-MS method for the determination of cholesterol oxidation products in food. *J Chromatogr A* 1067:207–211
- Rodriguez-Carpena JG, Morcuende D, Petróñ MJ, Estevez M (2012) Inhibition of cholesterol oxidation products (COPs) formation in emulsified porcine patties by phenolic-rich avocado (*Persea americana* Mill.) extracts. *J Agric Food Chem* 60:2224–2230
- Rose-Sallin C, Huggett AC, Bosset JO, Tabacchi R, Fay LB (1995) Quantification of cholesterol oxidation products in milk powders using [2H7] cholesterol to monitor cholesterol autoxidation artifacts. *J Agric Food Chem* 43:935–941
- Ryan L, O'Callaghan YC, O'Brien NM (2005) The role of the mitochondria in apoptosis induced by 7 β -hydroxycholesterol and cholesterol-5 β ,6 β -epoxide. *Brit J Nutr* 94:519–525
- Saldanha T, Sawaya ACHF, Eberlin MN, Bragagnolo N (2006) HPLC separation and determination of 12 cholesterol oxidation products in fish: comparative study of RI, UV, and APCI-MS detectors. *J Agric Food Chem* 54:4107–4113
- Sieber R (2005) Oxidized cholesterol in milk and dairy products. *Int Dairy J* 15:191–206
- Ubhayasekera SJKA, Verleyen T, Dutta PC (2004) Evaluation of GC and GCMS methods for the analysis of cholesterol oxidation products. *Food Chem* 84:149–157
- Vicente SJV, Sampaio GR, Ferrari CKB, Torres EAFS (2012) Oxidation of cholesterol in foods and its importance for human health. *Food Rev Int* 28:47–70