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Short communication

A new LC/APCI-MS method for the determination of cholesterol oxidation products in food

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Abstract

Cholesterol oxidation products (COPs) can be formed in the body or in animal foods from cholesterol during food processing. A new method for the extraction and quantification of cholesterol, 7-ketocholesterol, cholestane-3β-5α-6β-triol, 25-hydroxycholesterol, 5,6α-epoxycholesterol, and 7β-hydroxycholesterol by means of reversed-phase LC/atmospheric pressure chemical ionization mass spectrometry is presented. A baseline separation of all COPs was achieved, allowing a separate quantification also for isobaric compounds. The limits of detection were 15–30 ng/mL, quantification was performed from 100 ng/mL to 10 μg/mL with RSD < 2%. The method was applied successfully to the determination of cholesterol and COPs in processed foods such as pork, beef, chicken, and egg.

Keywords: Cholesterol oxidation products; Liquid chromatography; APCI; Evaporative light scattering; Food

1. Introduction

Cholesterol oxidation products (COPs) are a number of compounds that are formed from cholesterol during storage or processing, particularly under thermal treatment. As an unsaturated alcohol, cholesterol is susceptible to oxidation in the presence of light, oxygen and elevated temperatures [1,2]. The toxicological relevance of the COPs results from their potentially atherogenic, cytotoxic, mutagenic and possibly carcinogenic effects [3–10]. COPs are formed either endogenously due to the oxidation of unsaturated fatty acids or by uptake with food. Therefore, analytical methods to determine the amount of COPs in food are of great importance.

The analysis of COPs requires a chromatographic separation and a more or less selective and sensitive detection.

GC with electron impact ionization (El) [11–14] usually provides good separation and sensitivity. However, elevated temperatures can cause oxidation artefacts. Another well-known drawback is the need for derivatization to make the substances volatile for GC. When headspace injection is not available, non-volatile compounds can enter the column, so that a reliable routine analysis is not possible.

Liquid chromatography (LC) enables the analysis of compounds in solution without the need for volatility. COPs have been analyzed using normal-phase [15–20] as well as reversed-phase separation [21–24].

With UV detection, the sensitivity for COPs will be poor because of the lack of a chromophore. Nevertheless, such approaches have been reported [16,23]. One way to overcome this is derivatization [22], but this additional step is usually unwanted and may increase RSDs in quantification. LC/MS opens access to more selective detection. However, its prerequisite is efficient ionization. For the relatively non-polar COPs, standard electrospray ionization (ESI) is not very efficient. Atmospheric pressure chemical ionization is more suitable for these lipid-related compounds. Razzazi-Fazeli

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et al. have presented an LC/APCI-MS method for COPs [24].

Even though MS detection is considered more specific than UV, there are still a number of isobaric compounds that cannot be distinguished by using mass spectra alone. Therefore, a good, preferably baseline separation, is much more important than usual for LC/MS and GC/MS. In that respect, most of the cited references show there is a need for improvement. Therefore, the goal of the present work was to develop a sensitive, robust and reliable method allowing identification and quantification of COPs such as 7-ketocholesterol, cholestane-3β-5α-6β-triol, 25-hydroxycholesterol, 5,6α-epoxycholesterol, and 7β-hydroxycholesterol not only in model mixtures, but also in food samples.

2. Materials and methods

2.1. Chemicals

Acetonitrile, methanol and water of HPLC quality were from Baker (Deventer, The Netherlands). The standards 7-ketocholesterol, cholestane-3β-5α-6β-triol, 25-hydroxycholesterol, 5,6α-epoxycholesterol, 7β-hydroxycholesterol and cholesterol were all from Sigma (Deisenhofen, Germany). Betamethasone-17,19-dipropionate, which was used as internal standard for LC/MS analysis, was from Synopharm (Barsbüttel, Germany).

2.2. Sample preparation

For lipid extraction the method according to [25] was used. Five grams of the material to be extracted were weighed and extracted with *n*-hexane/isopropanol 3:2 for 18–20 h. The cholesterol fraction was isolated using preparative normal-phase LC on a 5 μm Chromasil 100 LC column (125 mm × 4.6 mm). The compounds were detected using an evaporative light scattering detector SEDEX 55 (SEDERE, Alfortville, France). Elution was done with *n*-hexane, *n*-hexane/dichloromethane, and methanol, consecutively. The flow rate was 1.4 mL/min for each run, 100 μL lipid solution were injected. The fraction containing cholesterol and COPs eluted from 21.1 to 26.1 min. It was evaporated to dryness in a glass vial and stored at −80 °C. Before analysis, the sample was carefully thawed and solved in mobile phase. The recovery rate was better than 90%.

2.3. LC/APCI-MS

LC was performed using a 5 μm ODS AQ C_{18} column (150 mm × 4 mm) from YMC Europe (Schermbeck, Germany). Elution was performed with isocratic methanol/acetonitrile/water (87:6:7, v/v) at a flow rate of 1 mL/min.

An HPLC pump Spectra System P 4000 with a vacuum degasser SCM 1000 and an autosampler AS 3000 was coupled to an ion trap mass spectrometer Finnigan LCQ equipped with an APCI interface (all from Thermo Electron, San Jose, CA, USA).

The COP samples were dissolved in mobile phase under cooling to avoid further oxidation.

For calibration purposes, an external calibration curve was set up every day. All measurements were repeated threefold. An internal standard was added to correct for matrix effects. The injection volume was 20 μL. The APCI conditions were: vaporizer temperature, 500 °C, corona discharge current, 5 μA.

3. Results and discussion

Next to cholesterol itself, the five most important cholesterol oxidation products (COPs) had to be analyzed: 7-ketocholesterol, cholestane-3β-5α-6β-triol, 25-hydroxycholesterol, and 5,6α-epoxycholesterol. Unfortunately, the latter three have the same molecular weight, and as isobaric compounds they cannot be distinguished from each other by means of MS detection.

Tandem mass spectrometry (MS/MS) based on collision-induced dissociation only results in non-specific fragmentations, namely loss of water. Cholesterol itself can lose one, the COPs also a second molecule of water (and cholestane-3β-5α-6β-triol even a third one), but it remains unclear from which position. The energy in CID is normally not sufficient to split stable carbon–carbon bonds; therefore, it is not useful for structure elucidation. Unequivocal identification with the help of APCI-MS is therefore currently not possible. In other words, a good LC separation is mandatory, in particular for the isobaric COPs.

In the course of separation method development and optimisation different modes of detection have been used, e.g. evaporative light scattering detection (ELSD). A mixture of methanol/acetonitrile/water turned out to be most promising and the composition was optimized.

Among the tested reversed-phase columns, an ODS-AQ column was found highly suitable. Columns with three i.d.s were tested, 1, 2, and 4 mm. Although 1 mm is best for separation efficiency and solvent consumption, this column and even the 2 mm i.d. column caused problems with increasing back pressure. Since robustness and compatibility with APCI-MS (which works best with flow rates around 1 mL/min) are crucial, the conventional 4 mm i.d. column was preferred.

A new LC/APCI-MS procedure was set up (see Section 2.3). Compared to the one used in [24], a different LC eluent was chosen which enabled baseline separation of the COPs. Furthermore, the quantification of cholesterol itself was possible in the same run, and the analysis time for the COPs was shorter. For optimal sensitivity, MS detection was performed in the selected ion monitoring (SIM) mode. The following

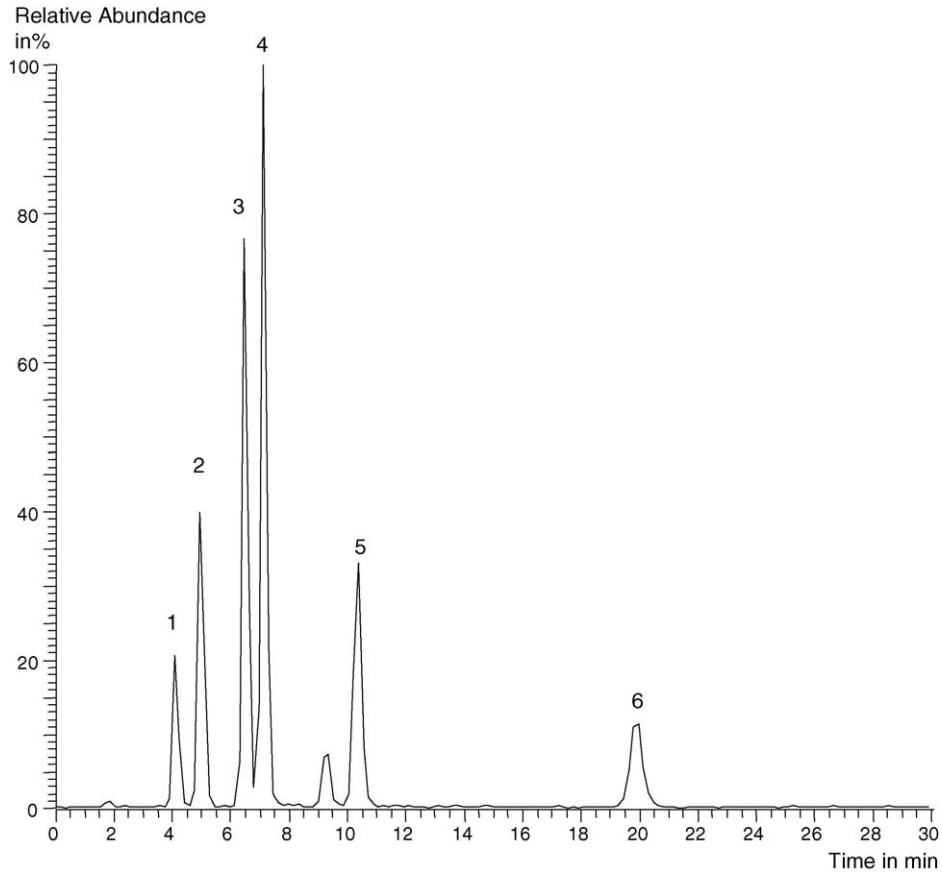

Fig. 1. LC/APCI-MS of COP standards. (1) 25-Hydroxycholesterol, (2) cholestane-3β-5α-6β-triol, (3) 7β-hydroxycholesterol, (4) 7-ketocholesterol, (5) 5,6α-epoxycholesterol, (6) cholesterol. See text for details.

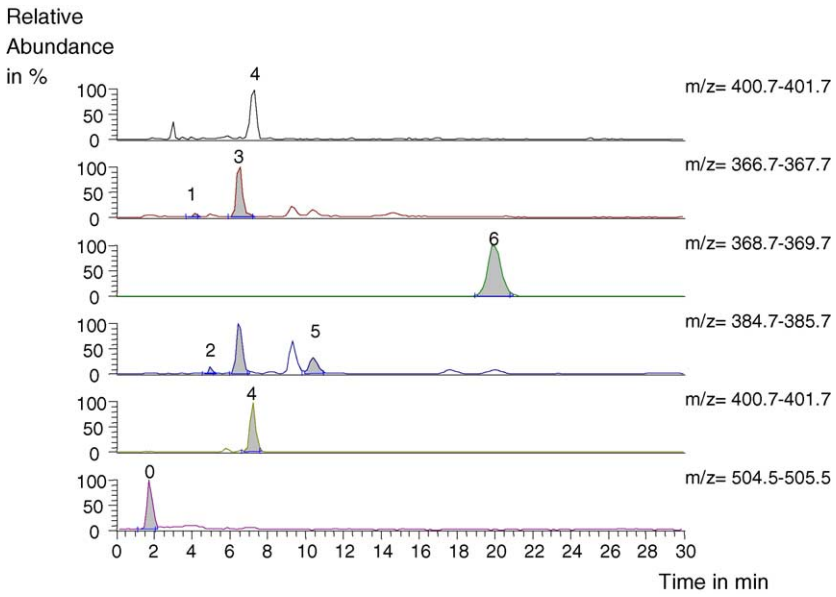

Fig. 2. LC/APCI-MS of COP's in egg extract. (0) Internal standard betamethasone-17,19-dipropionate, (1) 25-hydroxycholesterol, (2) cholestane-3β-5α-6β-triol, (3) 7β-hydroxycholesterol, (4) 7-ketocholesterol, (5) 5,6α-epoxycholesterol, (6) cholesterol. See text for details.

mass traces (*m*/*z*) were recorded:

m/*z*	Compound	Fragment
367.20	25-Hydroxycholesterol; 5,6-epoxycholesterol; 7ß-hydroxycholesterol	$[M+H-2H_2O]^+$
369.20	Cholesterol	$[M+H-H_2O]^+$
385.20	Cholestane-3β-5α-6β-triol	$[M+H-2H_2O]^+$
	25-Hydroxycholesterol	$[M+H-H_2O]^+$
	5,6-Epoxycholesterol	$[M+H-H_2O]^+$
	7ß-Hydroxycholesterol	$[M+H-H_2O]^+$
401.20	7-Ketocholesterol	$[M+H]^+$
505.00	Betamethasone	$[M+H]^+$

Fig. 1 shows chromatograms obtained with LC/APCI-MS of a standard mixture. The method turned out to be robust with biological samples; no instability was found. Betamethasone-17,19-dipropionate was chosen as internal standard since it does not interfere with cholesterol and COP elution and/or ionization.

The limits of detection (S/N 3:1) were 15–30 ng/mL. Calibration curves showed good linearity ($R^2 = 0.99$ or better) over two orders of magnitude. Quantification was performed in the range from 100 ng/mL (LOQ) to 10 μg/mL (HOQ) with a precision better than 2% (RSD; $n = 5$). The LC/APCI-MS method was used for the determination of cholesterol and COPs in extracts from heated bacon, muscle meat from chop (both from pork), heated chicken meat, fresh egg yolk, and heated freeze-dried egg (Fig. 2). Relevant results are given in Table 1. It shows that COPs were found to be present in all samples. As expected, the concentrations were particularly high in heated egg samples, while in meat samples there was not always an increase after processing.

4. Conclusion

Extraction of COPs using normal-phase LC on silica has several important advantages. It is easier to control and stan-

Table 1
COP concentrations typically found in food samples ($n = 3$, RSD 2–10%)

Sample	Compound	Concentration (μg/mL)	Concentration (ng/g tissue)
Pork, heated	7-Ketocholesterol	0.036	10.8
	Cholestanetriol	0.122	36.6
	25-OH-cholesterol	0.007	2.1
	α-Epoxycholesterol	0.066	19.8
	7β-OH-cholesterol	0.009	2.7
	Cholesterol	0.352	105.6
Pork chop (muscle meat), heated	7-Ketocholesterol	0.25	50.8
	Cholestanetriol	0.19	38.0
	25-OH-cholesterol	0.00	–
	α-Epoxycholesterol	0.20	40.2
	7β-OH-cholesterol	0.14	28.6
	Cholesterol	1.17	233.4
Pork chop (muscle meat), untreated	7-Ketocholesterol	1.73	345.4
	Cholestanetriol	0.55	109.6
	25-OH-cholesterol	0.17	34.0
	α-Epoxycholesterol	1.39	277.2
	7β-OH-cholesterol	2.78	556.4
	Cholesterol	1.52	304.2
Broiler (muscle meat), heated	7-Ketocholesterol	1.02	204.2
	Cholestanetriol	0.35	69.4
	25-OH-cholesterol	0.09	18.4
	α-Epoxycholesterol	0.00	–
	7β-OH-cholesterol	2.16	431.4
	Cholesterol	114.14	22827.2
Egg, freeze-dried and heated	7-Ketocholesterol	1.38	275.6
	Cholestanetriol	0.48	97.0
	25-OH-cholesterol	0.25	50.2
	α-Epoxycholesterol	1.55	309.8
	7β-OH-cholesterol	2.58	515.8
	Cholesterol	61.4	12282.2
Egg yolk, untreated	7-Ketocholesterol	0.15	30.4
	Cholestanetriol	0.38	76.2
	25-OH-cholesterol	0.10	19.2
	α-Epoxycholesterol	0.51	102.4
	7β-OH-cholesterol	0.60	120.2
	Cholesterol	219.55	43910.2

dardize and less time-consuming than SPE. The composition of the extraction solvent is kept constant and the fractionation can be controlled by using ELS detection. The analytes are protected from light that can catalyze oxidation. Contact to plastic surfaces is avoided, because extracted additives such as plasticizers can cause disturbing peaks in mass spectrometry.

The content of COPs can vary depending on the source of a particular tissue (species, anatomic location, even feeding). A careful tissue preparation (under cooling to avoid further oxidation) before extraction is recommended to improve extraction efficiency.

A new reversed-phase LC/APCI-MS method for the separation and quantification of cholesterol and the five most important COPs was applied successfully to their determination in processed food (bacon, pork meat, beef, chicken, egg). A baseline separation was achieved in 20 min which allowed the separate quantification also for isobaric COPs. The method is rugged, no derivatization is necessary and no artefact formation was observed.

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