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Article

Qualitative and Quantitative Determination of COPs in Cypriot Meat Samples Using HPLC Determination of the Most Effective Sample Preparation Procedure

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This research work describes the development of a fast high-performance liquid chromatography method for the simultaneous determination of five important cholesterol oxidation products (COPs). The influence of various experimental parameters, such as the composition of the mobile phase, the flow rate and the column temperature, were investigated. Baseline separation was achieved by using acetonitrile–methanol–water– isopropanol (67:27:5:1) as a mobile phase with a 10°C column temperature. The developed method demonstrated good linearity and high reproducibility, with relative standard deviation values below 1.26% for all the COPs that were examined. The method was then applied, for the first time, to Cypriot smoked-meat products (lountza and hiromeri). The presence of COPs in these products suggests that the preparation of the meat products, and particularly the smoking process, possibly favors the oxidation of cholesterol. Finally, three different sample preparation procedures were evaluated and the optimum procedure was determined based on recovery, precision and simplicity.

Introduction

Cholesterol is a monounsaturated alcohol, which can easily undergo oxidation. This, in turn, generates a large number of oxidation products, the so-called cholesterol oxidation products (COPs) (1–4). In recent years, COPs have drawn scientific interest, particularly due to their implications regarding human health. Many of these compounds have been demonstrated to be cytotoxic, mutagenic and carcinogenic. They play an important role in the development of atherosclerosis, and they have been associated with alterations in cell-membrane properties and inhibition of cholesterol biosynthesis (5–11).

The primary source of COPs is diet, particularly the consumption of cholesterol-rich foods (1, 8). This, therefore, raises questions about the safety of consumers and suggests the necessity for the development of a sensitive and a reliable analytical method to identify and quantify these components in food samples. Gas chromatography (GC) and high-performance liquid chromatography (HPLC) are the most widely used techniques for the analysis of COPs. In GC, due to the high temperatures used, derivatization of the analytes is required to stabilize some heat-sensitive diols. HPLC, which is considered to be the alternative method to GC, enables the analysis of these compounds in relatively low temperatures without the need for derivatization. Hence, it is a fast and simple analytical methodology (1, 5, 6, 12, 13).

In this report, a study is described regarding the presence of COPs in Cypriot traditional meat products, such as lountza (smoked pork loin fillet) and hiromeri (smoked pork leg matured in wine). Numerous studies have reported qualitative and quantitative analyses of COPs in various food products, particularly in meats. The B-ring oxides, such as 7-ketocholesterol and 7α- and 7β-hydroxycholesterol, are commonly found in meat, in contrast to the oxides that originate from the side chain (25-, 20- and 22- hydroxycholesterol) (14–16). However, 22R-hydroxycholesterol and 25-hydroxycholesterol are the most common cholesterol oxides found in the samples mentioned previously. Until now, 22R-hydrohycholesterol has not been significantly studied in meat.

Many studies have demonstrated that the content of COPs increases dramatically during food processing (1, 7, 17, 18). During their preparation, the products under study are subjected to smoking in specifically designed smoking rooms, using apple and pine-wood, for a period of 1–2 months. It is believed that this process increases the degree of cholesterol oxidation. This hypothesis can be confirmed by investigating the presence of the most common COPs in both lountza and hiromeri. In this study, COPs are therefore detected and quantified, for the first time, in these particular Cypriot meat products.

The quantification of COPs in food is difficult due to interruptions caused by triglycerides, phospholipids and other matrix lipids. Therefore, a sample preparation procedure is necessary prior to the chromatographic analysis to isolate the COPs from food that contains the previously mentioned interfering substances (1, 2, 5). Although different studies have been performed for the determination of COPs in processed foods, there is no standard method available for the extraction of all COPs from these samples. Therefore, in each case, extraction, purification and detection methods have to be developed for the isolation of different COPs from a particular matrix. In this study, the evaluation of different sample pretreatment methods is reported to find the one that is the most effective with regard to analyte recovery, time, difficulty and reproducibility.

Experimental

Reagents

COP standards, 20α-hydroxycholesterol, 7-ketocholesterol, 7β-hydroxycholesterol, 22R-hydroxycholesterol and 25-hydroxycholesterol, were purchased from Sigma-Aldrich (Steinheim, Germany). The chemical structures of the compounds, in elution order, are presented in Figure 1. Analytical standard stock solutions of the five analytes were prepared in methanol at concentrations of 0.1 mg/mL each to give a final concentration of 0.02 mg/mL in the mixture,

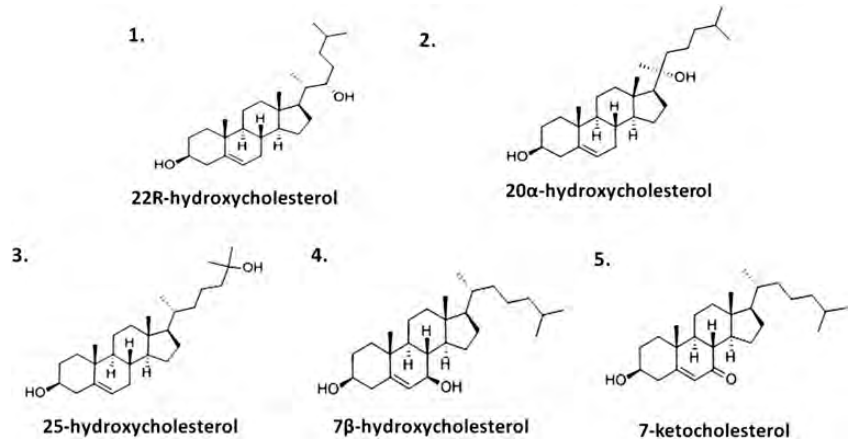

Figure 1. Chemical structures and numerical designations of cholesterol oxides.

and they were stored at 4°C. The HPLC-grade solvents were obtained from Merck (Hohenbrunn, Germany). They were filtered through a 0.45 μm Millipore membrane filter under vacuum and degassed for 5 min before use. Lountza and hiromeri were obtained from a local supermarket in Nicosia (Cyprus). All samples were ground thoroughly by using an electric blender.

Instrumentation and conditions

All experiments were conducted on a Shimadzu HPLC equipped with a diode-array detector (DAD) for ultraviolet (UV) detection. The DAD was set at 206 nm for the detection of the five COPs. LCsolution software was used for the collection and evaluation of all experimental data. The analytical column was a Brava C18-ODS, 250 × 4.6 mm, 5 μm column (Waters, Milford, MA), preceded by a Brava C18-ODS 7.5 × 4.6 mm, 5 μm guard column. The solid-phase extraction (SPE) cartridges, which were used for sample treatment, were Chromabond (500 mg SiOH-stationary phase). For the determination of the optimum set of separation parameters, the column temperature and the mobile phase flow rate were investigated. They ranged from 10 to 25°C and from 0.8 to 2.0 mL/min, respectively. The temperature was regulated by placing the HPLC column in a Shimadzu column oven, which uses an electronic cooler (Peltier effect). The injection volume was 10 μL.

Sample preparation

Three different preparative methods were used for the analysis of different Cypriot commercially meat products.

Method A

One gram of ground meat sample was treated with 4 mL of a 50% aqueous solution of potassium hydroxide and 6 mL of ethanol to perform saponification at room temperature for 22 h in the dark. The mixture was filtrated through filter paper with pore size 125 mm. Five milliliters of distilled water and 10 mL of *n*-hexane were added to the sample, which was then shaken, and the hexane fraction was separated. The extraction with 10 mL of *n*-hexane was repeated three times. The solution was dried in a rotary evaporator. The residue was dissolved in 5 mL of *n*-hexane and filtered through a 0.45 μm syringe filter for purification (1). The extract was loaded into a silica cartridge, which was preconditioned with 10 mL of hexane. After a washing step with 2 mL of *n*-hexane–diethyl ether (8:2, v/v) and 2 mL of *n*-hexane–diethyl ether (1:1, v/v), the COPs were eluted with 2 mL of methanol.

Method B

One gram of ground meat sample was mixed with 10 mL of chloroform–methanol (2:1 v/v) and shaken for 30 min. The mixture was filtrated through filter paper and then evaporated to dryness under vacuum. The residue was dissolved in 3 mL of hexane and filtered through a 45 μm syringe filter to remove the impurities (19). The extract was loaded into the extraction cartridge, which was previously washed with 10 mL of *n*-hexane. Then, the cartridge was washed with 2 mL of *n*-hexane–diethyl ether (8:2, v/v) and 2 mL of *n*-hexane–diethyl ether (1:1, v/v). The COPs were eluted with 2 mL of methanol.

Method C

This process differs from the Method B only in the organic solvents that were used for fat extraction. In Method C, a mixture of *n*-hexane and 2-propanol at a ratio of 3:2 v/v was used, according to Radin's method (20).

Results and Discussion

Method development

The first objective of this research was to develop an analytical method allowing the simultaneous separation of cholesterol oxidized derivatives. The optimum experimental conditions were determined by evaluating several parameters, such as the composition of the mobile phase, the flow rate and the column temperature.

In preliminary experiments, the chromatographic behavior of the oxidation products was investigated by varying the composition of the mobile phase and the percentage of these solvents. A mobile-phase modification results in a difference

in selectivity and resolution. In particular, a gradual decrease in acetonitrile (ACN) from 78 to 67% (v/v), an increase in methanol (MeOH) and water (H_2O) from 20 to 27% (v/v) and from 2 to 5% (v/v), respectively, and an addition of 1% (v/v) isopropanol (IPA), provided remarkably better separation between peaks 2–3 and 4–5, which correspond to the analytes 20α-hydroxycholesterol–25-hydroxycholesterol and 7β-hydroxycholesterol–7-ketocholesterol. Specifically, the resolution values increased from 1.01 to 1.17 and from 0.68 to 1.31, respectively. Thus, an isocratic solvent system of ACN–MeOH–H_2O–IPA (67:27:5:1) was selected as the optimum composition of the mobile phase (data not shown). In addition, it was generally observed that the relatively hydrophilic nature of the mobile phase provided a significantly better separation of the cholesterol oxides.

The effect of the column temperature on resolution was then investigated (Figure 2). As anticipated, from 25 to 10°C, a long analysis time was observed, possibly due to a decrease in mobile-phase viscosity. The greater the retention of the analytes on the stationary phase, the better the separation resolutions of the peak pairs 2–3 and 4–5. The resolution values increased from 1.17 to 1.45 and from 1.31 to 1.54, respectively. Taking into consideration the sufficient resolution, the temperature of 10°C was chosen as the optimum.

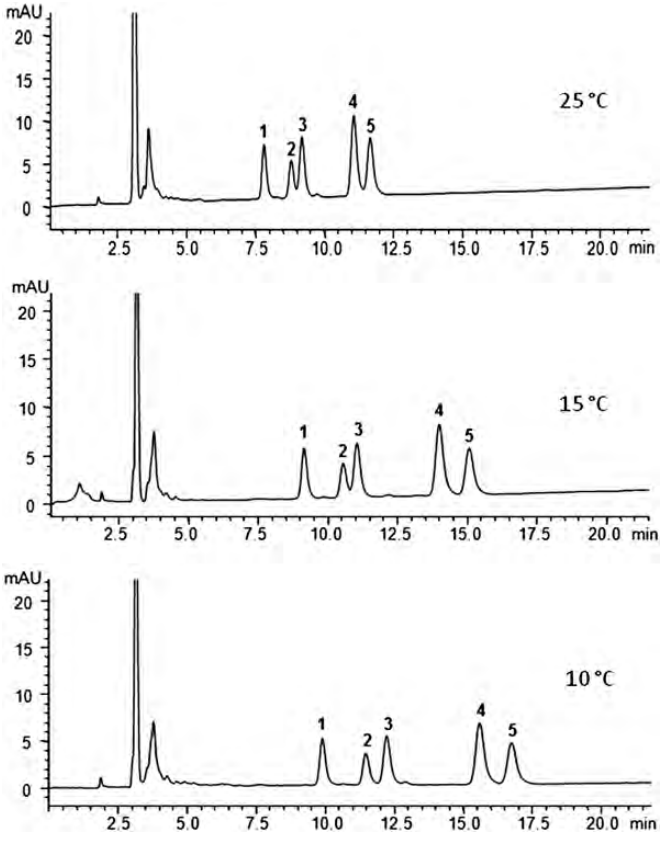

Figure 2. Effect of column temperature on the separation of five cholesterol oxides. Conditions: mobile phase, ACN–MeOH–H2O–IPA 67:27:5:1; flow rate, 1 mL/min; injection volume, 10 μL; detection, 206 nm. Peak numbers correspond to those present in Figure 1.

The flow rate of the mobile phase was finally examined to optimize the separation conditions. As shown in Figure 3, a decrease of the flow rate from 2 to 0.8 mL/min resulted in longer migration times of all the analytes. This, in turn, significantly improved the resolution (Rs: 1.56 and 1.61 of the peak pairs 2–3 and 4–5, respectively), and a baseline separation of all COPs was achieved within 22 min. Therefore, a flow rate of 0.8 mL/min was maintained throughout the separation. A reduction in the flow rate and the column temperature did not significantly influence the efficiency.

Method validation

The optimum HPLC method was evaluated in terms of linear response range, limit of detection (LOD), limit of quantification (LOQ), and precision. The results are demonstrated in Table I.

Calibration curves, which were varied from 1.5 to 50.0 μg/mL (seven points), were constructed for each analyte by plotting the chromatographic areas versus analyte concentration (μg/mL). All analytes under study demonstrated a good linearity response in the investigated range of concentrations, 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 were in the ranges of 0.99–1.28 and 2.99–3.89 μg/mL, respectively. These results, as

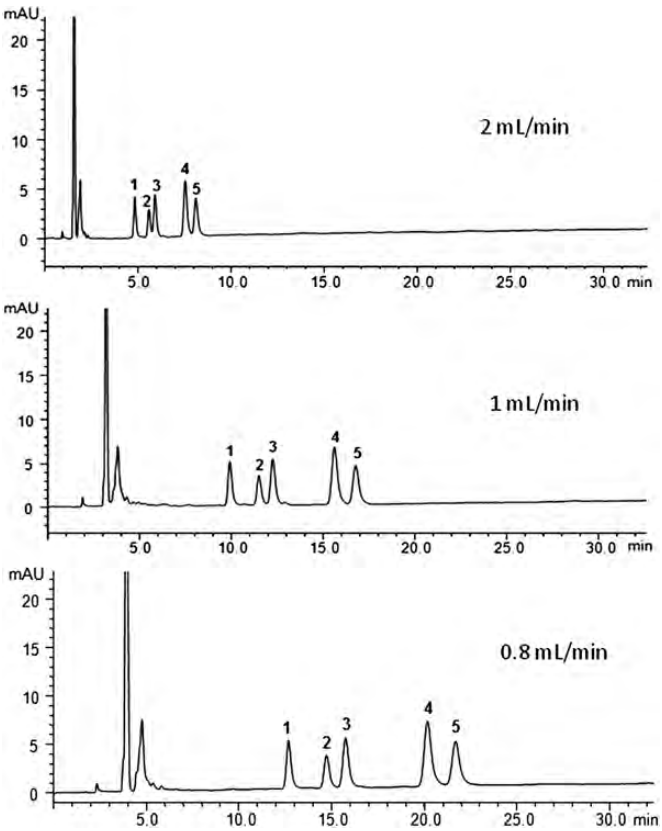

Figure 3. Effect of flow rate on the separation of five cholesterol oxides. Conditions: temperature, 10°C; other experimental conditions are the same as in Figure 2. Peak numbers correspond to those present in Figure 1.

Table I
Linearity, LOD and LOQ, and Precision of Cholesterol Oxidized Derivatives under Optimum Conditions

Compound	Regression equation	r^2	LOD (μg/mL)	LOQ (μg/mL)	RSD (time) (%)
22R- hydroxycholesterol	$y = 1.05e7x - 5264.1$	0.9997	1.11	3.37	0.558
20α- hydroxycholesterol	$y = 8.57e6x + 5562.4$	0.9996	1.28	3.89	0.840
25-hydroxycholesterol	$y = 1.30e7x - 1261.9$	0.9998	0.99	2.99	0.373
7β-hydroxycholesterol	$y = 1.26e7x + 13573$	0.9997	1.09	3.31	0.127
7-ketocholesterol	$y = 1.75e7x - 1620.7$	0.9998	1.01	3.09	1.260

demonstrated, provided the opportunity for reliable determination of COPs in meat products under the optimum experimental conditions.

The analytical repeatability of the method was calculated as the relative standard deviation (RSD) of the migration times of the analytes for five successive injections of the standard samples. The RSD values for each analyte were below 1.26%, which demonstrates the very good precision of the methodology.

Optimization of meat sample preparation procedure

As previously described, the primary objective of the study was to evaluate different sample pre-treatment methods to determine the most effective method allowing a reliable quantitation of COPs in traditional Cypriot meat products. Although there are numerous publications regarding methods used for the extraction and purification of COPs, the reported results are significantly different. No method has, so far, been established as a routine analysis method for extraction, detection and quantitation of cholesterol oxides in foods. Therefore, it was considered important to compare different preparative methods to determine the appropriate process for the isolation of COPs from the samples under study.

In the present research work, three different preparation methods were evaluated and the optimum method was determined, based on recovery, precision and relative simplicity. In Method A, which was described earlier, the extraction of the COPs was performed by direct saponification of the sample, followed by SPE for cleanup of the COPs. In Methods B and C, the extraction of the fat was first performed by using a mixture of organic solvents, followed by isolation of the COPs from other components that exist in the lipid fraction by use of a silica-SPE cartridge.

As illustrated in Figure 4, the chromatogram, which was obtained by using Method A, was very complex and the background was unstable at the time range at which 22R-hydroxycholesterol was eluted. Therefore, the identification of this important component was impossible by using this sample pre-treatment method. When Methods B and C were examined, the background improved remarkably, making possible the quantification of the compounds under study. The chromatograms obtained by using these last two sample preparation procedures were almost the same. However, by using Method C, the COP recovery was dramatically decreased ($20 \pm 4\%$), which was probably due to the apolar solvents that were used

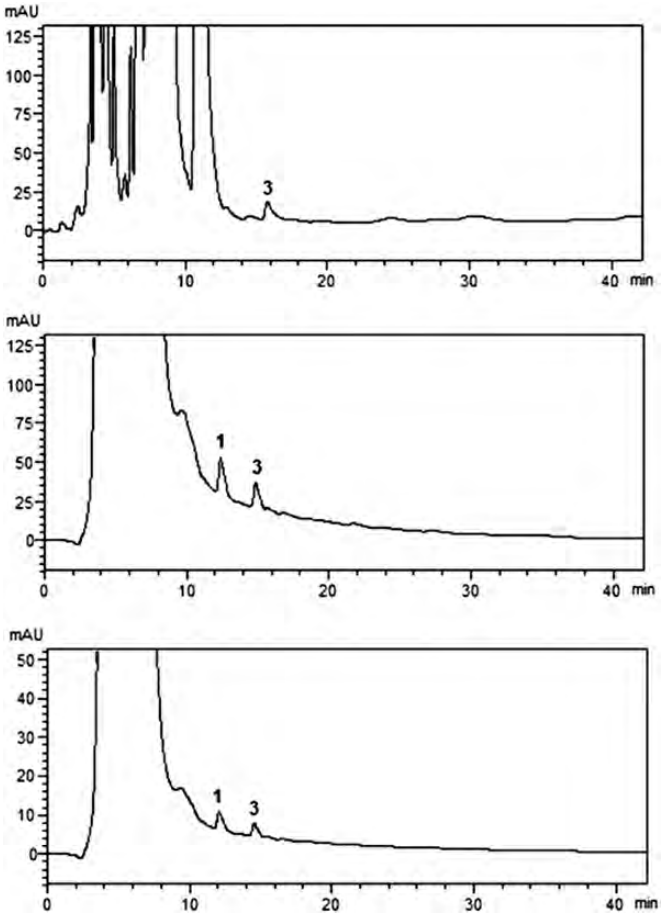

Figure 4. Chromatograms of the lountza samples. They were obtained by using three different sample preparation procedures under the optimum conditions. Note: The chromatograms are not in the same grade.

in fat extraction. The optimum sample pre-treatment method (Method B) provided a very good recovery value ($106 \pm 8\%$). Recoveries were evaluated by using a COP that was not present in any of the meat samples under investigation. This study was also performed to confirm that the particular COP was not present in the meat samples. In particular, the standard of 7β-hydroxycholesterol was added into the Cypriot meat samples at a concentration of 0.1 mg/mL. All samples were then prepared according to Methods A, B and C, and all pretreated samples were injected separately into the HPLC system. The chromatograms that were obtained demonstrated that the amount of 7β-hydroxycholesterol that was added into each meat sample was able to be detected and separated from the other components present in the meat sample.

Another major advantage of Method B, which makes it superior to the others, was the sample preparation time, which is remarkably low in comparison with Method A. Particularly, Method B is completed within 40 min, while in Method A, the sample saponification step requires 22 h.

Another important consideration for determining the optimum sample preparation procedure was reproducibility. This study was performed by using Method B. The reproducibility was evaluated by computing the RSD values of the migration times and the areas of the five identified analyte peaks. The

Table II
The Content of Cholesterol Oxides in Lountza and Hiromeri Samples (μg/g)*

Compound	Lountza 1	Lountza 2	Lountza 3	Hiromeri 1	Hiromeri 2
22R-hydroxycholesterol	29.8	9.0	UQ	13.7	28.4
20α- hydroxycholesterol	–	–	UQ	–	–
25-hydroxycholesterol	17.4	5.3	–	11.6	13.3
7-ketocholesterol	–	3.6	–	–	–

*Note: UQ = under quantification limits, but above detection limit.

run-to-run RSD values were obtained from five consecutive chromatographic runs. All RSD values of the migration times and the areas were below 1 and 5%, respectively, indicating very good reproducibility.

Because Method B proved to be the optimum sample pre-treatment method, it was further applied, for the first time, to Cypriot meat products for the determination of COPs. The presence and the amount of COPs in lountza and hiromeri have been unknown thus far. Four COPs were detected and quantified in the meat samples that were examined. The highest concentrations primarily corresponded to 22R-hydroxycholesterol and 25-hydroxycholesterol. This observation was not surprising because during their preparation, these products are exposed to smoking for a long period of time, which probably favors the oxidation of cholesterol. This observation was confirmed by performing the same experimental procedure with a non-smoked lountza. The chromatogram obtained did not demonstrate any peaks that correspond to the COPs (data not shown).

A total of three lountza samples and two hiromeri samples were investigated, and the amounts determined from the calibration curves are demonstrated in Table II. As observed, 22R-hydroxycholesterol and 25-hydroxycholesterol, which is generally considered to be the most atherogenic COP (2, 5, 21, 22) exist in almost all samples. The COPs 7-ketocholesterol and 20α-hydroxycholesterol were found in very small quantities in only one sample, particularly lountza samples 2 and 3, respectively. As mentioned earlier, the presence and amount of COPs in the particular smoked samples have thus far been unknown, therefore, no comparisons can be performed.

Conclusions

In this study, a fast and a simple HPLC method was developed for the separation of the five most important COPs. A baseline separation of all COPs was achieved in 22 min by using a mobile phase of ACN–MeOH–H_2O–IPA (67:27:5:1), a flow rate of 0.8 mL/min and a column temperature of 10°C. The method provided good linearity response, LODs and precision. The primary achievement of this work was the development of a sample pre-treatment method that allowed the unambiguous determination of COPs in lountza and hiromeri. The presence of COPs in these products suggests that the preparation of the meat products, particularly the smoking process, possibly favors the oxidation of cholesterol. In addition, these results highlight the need for controlling the oxidation of cholesterol in commercial food products, particularly smoked-meat products.

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