Research Article

Novel approach to fast determination of cholesterol oxidation products in Cypriot foodstuffs using ultra-performance liquid chromatography-tandem mass spectrometry

This paper reports the development and validation of a new method based on ultra-performance LC coupled to MS/MS for the simultaneous determination of four cholesterol oxidation products (COPs) in foodstuffs in only 4.1 min. The COPs were detected by ESI in positive-ion mode with multiple reaction monitoring, and the mass spectrometric conditions were optimized in order to increase sensitivity. The developed method was validated in terms of linearity, precision, LODs, and LOQs. Recoveries of the extraction process ranged from 86 to 98.5% when the samples were fortified at 100, 500, and 1500 ng/mL. The applicability of the method was confirmed by analyzing different food samples. Considering the paucity of data regarding the content of COPs in Cypriot foods, particular attention was devoted, for the first time, to the determination of the profile of the main COPs in widely consumed, traditional Cypriot foodstuffs (halloumi cheese, hiromeri, snails, etc.)

Keywords: Cholesterol oxidation products / Cypriot food products / Tandem mass spectrometry / Ultra-performance liquid chromatography DOI 10.1002/elps.201500196

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1 Introduction

Cholesterol oxidation products, commonly known as cholesterol oxidation products (COPs), comprise a variety of compounds deriving from oxidation of cholesterol. In the last decades, COPs have been one of the most intense topics in food science due to their link to a series of biological effect [1]. These compounds have been well documented for being potentially cytotoxic, mutagenic, carcinogenic, and they have been associated with the promotion of atherosclerosis [2–7]. It is thought that COPs modulate the structure and function of the cellular membrane and inhibit the activity of enzymes involved in cholesterol biosynthesis [8–13].

COPs are present in our diet through cholesterol-rich foods, such as meat, eggs, and dairy products [14, 15]. Although there are no established safety levels for COPs ingestion, these compounds can be a risk for human health due to chronic exposure. This, therefore, raises questions about the safety of the consumers, and it suggests that it is imperative to develop a sensitive and a reliable analytical method in order to guarantee the strict quality and authenticity of food supply [16].

In view of the health implications of dietary COPs, there is a continuous interest for the determination of COPs in different kinds of foods. However, there is a substantial lack of knowledge concerning the content of COPs in Cypriot food products. Based on this data, the objective of the present study was to estimate the extent of cholesterol oxidation and identify the profile of the main COPs in widely consumed, traditional Cypriot foodstuffs. These products were chosen because their preparation procedures involve treatments and numerous prooxidants factors, such as extensive salting, smoking, and

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Abbreviations: COPs, cholesterol oxidation products; MRM, multiple-reaction monitoring mode; UPLC, ultra-performance LC

Colour Online: See the article online to view Fig. 2 in colour.
exposure to air, which tend to increase the degree of cholesterol oxidation.

A study by Clariana M. et al. reported the determination of COPs in dry-cured shoulder, a traditional Spanish meat that is similar to Cypriot cold meats [1]. During the curing process, an abusive condition, such as extensive salting, dehydration, and exposure to air, occurred. 7-Keto and 7-OH were the predominant COPs, and their concentrations were 0.591 and 0.446 μg/g sample, respectively. In another study, Sampai et al. [15] evaluated fifty samples of salted-dried shrimp, which went through several processing stages: immersion in brine, drainage, drying under the direct incidence of the sunlight, addition of salt, and boiling. The COPs 7β-OH (34.63–72.56 μg/g), 7α-OH (5.02–12.12 μg/g), 7-keto (7.44–32.68 μg/g), and 25-OH (2.37–22.88 μg/g) were determined in all analyzed samples. This indicated the oxidation of this product, which was probably initiated by inadequate processing conditions.

GC and HPLC are the most widely used techniques for the analysis of COPs. The former can provide better resolution, but it requires a time-consuming derivatization of the analytes in order to enhance their volatility and thermal stability [17]. In consequence, HPLC is preferred as a faster and a milder analytical methodology [18, 19]. However, these conventional chromatographic techniques need to be replaced by highly powerful and discriminating analytical tools, bearing in mind the requirements of modern food analysis. Nowadays, the wide variety of food products available, as well as the high number of target analytes, demands the development of ultra-fast analytical techniques, which should be able to provide reliable results in a short time with extremely low detection limits. The conjunction of ultra-performance LC (UPLC) detector appears to be an ideal approach that fulfills key requirements, in terms of speed, sensitivity, and selectivity [20, 21]. In the light of the above, it was considered interesting to develop and establish an analytical method based on UPLC and MS/MS for the determination of the profile of the main COPs in widely consumed, traditional Cypriot food-stuffs (halloumi and feta cheese, anari, hiromeri, snails, etc.). Nothing has been reported, so far, on the analysis of COPs in the above-mentioned samples and by using this particular type of chromatography.

2 Materials and methods

2.1 Equipment

All analyses were performed on an ACQUITY TQD UPLC-MS/MS system (Waters) by using a method specifically developed for this application. A triple quadrupole mass spectrometer TQD (serial number QBA012) coupled with ESI source running in positive-ion mode was used for the detection of target compounds. In order to achieve sufficient sensitivity for the quantitative analysis, data acquisition was performed in multiple-reaction monitoring mode (MRM), recording the transitions between the precursor ion and the most abundant fragment ions. Instrument control and data processing were carried out by means of MassLynx 4.1 software. The ionization source parameters were: capillary voltage, 3.5 kV; extractor voltage, 3 V; source temperature, 110°C; desolvation temperature, 200°C; cone gas flow, 50 L/h, and desolvation gas flow, 850 L/h (both gases were nitrogen).

Analytical conditions employed consisted of the use of a BEH Shield RP18 column (50 × 2.1 mm id, 1.7 μm particle size) using 0.1% v/v formic acid in MilliQ water as mobile phase A and methanol as mobile phase B. Elution was carried out according to the following gradient: 0–1 min 15% B; 1–2 min 25% B; 2.1–3 min 80% B; 3–4.5 min B. The optimum flow rate was 0.4 mL/min, whereas the injection volume was 10 μL. Column and autosampler compartments were thermostated at 40 and 10°C, respectively.

2.2 Samples and sample preparation

The food samples were purchased from local supermarkets in Nicosia (Cyprus). COPs were extracted from different food matrices using a procedure that was reported elsewhere [22]. Briefly, 5 g of the samples were subjected to total lipid extraction with 120 mL of Folch’s solution, consisting of chloroform and methanol (2:1, v/v). The samples were homogenized with this mixture for 3 min, and then, the entire content was filtered through filter paper (Whatman No.1) to eliminate the solid residue. The filtrate was mixed thoroughly with 30 mL of a NaCl solution (0.88%, w/w) and left overnight at 4°C in order to obtain phase separation. The lower phase, containing the lipids was collected and dried in a vacuum evaporator. Two hundred fifty milligrams of lipid portion was treated with 10 mL of 1 M KOH solution in 60 % aqueous ethanol, to perform a cold saponification at room temperature for 18 h in darkness and under continuous agitation. After the addition of 10 mL water, the unsaponifiable matter was extracted four times in succession with 10 mL of hexane. The extracts were filtered using filter paper (Whatman No1.) through a bed of anhydrous sodium sulfate, and the material retained after filtration was washed with 10 mL hexane. Subsequently, the solution was dried in a rotary evaporator, and the residue was dissolved in hexane/ethyl acetate (95:5, v/v). COPs were enriched and purified from apolar materials and cholesterol by SPE, employing NH2 column. Each column was pre-equilibrated with 3 mL of hexane and, after sample loading, it was eluted with a series of three solvents: 6 mL hexane/ethyl acetate (95:5, v/v), 10 mL hexane/ethyl acetate (90:10, v/v), and 10 mL acetone. The first two fractions were discarded, whereas the last fraction, containing COPs, was dried under a nitrogen steam.

2.3 Reagents and standards

Reference standards of 22R-hydroxycholesterol (22R-OH), 7-hydroxycholesterol (7-OH) were purchased from Steraloids (Newport, RI, USA), whereas 5,6-cholesterol epoxide (5,6-EP) and 7-ketocholesterol (7-keto) were acquired from
Sigma-Aldrich Co. (St. Louis, MO, USA). All solvents for use in the sample extraction protocol (hexane, methanol, ethanol, chloroform, ethyl acetate, and acetone) were of at least HPLC grade, and were all provided by Merck (Darmstadt, Germany). Potassium hydroxide and sodium chloride were purchased from Merck, and sodium sulfate from Sigma-Aldrich. NH₄OH, Potassium hydroxide and sodium chloride were purchased from Merck. The solvents for use (chloroform, ethyl acetate, and acetone) were of at least HPLC grade. All solvents for use were provided by a Millipore purification system (Milli-Q, Milipore, Bedford, USA).

3 Results and discussion

Chromatographic separation might not be a crucial issue when MS/MS is used for detection, because the possibility of finding two compounds with the same MR transitions is fairly low [23]. However, the sterol molecules under study share a common fragmentation pattern due to the similarity in their chemical structures (same steroid backbone) [24]. This clearly implicates that it is not possible to discriminate between COPs solely on their fragmentation spectra, and that chromatography is an indispensable tool. Chromatographic separation was performed by using methanol as the organic phase and water as the polar phase with the addition of 0.1% formic acid. The application of the elution gradient program, described in Section 2.1, achieved efficient separation in less than 4.5 min with good peak shapes and resolutions (Fig. 1).

The MS/MS method was optimized by: (i) selecting candidate precursor ions in full scan spectra, (ii) fragmenting of selecting precursor ions in product ion scan mode, and (iii) selecting the MRM transitions for each analyte. Data acquisition was performed in full scan to determine an abundant precursor ion. Initially, the spectra were characterized by a few fragment ions, due to the loss of water molecules. The protonated molecular ion [M + H]⁺ was the main peak, only in the case of 7-keto, and this is consistent with the findings of Razzazi-Fazeli et al. [25]. This enables the mass separation of 7-keto, since the m/z 401.5 mass was not detectable in other mass spectra of COPs. The main fragments in the mass spectra of the other compounds were m/z 385.3 [M-H₂O+H]⁺ and 367.3 [M-2H₂O+H]⁺.

Next, tandem MS experiments were performed in order to obtain valuable information regarding the fragmentation pathway of target analytes. Particularly, a product ion scan was performed for selected product ions to identify the appropriate fragment ions. The results obtained attest that the dominant breaking sites of the COPs are found in the sterol ring system. This observation is consistent with reports by Rossmann et al. [24]. Table 1 shows the precursor and daughter ions for target compounds as well as the optimum values of MS/MS parameters: voltage of the first quadrupole for isolation of the precursor ion and collision energy for efficient fragmentation [26]. In the study, two transitions per compound were routinely monitored. The most sensitive MRM transition was typically selected for quantification, whereas the additional transition was acquired to render a highly reliable confirmative method. This approach provides four identification points, which are sufficient to fulfill the criteria for unequivocal identification and confirmation of the presence of a target analyte in food matrices. Furthermore, retention time (tR) was monitored against standard solutions for additional confirmation.

Special effort was also made to optimize two key parameters in MS operation, the desolvation gas flow rate, and the temperature of ionization source, in order to raise the maximum signal for each COP. Bearing in mind that these are two factors that favor the evaporation process and can affect the ionization efficiency, they were investigated, in order to find the optimum values, which provided the best total ion intensities. The optimization was achieved by analyzing a multistandard solution, which contained 500 ng/mL of each analyte.

The effect of the desolvation gas flow on the COPs signal was first examined by varying the flow rate between 650 and 850 L/h (three injections per flow rate) and by keeping all other parameters constant. As anticipated, an increase in the flow rate resulted in a remarkable enhancement of the signal for all analytes, due to improved desolvation efficiency. Considering the high solvent flow rate and aqueous nature of the mobile phase, a higher flow rate of desolvation gas was applied.

Another important factor to consider was the optimization of the source temperature (Supporting Information Fig. 1). Increasing signal responses of analytes from 90 to 110°C confirmed the expectation that high temperatures favor desolvation and transition of analyte to the gas phase. However, its signal responses demonstrate a strong decrease at temperatures above 110°C, possibly due to the thermal instability of target compounds. Hence, the optimum intensity was obtained when a temperature of 110°C was used.

3.1 Method validation

After separation and optimization of MS/MS conditions, a validation procedure was carried out to evaluate several performance characteristics of the method: sensitivity, linearity, intra- and interday precision, matrix effect, and recovery. The sensitivity of the method was evaluated by determining the LOD and LOQ.

First, the method precision was assessed by performing replicate analyses of a mixture of analyte standard solutions at 150 and 500 ng/mL, on the same day and in three consecutive days. Precision was expressed in the term of %RSD of the peak area. The intraday precisions, which were obtained
Figure 1. LC-MS/MS chromatograms of (1) 22R-OH, (2) 7-OH, (3) 5,6-EP, and (4) 7-keto, obtained from a standard mixture at 500 ng/mL.

Table 1. Retention time and MS/MS parameters for target analytes

| Analytes  | \( t_R \) (min) | Quantifier transition | Qualifier transition | Cone voltage (V) | Collision energy (eV) |
|-----------|-----------------|-----------------------|----------------------|------------------|----------------------|
| 7-Keto    | 3.74            | 401.6 > 383.3         | 401.6 > 365.2        | 50               | 24                   |
| 5,6-EP    | 4.11            | 385.6 > 159.4         | 385.4 > 95.1         | 42               | 30                   |
| 7-OH      | 3.67            | 367.6 > 81.2          | 367.8 > 95.2         | 48               | 30                   |
| 22R-OH    | 3.23            | 367.7 > 147.2         | 367.8 > 95.2         | 50               | 27                   |

from ten consecutive runs, were lower than 1.9%, while the day-to-day RSD values were slightly higher (1.4–4.2 %). The results indicated that the proposed method could be applied to the determination of COPs in food samples with satisfactory precision.

The linearity of the method was assessed by analyzing the standard solutions and the different matrix-matched standard solutions in triplicate at five concentrations, ranging from 30 to 150 ng/mL. The parameters of the linear regression equations: slope, intercept, their SDs, and the determination coefficient \( R^2 \) are summarized in Table 2. The results exhibited a strong linear correlation, with \( R^2 \) values greater than 0.99 for all the analytes under study.

Sensitivity was evaluated by means of LOD and LOQ values. Both parameters were determined using the following equations:

\[
LOD = \frac{(3.3S_D)}{m} \\
LOQ = \frac{(10S_D)}{m}
\]

where \( S_D \) represents the residual SD of the regression line and \( m \) stands for the slope of the calibration curve. The LOD and LOQ values, which were obtained for the COPs in all matrices, ranged from 0.03 to 0.16 μg/250 mg and 0.12 to 0.49 μg/250 mg fat, respectively (Table 2). These were sufficient for quantification of the compounds in real samples.

3.1.1 Evaluation of matrix effect

For the quantification of the target compounds, it must be taken into account that food samples are complex matrices with large amounts of compounds that can interfere with the analyte signal, providing matrix effect. Consequently, the study of this effect has become crucial as it can seriously compromise the integrity of an analytical method. Bearing in mind that no blank food samples were available, samples were spiked, after the extraction, with the selected compounds at different concentrations (from 30 to 150 ng/mL), and the slopes of the calibration curves were compared with results obtained when standard solutions of the COPs compounds were analyzed. Table 2 shows slope ratios matrix/solvent for each compound, considering a tolerable signal suppression or enhancement effect if the slope ratio ranged between 0.8 and 1.2, whereas lower values than 0.8 or higher than 1.2 implied a strong effect on signal suppression or enhancement, respectively [27]. It was observed that matrix significantly affected the response for all compounds under study. Bearing in mind that slopes depend on the matrix, standard addition methodology was used during the quantification step in order to obtain accurate results. It is worth here to note that all compounds required the construction of two calibration curves for the concentration range studied.
3.1.2 Recovery test

The recovery of the extraction step of each COP at three fortification levels (100, 500, and 1500 ng/mL) in all samples was studied. Particularly, the recovery values were calculated as $C_{\text{obs}}/C_{\text{ref}}$, where $C_{\text{obs}}$ is the difference between the concentration determined for the spiked sample and native concentration in the same sample, and $C_{\text{ref}}$ is the spiked concentration.

As it can be appreciated, the method afforded recoveries between 86 and 98%. These results, which are demonstrated in Supporting Information Table 1, illustrate clearly the applicability of the developed method to extract COPs from real samples. It is important here to note that the recovery values obtained for the three concentration levels were comparable and indicated that, in the explored concentration range, recovery does not depend on analyte concentration.
Figure 2. Effect of salting duration on the concentration of COPs in raw pork meat.

Table 3. Levels of COPs detected in Cypriot food products

| Samples          | 7-OH (ng/250 mg of fat) | 7-Keto (ng/250 mg of fat) | 5,6-EP (ng/250 mg of fat) | 22R-OH (ng/250 mg of fat) | Total COPs (ng/250 mg of fat) |
|------------------|-------------------------|---------------------------|---------------------------|---------------------------|-----------------------------|
| **Dairy products** |                         |                           |                           |                           |                             |
| Yogurt           | 41 (1.7)                | 57 (5.2)                  | 1314 (1.7)                | n.d                       | 1412                        |
| Feta cheese      | 1430 (1.8)              | 73 (2.8)                  | n.d                       | n.d                       | 1503                        |
| Halloumi cheese  | 616 (1.1)               | 82 (3.0)                  | n.d                       | n.d                       | 698                         |
| Anari cheese     | 105 (2.4)               | 1099 (2.7)                | n.d                       | n.d                       | 1204                        |
| **Meat products** |                         |                           |                           |                           |                             |
| Snails           | 1239 (4.7)              | 1424 (3.0)                | n.d                       | n.d                       | 2663                        |
| Hiromeri         | 395 (2.2)               | 155 (2.8)                 | 105 (2.7)                 | n.d                       | 655                         |
| Sausage          | 444 (1.7)               | 490 (4.7)                 | n.d                       | n.d                       | 934                         |
| Salami           | 972 (2.5)               | 141 (3.7)                 | 442 (3.0)                 | n.d                       | 1555                        |
| Caul fat         | 122 (1.7)               | 331 (3.6)                 | 1406 (2.3)                | n.d                       | 1859                        |
| Bacon (raw)      | 1104 (1.5)              | 158 (3.0)                 | n.d                       | n.d                       | 1262                        |
| Bacon (fried)    | 2166 (1.5)              | 237 (1.8)                 | 232 (2.8)                 | n.d                       | 2635                        |
| Rabbit (fried)   | 843 (1.8)               | 681 (1.4)                 | 202 (2.6)                 | n.d                       | 1726                        |
| Rabbit (in oven) | 2530 (0.3)              | 938 (1.8)                 | 1594 (2.7)                | n.d                       | 5062                        |

RSD values are given in brackets (n = 4).

n.d.: not detectable.

3.2 Analysis of real samples

As it has been already stated, there is scarcity of data about the content of COPs in Cypriot food products. In this work, considerable attention was provided for the quantitation of the most important COPs in widely consumed traditional Cypriot food products (halloumi cheese, feta cheese, hiromeri, snails, etc.). Table 3 summarizes the amount of COPs found in all of the investigated food samples. As demonstrated, 7-keto is the only COP that was detected in all analyzed samples. This finding confirms further that 7-keto can be employed as a suitable marker of cholesterol oxidation in food systems. However, it is important to note that the most concentrated oxide was the 7-hydroxy derivative, while the harmful epoxides were only observed in a few samples.

Particular interest was devoted to the results obtained from the analysis of the marinated hiromeri in red wine, which contains significantly lower concentration of COPs in comparison to the majority of the other food samples. This outcome was possibly attributed to the antioxidant activity of polyphenols, which are found in red wine [28]. Bearing in mind the scant of information regarding the protective effects of polyphenols against cholesterol oxidation, it is considered an interesting topic for future research.

The concentration of COPs in fried bacon was two times higher in respect to the corresponding raw product, confirming that thermal processing influences the production...
of these oxides. Moreover, the content of COPs in rabbit prepared in oven (16 min) was significantly higher than in fried rabbit (9 min). The formation of the lower amount of COPs in the latter may be attributed to the shorter time that the sample was subjected to thermal process. As confirmed by the results of other authors, heating time is one of the basic parameters affecting oystersterol formation [29].

Sodium chloride, which is an important additive for the preparation of the products under study, has been reported to act as a prooxidant [30]. Hence, to further investigate the effect of salting on oxidation, different salting times (0, 2, 4, 8, 12, 24, and 48 h) in raw pork meat were studied. The results demonstrated that an increase in salting duration significantly affected the lipid oxidation. The amount of COPs showed little change over a 12-h salting period, while a sharp increase was observed after that. Particularly, the levels of total COPs increased about threefold after 24 h and tenfold after 48 h, with 7-keto and 7-OH being the predominant COPs (Fig. 2).

In the overall picture, the presence of high levels of COPs in the samples under study represents an important toxicological risk and should raise concerns. Considering the daily intake of these food products, a large part of Cypriot population is exposed to COPs, and this could be associated to the incidence of atherosclerosis and other health problems.

4 Concluding remarks

This work represents the first application of UPLC-MS/MS for the analysis of COPs in foodstuffs. The good performance of the particular analytical methodology comes from different relevant aspects. The use of UPLC has allowed chromatographic run time down to 4.2 min per sample, while the use of MS/MS with triple quadrupole analyzer has allowed the acquisition of two SRM transitions per compound with good sensitivity, providing, in turn, a reliable confirmation of COPs detected in samples. The optimized method demonstrated satisfactory validation parameters, including linearity range, precision, LOD, LOQ, and recovery. Finally, the applicability of the method was confirmed by analyzing different food samples. Particularly, this study represented a first evaluation of the extent of cholesterol oxidation in traditional Cypriot foodstuffs. In all the analyzed samples, large amounts of COPs were found, with the 7-keto and 7-OH being the most abundant COPs. The results underlined that the regular consumption of the aforementioned foods can be a source of considerable amounts of COPs in the diet of the local population in Cyprus.

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