European Food Research and Technology (2024) 250:483–493
https://doi.org/10.1007/s00217-023-04404-9

ORIGINAL PAPER

Determination of cholesterol oxidation products in cheese under photo-oxidative stress using QuEChERS and LC–MS

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Received: 25 July 2023 / Revised: 25 October 2023 / Accepted: 27 October 2023 / Published online: 18 November 2023

Abstract

The QuEChERS approach was optimized for extracting cholesterol oxidation products in cheese, followed by LC-APCI-MS/MS analysis. Optimization of the method, including evaluations of saponification step, sample weight, and d-SPE purification resulted in good recoveries for each analyte, ensuring a reliable determination of these contaminants in cheese samples. In addition, the method was successfully validated by testing linearity of response, analytical limits (LOD and LLOQ), and precision. Sliced cheese samples wrapped in various packaging materials underwent a challenging test to simulate refrigerated storage conditions under fluorescent light, inducing photo-oxidative stress. The validated QuEChERS method revealed that only seasoned hard cow's cheese showed an increase in the concentration of 7-ketocholesterol and its chemical precursors, 7β-hydroxycholesterol, and 7α-hydroxycholesterol, reaching levels of 0.45, 0.35, and 0.35 μg g^{-1}, respectively. Conversely, opaque packaging and the use of a double film were found to be effective in preventing the formation of COPs in cheese samples subjected to photo-oxidative stress, such as smoked cheese and melted cheese slices (*sottilette*). A trade-off must be found between ensuring cheese protection and meeting the consumer's desire to see the product.

Keywords Dairy products contaminants · Cheese · Packaging · Food safety

Abbreviations

ACN	Acetonitrile
APCI	Atmospheric pressure chemical ionization
CAD	Collision-activated dissociation
CE	Collision energy
CEP	Collision cell entrance potential
COP	Cholesterol oxidation product
CUR	Curtain gas
CXP	Collision cell exit potential
DP	Declustering potential
d-SPE	Dispersive solid-phase extraction
EP	Entrance potential
ESI	Electrospray ionization
FIA	Flow injection mode
GC	Gas chromatography
GCh	Grated cheese
GS1	Nebuliser gas
GS2	Heater gas
HPLC	High-performance liquid chromatography
LC	Liquid chromatography
LLOQ	Lower limit of quantification
LOD	Limit of detection
MS/MS	Tandem mass spectrometry
MS^2	Product ion scan
NC	Nebuliser current
PDO	Protected designation of origin
PES	Polyethylene sulfone
PSA	Primary secondary amine
QuEChERS	Quick, easy, cheap, effective, robust or rugged and safe
R^2	Coefficient of determination
RP	Reversed phase
RSD	Relative standard deviation
Q	Quadrupole

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SD	Standard deviation
SHCC	Seasoned hard cow's cheese
S/N	Signal to noise ratio
SPE	Solid-phase extraction
SRM	Selective reaction monitoring
ST	Standard
TEM	APCI temperature

Introduction

Cholesterol, a steroid molecule with a double bond on Carbon-5, is susceptible to oxidation, resulting in the formation of cholesterol oxidation products (COPs). These COPs have been associated with various health conditions, including atherosclerosis, coronary heart disease, apoptosis, and neurodegenerative diseases [1, 2]. Their cytotoxic, mutagenic, and potential carcinogenic effects are a cause for great concern [3].

COPs can be produced through non-enzymatic mechanisms, including rapid autoxidation of cholesterol and cholesterol esters. In vivo, they can also be generated by monooxygenases, primarily from the cytochrome P450 family [4]. Food processing and food storage involve physical and chemical factors, such as light, radiation, heat, oxygen, free radicals, metal ions, and enzymes, which contribute to COPs formation [3, 5–7]. Photosensitizers such as chlorophyll, phaeophytin, myoglobin, riboflavin, and porphyrins under visible light activation can further promote the generation of COPs [8–12]. For these reasons, cholesterol, as well as unsaturated fatty acids including polyunsaturated fatty acids (PUFA), are ideal substrates for this degradation [13, 14].

A detailed description of the mechanism of COP production and evolution has been given by many authors [5, 15–18]. Given the complexity of this oxidative process, the studies on COPs are now focused on kinetic modeling as a predictive tool to monitor the thermodynamically governed reaction pathways [14, 19, 20]. The application of predictive models to specific food processing represents a strategy to monitor and, in turn, to reduce COP accumulation in the final products [19].

Cheese, depending on factors such as milk origin, fat content, and ripening stage, may contain moderate amounts of COPs [9, 16, 21, 22]. Riboflavin naturally present in cheese [10, 12, 23], along with processes such as salting, smoking, and air exposure [24], can contribute to COPs accumulation. Packaging materials used for cheese storage offer some protection against photo-oxidative stress caused by fluorescent lamps in supermarket refrigerators [25]; however, prolonged exposure can still lead to COPs accumulation [26].

Many efforts were made to improve the detectability of COPs concentrations in food. Since COPs mainly occur in very low concentrations, extraction and clean-up are the critical steps in ensuring efficient recovery and minimizing the formation of artifacts [27, 28]. Moreover, COPs are liposoluble compounds, so they need to be extracted by an organic solvent. However, a single non-polar solvent, such as hexane, is not suitable. The best choice would be a relatively polar solvent or a combination of some [29, 30].

To accurately detect low concentrations of COPs in food, efficient extraction and clean-up steps are essential. An ideal approach is the Quick, Easy, Cheap, Effective, Robust or Rugged, and Safe (QuEChERS) technique. It has been successfully applied for the determination of pesticide residues [31], drugs in blood, veterinary drugs in animal tissue [32, 33], hormones in meat [34], and acrylamide and *Alternaria alternata* mycotoxin detection in food [35–37]. For COPs determination, gas chromatography (GC) and high-performance liquid chromatography (HPLC) are commonly used analytical techniques. HPLC coupled with tandem MS/MS offers selective detection and improved signal-to-noise ratio [38], without requiring the derivatization step needed in GC [39, 40].

The objective of this study was to set up a reliable analytical method based on the QuEChERS approach to detect COPs in cheese. In addition, through an application experiment that emulated in-store conditions, the study also aimed to assess the effectiveness of various packaging materials in safeguarding cheese from surface cholesterol degradation caused by photo-oxidation.

Materials and methods

Sampling

Five different types of cheese were acquired from the Italian market for this study: grated cheese (GCh), two different protected designation of origin (PDO) seasoned hard cow's cheeses, melted cheese slices (*sottilette*), and smoked cheese. GCh was chosen as a matrix model to evaluate the method's performance for dairy products due to its conditions (high surface-to-volume ratio exposed to air) that promote cholesterol oxidation reactions [41]. Therefore, it was selected for all the experiments to set up and optimize the method.

Chemicals and instruments

High purity standards of 3,5-cholestadiene-7-one (7-K-3,5-CD), 5α-cholestane-3,5,6-triol (CT), 5-cholesten-3β-ol-7-one (7-ketocholesterol, 7-KC), cholesterol, cholesterol-5α-6α-epoxide (α-CE), cholesterol-5β-6β-epoxide (β-CE), 7α-hydroxycholesterol (7α-HC), 7β-hydroxycholesterol (7β-HC), and 25-hydroxycholesterol

(25-HC) were obtained from Sigma-Aldrich® (Merck S.p.A., Milan, Italy).

The solubility of COPs was verified in acetonitrile (ACN) and methanol (MeOH). Standard stock solutions (100 mg L^{-1}) for each compound were prepared using ACN as the solvent. Working standard solutions were created by diluting the stock solutions with ACN and stored at -18 °C, protected from light.

All solvents and reagents used were of analytical reagent grade. Acetonitrile (Chromasolv purity for LC–MS), methanol (Chromasolv purity for LC–MS), *n*-hexane, and diethyl ether were purchased from Fluka-Sigma-Aldrich® (Merck S.p.A., Milan, Italy). Anhydrous sodium sulfate and potassium hydroxide (KOH) were obtained from Carlo Erba Reagents S.p.A. (Rodano, Milan, Italy). Deionized water was obtained through an Elix 3UV purification system (Merck S.p.A., Milan, Italy).

COPs extraction

Optimization of QuEChERS protocol for COPs extraction

Recovery tests were conducted under various conditions to optimize the QuEChERS procedure. The general QuEChERS process involves grinding and homogenization of solid samples, which are then suspended in an aqueous medium and extracted with an organic solvent that is miscible with water. Dehydrating salts are introduced to the sample to remove water, making it unavailable for dissolving less polar analytes. During the extraction process, acetonitrile separates and forms a distinct layer on top, effectively dissolving the analytes. After centrifugation, further sample clean-up is performed using the d-SPE technique.

Considering that cholesterol oxidation products (COPs) can be bound to ester bonds and are present in the lipid layer, a preliminary saponification step was evaluated to optimize the QuEChERS extraction protocol. The objective was to achieve the best performance in terms of percentage recoveries of the analytes while minimizing artifact formation due to strong saponification conditions. Two saponification times (5 min and 20 min) were tested using 40% KOH at room temperature on GCh as a reference sample. Acetonitrile was chosen as the extraction solvent since all standard compounds were soluble in it. In addition, the sample weight, and the effects of purification by dispersive SPE (d-SPE) were carefully assessed.

To determine quantitative data on recoveries, GCh was spiked with a mixture of all pure reference standard COPs (250 µg mL^{-1}, each dissolved in ACN) before saponification and allowed to stand for 30 min to enhance the interaction between the matrix and the analytes. The analysis was conducted in triplicate for all samples.

Optimized QuEChERS and d-SPE protocol for COPs extraction

After conducting the optimization tests, the adopted extraction protocol is as follows: the finely ground cheese sample (1 g) was weighed precisely and placed in a 50 mL-Falcon tube along with 10 mL of a 40% KOH aqueous solution and a ceramic homogenizer. The tube was protected from light during the 20-min saponification process (under shaking), after which 10 mL of acetonitrile was added and manually shaken for 1 min. Subsequently, a mixture of salts (8.0 g $MgSO_4$ + 2.0 g CH_3COONa) from tube 1 (DisQue QuEChERS kit, Waters S.p.A., Milan, Italy) was added to the Falcon tube, stirred for 1 min, and then centrifuged for 5 min at 3000 rpm. An aliquot (1.5 mL) of the supernatant was transferred to tube 2 (DisQue QuEChERS kit, Waters S.p.A., Milan, Italy), containing 25 mg of primary/secondary amine (PSA) + 150 mg of $MgSO_4$, for further purification with d-SPE. The extract was again centrifuged for 5 min at 3000 rpm, and the supernatant was filtered using a 0.2-µm syringe filter before being transferred into HPLC vials for COPs determination.

Chromatographic method

Optimization of mass spectrometry parameters

Prior to chromatographic optimization, the mass spectrometry parameters were fine-tuned. The most abundant fragments for each analyte were used to optimize the MS/MS "compound dependent" (declustering potential, DP; entrance potential, EP; collision energy, CE; collision cell entrance potential, CEP; collision cell exit potential, CXP) and "source dependent" (curtain gas, CUR; collision-activated dissociation, CAD; nebulizer current, NC; nebulizer gas, GS1; heater gas, GS2; and source temperature, TEM) parameters using standard solutions of analytes individually introduced into the detector. Flow injection mode (FIA) was used to optimize "source-dependent" parameters using MeOH/H_2O (90:10 v/v) as the mobile phase. Two precursor-to-product ion transitions (selective reaction monitoring—SRM) were used for each compound in the 200–450 *m*/*z* range. The most intense SRM was used as a quantifier ion (SRM_1), while the least intense was used as a qualifier ion (SRM_2). Nitrogen was used as the desolvation and vaporization gas.

LC-APCI-MS/MS optimized analytical conditions

COPs determination was performed using a liquid chromatography–mass spectrometry (LC–MS/MS) system (Agilent, Waldbronn, Germany) consisting of a gas generator (API; Peak Scientific Billerica, MA, USA), an HPLC

(Agilent 1200) equipped with a degasser, a binary pump, an autosampler, a thermostatic column compartment, and a triple quadrupole mass spectrometer (API 3200, AB Sciex Italia Srl, Milano, Italy). Samples (5 µL) were injected after filtration (0.45 µm, PES syringe filter) into a 150 mm × 3.0 mm i.d. × 3 µm particle size RP-C_{18} column (Luna, Phenomenex, Torrance, CA, USA).

The solvent system consisted of $(NH_4)_2CO_3$ (3 mM; solvent A) and methanol (solvent B), and the elution was carried out with the following gradient: 90% B (0.00 min), 90% B (22.00 min), 91% B (22.01 min), 91% B (32.00 min), 96% B (32.01 min), 96% B (40.00 min), 100% B (40.01 min), 100% B (50.00 min), 90% B (50.01 min), and 90% B (60.00 min), with a flow rate of 0.4 mL min^{-1} at room temperature.

The approach used to identify the peaks included the comparison of their retention times with those obtained using solutions of chemical standards and said retention times were confirmed using both the SRMs. The relative intensity of each peak (SRM_2 to SRM_1 percentage ratio) was also evaluated and verified as being within an uncertainty range of ± 10%. Quantification was performed by external standard calibration. The chromatograms were acquired and processed by Analyst software version 1.5 (AB Sciex Italia Srl, Milan, Italy).

Validation of COPs extraction method

To validate the COPs extraction method, increasing concentration solutions of COPs (ranging from 0.10 to 2.50 µg g^{-1} for each COP) and cholesterol (ranging from 0.01 to 0.10 mg g^{-1}) were prepared using ACN as the solvent. The linearity of the instrumental response within the expected concentration range for each substance was evaluated. The limit of detection (LOD) and the lower limit of quantification (LLOQ) were calculated based on the signal-to-noise ratio data [42]:

$$y_{LOD} = y_{Noise} + 3s_{Noise} \text{ and } y_{LLOQ} = y_{Noise} + 6s_{Noise}.$$

Precision was assessed through an intra-day repeatability test, where four extractions and injections were performed on both seasoned hard cow's cheese samples spiked with a mixture of COPs standard solution (250 µg mL^{-1} each in ACN, added before saponification and allowed to stand for 30 min to enhance the interaction between the matrix and the analytes).

The trueness was evaluated through recovery tests, where all types of cheese were spiked with known amounts of the COPs standard solution (250 µg mL^{-1} each in ACN) to optimize the QuEChERS, d-SPE extraction protocol, and determination by LC–MS/MS. The analysis was performed in triplicate for all the samples. These samples were subjected to extraction and LC–MS/MS determination, as described above.

Experiment of photo-oxidative stress exposure of various types of cheese

To assess the effectiveness of packaging materials in maintaining the quality and safety of the cheese by minimizing COPs formation, a certain number of pre-sliced samples, i.e., seasoned hard cow's cheese, smoked cheese, and *sottilette* were exposed to fluorescent light in their original packaging materials for 40 days at 4 °C, simulating typical supermarket storage conditions (approximately 27 cm distance from the light source). The light source was a fluorescent tube lamp (Master TL-D, Philips; length 59 cm × diameter 26 mm) of 18 W of power, 1300 Lm of luminous flux, and 4300 lx of light intensity. Fluorescent light has a spectral power distribution in the visible region. Controls of each of the same cheese samples were not subjected to the photo-oxidation experiment and were stored in the dark for 40 days at 4 °C.

As for the seasoned hard cow's cheese, two different types of it packaged with stretched film were chosen for the experiment. This packaging material was mostly transparent, composed of biaxially oriented polyamide coupled with polyethylene/ethylene–vinyl acetate (PE/EVA). The *sottilette* slices were enclosed in an opaque external packaging material (polyester coupled with polyethylene/ethylene–vinyl alcohol, PE/EVOH) fully covered by images, providing shielding. In addition, each slice had an extra protective transparent film (polypropylene). Finally, the smoked cheese was stored in a tray made of amorphous polyethylene terephthalate coextruded with PE/EVOH, covered by a partially transparent packaging film (polyester coupled with polyethylene).

A square section of 4 cm^2 × 1 mm of thickness (about 1 g, exactly weighed) was used for each determination. The sections were always taken superficially but from different parts of the cheese slices to obtain representative samples for each different light exposure. Analyses were performed in triplicate for each sample.

Results and discussion

Effect of saponification time on COPs recoveries

To optimize COPs recoveries, different saponification times were tested. Saponification time was considered a critical parameter affecting recoveries for two reasons [43]: (i) short saponification time might not be sufficient to completely release COPs from the matrix; (ii) the longer the time of saponification, the higher the artifacts formation.

In the present study (Table 1), it was observed that longer saponification time (20 min) resulted in better recoveries for

Table 1 (A) Recoveries of COPs (%) carried out on GCh using two different saponification times (5 min and 20 min; KOH 40%), (B) Recoveries of COPs (%) carried out on samples (1 g and 2.5 g grated cheese—GCh) extracted with QuEChERS device (addition of 8.0 g $MgSO_4$ + 2.0 g of CH_3COONa—tube 1—and extraction with ACN), following saponification with KOH (40%; 5 min). Recoveries obtained following purification with d-SPE (tube 2) are also showed for both weights, (C) Recoveries of COPs (%) carried out with QuEChERS and d-SPE method and applied to two different seasoned hard cow's cheeses, smoked cheese, and melted cheese slices (*sottilette*)

	A		B				C					
	Saponification (recovery %)		Sample weight and d-SPE (recovery %)				Type of cheese (recovery %)					
Analyte	5 min	20 min	1 g GCh		2.5 g GCh		Seasoned hard cow's cheese 1	Seasoned hard cow's cheese 2	Smoked cheese	*Sottilette*	Mean	RSD (%)
			Tube 1	Tube 2	Tube 1	Tube 2						
25-HC[a]	61	109	58	61	51	67	96	111	107	101	105	6
CT[b]	73	92	70	73	54	50	99	98	98	86	95	6
7β-HC[c]	39	95	55	39	41	62	119	108	124	111	113	13
7α-HC[d]	80	85	74	80	68	58	112	111	103	108	104	11
7-KC[e]	82	118	62	82	61	65	112	106	112	95	111	11
β-CE[f]	47	108	50	47	44	43	99	98	84	94	97	9
α-CE[g]	26	76	24	26	14	15	64	64	60	68	66	9
7-K-3,5-CD[h]	98	103	69	98	37	28	78	67	66	90	81	19

RSD relative standard deviation

[a]25-Hydroxycholesterol

[b]5α-Cholestane-3,5,6-triol

[c]7β-Hydroxycholesterol

[d]7α-Hydroxycholesterol

[e]5-Cholesten-3β-ol-7-one (7-ketocholesterol)

[f]Cholesterol-5β-6β-epoxide

[g]Cholesterol-5α-6α-epoxide

[h]3,5-Cholestadiene-7-one

all cholesterol oxidation products (COPs), whereas shorter saponification time (5 min) might not have been sufficient to fully release COPs from the matrix. Indeed, under the latter conditions, the recoveries were from 4.9% to 65.8% lower compared to those obtained with 20-min saponification time. After having confirmed this hypothesis, it was also essential to explore the potential presence of artifacts.

Busch and King [43] have reported the effect of saponification on 7-KC and cholesterol stability in several conditions, i.e., low and high alkaline (1 M and 3.6 M KOH, respectively), low, medium, and high temperature (24 °C, 37 °C, and 45 °C, respectively), and short and long time (3 h and 18 h, respectively). Using short saponification time at high temperature, 7-KC has been observed to be converted into its dehydration product (7-K-3,5-CD) in higher amount compared to saponification carried out for longer times at medium temperatures.

This observation led to the evaluation of sample temperatures. It was noted that under the QuEChERS protocol, the addition of anhydrous $MgSO_4$ induced an exothermic-type reaction. However, it was deemed not detrimental for sample stability as it only lasted for a few seconds. Moreover, it has been confirmed by Park et al. [44] that 7-KC, the most susceptible COP to degradation by alkali, experienced negligible degradation during saponification at room temperature.

Despite these findings, we decided to conduct a more in-depth investigation to verify the presence of 7-K-3,5-CD, as an artifact resulting from the degradation of 7-KC, following the conditions specified in the QuEChERS protocol. Interestingly, 7-K-3,5-CD was not detected in any of the samples, including both GCh samples (1 g and 2.5 g) fortified with 7-KC (0.25 μg g^{-1}) alone or with a reference standard solution (0.25 μg g^{-1} each) containing all COPs except 7-K-3,5-CD.

Effects of sample weight and d-SPE purification on recoveries

Since GCh is a mixture of unknown cheese types, it was selected as the reference sample due to its high variability. In addition, GCh is more prone to degradation, making it suitable for testing optimization parameters [22, 45]. The recoveries of the test ranged from 24 to 74% for 1 g of sample and from 14 to 68% for 2.5 g of sample (Table 1).

Despite the unsatisfactory recoveries for 7β-HC, β-CE, and α-CE, using 1 g of the sample yielded better results for each analyte. Consequently, the weight of 1 g was chosen for further optimization steps.

For most analytes (using 1 g of sample), d-SPE (tube 2, PSA sorbent) improved the recovery, proving to be a necessary step during COPs extraction (Table 1). It effectively removed matrix components such as free fatty acids and pigments. The presence of a ceramic homogenizer in the tube throughout the analysis helped break up salt agglomerates, promoting consistent sample extraction.

Effect of cheese matrix on recoveries by spiking

Based on the satisfactory recovery data obtained for GCh and the absence of 7-K-3,5-CD as an artifact, a saponification at low temperature with 40% KOH for 20 min was selected using QuEChERS and d-SPE devices. This method was tested on all types of cheese used in the experimentation to evaluate the performance of QuEChERS and d-SPE. The recoveries were slightly influenced by the different cheese matrices (Table 1). The QuEChERS and d-SPE method demonstrated good recoveries, ranging on average from 66 to 113%, with most analytes above 80%. Correction factors based on the recoveries for each type of cheese were applied for calculations on real samples. Cholesterol was present in the QuEChERS extracts, but not in a quantitative way. For this reason and because cholesterol determination was not the goal of this work, it was no longer considered.

Mass spectrometry parameters and chromatography setup

APCI was chosen as the ion source over ESI because it had provided a higher response in COPs determination [46]. The similarity among COPs chemical structures made the spectrometric optimization rather challenging, and the chromatographic separation of analytes became crucial. TEM was the parameter that most affected the COPs response and was set at 550 °C for all COPs. High temperature was necessary to avoid the condensation of both solvent and sample in the orifice plate. Although the best TEM for 7-KC and cholesterol were 400 °C and 350 °C, respectively [46], all the other COPs gave a better response at 550 °C. Some conditions were kept constant for all analytes: CUR, 103 kPa; CAD, 48 kPa; NC, 28 kPa; GS1, 310 kPa; GS2, 172 kPa.

The Q1 multiple ion was used to optimize the analyzer for specific ions, setting "compound dependent" parameters such as DP, EP, CE, CXP, along with CEP (Table 2). This maximized the signal in preparation for the MS^2 (product ion scan) and SRM optimization mode. For most COPs, the parent ion resulted from the loss of one or two water molecules (Table 2). Conversely, 7-KC and 7-K-3,5-CD showed the precursor $[M+H]^+$ (Table 2). The selection of the quantifier and qualifier transitions was based on transitions from the molecular ion to the most and second most predominant fragment ions, respectively (Table 2).

The RP-C_{18} Luna column performed well with 10% water in the mobile phase, stabilizing at a pressure of 18,000–19,000 kPa. The addition of water solution provided a better separation, although with an inevitably longer run time. The solvent system was optimized to achieve the best separation of COPs, although there were two overlapping peaks (7β-HC and 7α-HC) (Fig. 1). This drawback was solved by introducing $(NH_4)_2CO_3$ (3 mM in water) in the solvent system. This salt helped maintain pH control and reduced noise.

Validation of COPs extraction method by QuEChERS and d-SPE (evaluation of linearity, detectability, and precision)

The linearity of the method was evaluated by building calibration curves using COP standard solutions [47] ranging from 0.10 to 2.50 μg g^{-1}. All calibration curves showed high coefficients of determination (R^2) higher than 0.999, indicating excellent linearity.

Detectability was assessed by calculating the limit of detection (LOD) and the lower limit of quantification (LLOQ) using standard solutions of each COP and considering the level of noise (Table 3). However, the method included a tenfold dilution of the sample, and for this reason, the detectability limits were multiplied by that factor.

In the literature, the matrix models to evaluate the method performance for dairy products are usually milk powder and grated cheese. Both products present conditions that enhance cholesterol oxidation reactions [41]. The LODs and LLOQs were determined based on the level of noise in the data and were found to be lower (0.05–0.25 μg g^{-1}, and 0.10–0.50 μg g^{-1}, respectively) than those reported in experiments on grated cheese [48–50] and other types of cheese. [24]. In addition, none of these studies included 7-K-3,5-CD in the experiment.

Precision was evaluated through an intra-day repeatability test. Four repetitions of the extraction procedure (QuEChERS-d-SPE) and LC–MS determination were performed on two seasoned hard cow's cheeses. The relative standard deviations (RSDs) were all lower than 25% for all COPs detected, indicating good precision.

Results of the photo-oxidative experiment

Table 4 shows the results of the photo-oxidative stress experiment, which was conducted to evaluate the protective effect of packaging materials on cheese under such stress. Control samples (not exposed to light) of seasoned hard cow's cheese

Table 2 List of the molecular weights (MW), retention times (t_R), parent and products ions obtained after MS parameters optimization and main MS parameters optimized for each compound

Analyte	Molecular weight (g mol^{-1})	t_R (min)	Parent ion (*m/z*)	Quantifier ion (*m/z*)	Qualifier ion (*m/z*)	Transition (*m/z*)	DP[a] (V)	CE[b] (V)	CXP[c] (V)	EP[d] (V)	CEP[e] (V)
25-Hydroxycholesterol (25-HC)	402.65	9.95	385.5 $[M+H—H_2O]^+$	367.5 $[M+H—2H_2O]^+$	213.2	385.5/367.5 Q[f]	40.75	22.53	6.37	8.47	32.18
						385.5/213.2 q[g]		32.57	5.20		
5-α-Cholestane-3,5,6, triol (CT)	420.68	15.39	385.4 $[M+H—2H_2O]^+$	367.4 $[M+H—3H_2O]^+$	123.10	385.4/367.4 Q	51.62	26.41	6.19	8.05	32.18
						385.4/123.1 q		36.18	4.00		
7-β Hydroxycholesterol (7β-HC)	402.70	21.00	385.4 $[M+H—2H_2O]^+$	367.4 $[M+H—3H_2O]^+$	159.2	385.4/367.4 Q	34.80	24.71	6.02	8.48	32.17
						385.4/159.2 q		39.66	4.00		
7-α Hydroxycholesterol (7α-HC)	402.70	21.46	385.4 $[M+H—2H_2O]^+$	367.5 $[M+H—3H_2O]^+$	159.1	385.4/367.5 Q	43.10	19.16	6.04	8.60	32.18
						385.4/159.1 q		34.40	4.00		
5-Cholesten-3β-ol-7-one (7-KC)	400.64	23.28	401.5 $[M+H]^+$	383.3 $[M+H—H_2O]^+$	365.2	401.5/383.3 Q	61.70	28.95	4.00	6.13	32.61
						401.5/365.2 q		34.10	6.32		
Cholesterol 5β-6β-epoxide (β-CE)	402.65	30.83	385.4 $[M+H—H_2O]^+$	367.4 $[M+H—2H_2O]^+$	159.3	385.4/367.4 Q	47.00	22.20	7.14	8.49	32.18
						385.4/159.3 q		37.41	4.00		
Cholesterol 5α-6α-epoxide (α-CE)	402.65	34.00	385.5 $[M+H—H_2O]^+$	367.3 $[M+H—2H_2O]^+$	213.2	385.5/367.3 Q	48.55	25.25	6.00	8.67	32.18
						385.5/213.2 q		35.16	6.25		
3,5 Cholestadiene-7-one (7-K, 3,5-CD)	382.6	42.16	383.4 $[M+H]^+$	247.4	157.3	383.4/247.4 Q	68.16	35.72	4.60	8.79	32.12
						383.4/157.3 q		40.59	4.00		
Cholesterol	386.65	45.00	385.3 $[M—H]^-$	367.2 $[M—H—H_2O]^-$	159.2	385.3/367.2 Q	40.40	23.34	5.53	8.31	32.18

[a]Declustering potential

[b]Collision energy

[c]Collision cell exit potential

[d]Entrance potential

[e]Collision cell entrance potential

[f]Quantifier transition (SRM_1)

[g]Qualifier transition (SRM_2)

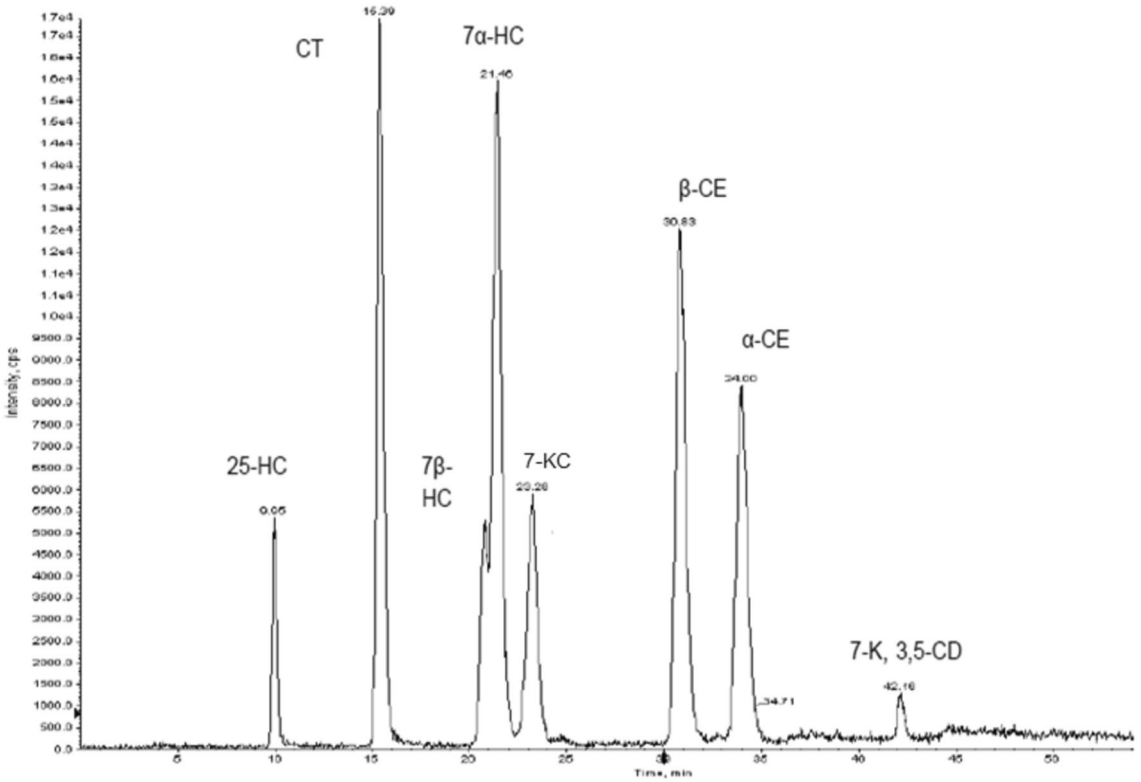

Fig. 1 Chromatogram of a reference standard solution containing COPs. 25-HC: 25-Hydroxycholesterol; CT: 5α-Cholestane-3,5,6-triol; 7β-HC: 7β-Hydroxycholesterol; 7α-HC: 7α-Hydroxycholesterol; 7-KC: 5-Cholesten-3β-ol-7-one (7-Ketocholesterol); β-CE: Cholesterol-5β-6β-epoxide; α-CE: Cholesterol-5α-6α-epoxide; 7-K-3,5-CD: 3,5-Cholestadiene-7-one

Table 3 Main parameters of the method validation for standard compounds. Evaluation of linearity (R^2), straight-line equations, limits of detection (LOD), and lower limits of quantification (LLOQ)

Analyte	Calibration range (μg g^{-1})	R^2	LOD (μg g^{-1})	LLOQ (μg g^{-1})
25-HC[a]	0.10–2.50	0.9998	0.10	0.25
CT[b]	0.10–2.50	0.9994	0.10	0.25
7β-HC[c]	0.10–2.50	0.9998	0.10	0.25
7α-HC[d]	0.10–2.50	0.9998	0.10	0.25
7-KC[e]	0.10–2.50	0.9992	0.05	0.10
β-CE[f]	0.10–2.50	0.9998	0.10	0.50
α-CE[g]	0.10–2.50	0.9996	0.10	0.25
7-K-3,5-CD[h]	0.10–2.50	0.9990	0.25	0.50

[a]25-Hydroxycholesterol
[b]5α-Cholestane-3,5,6-triol
[c]7β-Hydroxycholesterol
[d]7α-Hydroxycholesterol
[e]5-Cholesten-3β-ol-7-one (7-ketocholesterol)
[f]Cholesterol-5β-6β-epoxide
[g]Cholesterol-5α-6α-epoxide
[h]3,5-Cholestadiene-7-one

and smoked cheese contained only 7-KC, with concentrations around 0.10 μg g^{-1}, while the sections of seasoned hard cow's cheese most exposed to light showed higher concentrations of 7-KC and its chemical precursors, 7β-HC and 7α-HC, ranging from 0.30 to 0.45 μg g^{-1}. However, the concentrations decreased in sections less exposed to light.

Smoked cheese exhibited a different behavior, as the concentration of 7-KC remained almost unchanged compared to the control (0.11 μg g^{-1}), regardless of the different exposure areas. Finally, *sottilette* samples did not show COPs concentrations higher than their LODs (Table 4), in both the control and the samples subjected to photo-oxidative stress.

Cheese is usually sold in small pieces with large surface areas exposed to light, and it is wrapped in transparent packaging materials that allow consumers to observe the product [26]. This transparency is reduced by printed text and pictures, although these opaque areas protect the cheese from light [25].

In this experiment, seasoned hard cow's cheese was the cheese most exposed to light due to the packaging system's features and the scarcity of pigmented areas. Indeed, aside from 7-HC, 7β-HC and 7α-HC were also found in quite high concentrations. Conversely, the larger opacity of the

Table 4 Concentration of COPs (μg g^{-1}) ± standard deviation (n = 3) found in the experiment of photo-oxidative stress on three types of cheese

Sample	Treatment	7β-HC[a]	7α-HC[b]	7-KC[c]	Sum
Seasoned hard cow's cheese (section of 4 cm^2 to 1 mm of thickness—about 1 g)	Control stored in the dark	< LOD	< LOD	0.09 ± 0.02	0.09
	On the side of the cheese most exposed to light	0.35 ± 0.07	0.35 ± 0.07	0.45 ± 0.09	1.15
	On the side of the cheese most exposed to light	0.40 ± 0.08	< LLOQ	0.31 ± 0.06	0.71
	On the side of the cheese most exposed to light, but covered by an image	< LOD	< LLOQ	0.19 ± 0.04	0.19
	On the side of the cheese not exposed to light	< LOD	< LOD	0.23 ± 0.05	0.23
Smoked cheese (section of 4 cm^2 to 1 mm of thickness—about 1 g)	Control stored in the dark	< LOD	< LOD	0.11 ± 0.02	0.11
	On the side of the cheese most exposed to light	< LOD	< LOD	0.15 ± 0.03	0.15
	On the side of the cheese most exposed to light	< LOD	< LOD	0.11 ± 0.02	0.11
	On the side of the cheese most exposed to light, but covered by an image	< LOD	< LOD	< LLOQ	=
	On the side of the cheese not exposed to light	< LOD	< LOD	0.11 ± 0.02	0.11
Sottilette (section of 4 cm^2 to 1 mm of thickness—about 1 g)	Control stored in the dark	< LOD	< LOD	< LOD	=
	Left side of the topmost *sottiletta* exposed to light	< LOD	< LOD	< LOD	=
	Right side of the topmost *sottiletta* exposed to light	< LOD	< LOD	< LOD	=
	Central part of the topmost *sottiletta* exposed to light (part covered by an image)	< LOD	< LOD	< LLOQ	=
	Section of the second *sottiletta* (therefore, not exposed to light)	< LOD	< LOD	< LLOQ	=

Control samples were not subjected to the photo-oxidation experiment and were stored in the dark. The analyses were performed in triplicate

[a]7β-hydroxycholesterol

[b]7α-hydroxycholesterol

[c]5-Cholesten-3β-ol-7-one (7-ketocholesterol)

packaging materials used for smoked cheese and *sottilette* proved to be effective in avoiding photo-oxidative occurrences of cholesterol.

7-KC was confirmed to be the main chemical marker of photo-oxidation damage in dairy products [20, 51]. It accumulates during storage, although at a different rate as a consequence of different conditions. In addition, at low temperature, 7-KC concentration is stable for a long time (even after 6 months) [49].

Conclusions

In conclusion, the QuEChERS approach combined with LC-APCI-MS-Triple Quadrupole equipment proved to be a powerful and efficient method for determining COPs in cheese. It offers several advantages, including fast and reliable analysis, reduced solvent usage, and direct injection of purified samples without the need for derivatization as required in GC determination of COPs.

The validated extraction method allowed to demonstrate that different types of cheese contain varying amounts of COPs due to storage conditions, exposure to fluorescent light in refrigerators, and packaging materials. Opaque packaging and the use of a double film were found to be effective in preventing the formation of COPs in cheese samples subjected to photo-oxidative stress. This research study provides valuable insights into the levels of COPs in different cheese types and their susceptibility to photo-oxidation, which can have implications for cheese freshness, safety, and compliance with food regulations, benefiting both consumers and the dairy industry.

Cheese factories can consider using packaging materials that strike a balance between transparency and protection, such as utilizing a double-layered film or incorporating windows into the packaging. In this way, consumers can still view the product while ensuring adequate protection from light-induced COPs formation, maintaining both product appeal and safety.

Acknowledgements The authors wish to thank Dr. Laura Munari (English-language reviewer), Dr. Filippo Genovese and Diego Pinetti (C.I.G.S. of the University of Modena and Reggio Emilia), Prof. Fabio Licciardello (Dept. Life Science of the University of Modena and Reggio Emilia) and Rita Rivola (Coop Italia) for their valuable contribution in the drafting of the present article.

Funding This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Data availability All data included in this manuscript are available upon request by contacting with the corresponding author.

Declarations

Conflict of interest The authors have no competing interests to declare that are relevant to the content of this article.

Compliance with ethics requirements This article does not contain any studies on human or animal subjects.

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