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MS–MS Fragmentation Patterns of Cholesterol Oxidation Products

Birgit Rossmann, Karin Thurner, and **Wolfgang Luf** *

Department of Veterinary Public Health, Veterinary University of Vienna, Vienna, Austria

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Summary. This study was aimed at analyzing the daughter ion spectra of 7 toxicologically relevant cholesterol oxidation products (*COP*s) *i.e.* cholestanetriol (cholestane-3β,5α,6β-triol), 7-ketocholesterol (cholest-5-en-3β-ol-7-one), 7α-hydroxycholesterol (cholest-5-en-3β,7α-diol), 7β-hydroxycholesterol (cholest-5-en-3β,7β-diol), 25-hydroxycholesterol (cholest-5-en-3β,25-diol), α-epoxycholesterol (cholestane-5α,6α-epoxy-3β-ol) and β-epoxycholesterol (cholestane-5β,6β-epoxy-3β-ol). In addition, 19-hydroxycholesterol (cholest-5-en-3β,19-diol) was analyzed as this serves as internal standard in *COP*s determination by HPLC-MS.

Mass spectrometry was performed using a triple quadrupol mass spectrometer that was equipped with an APCI ion source.

Our results indicate a common fragmentation pattern for *COP*s. The main breaking sites identified were in the sterol ring system between the carbon atoms with the position numbers 11–12, 12–13, and 8–14. Typical daughter ions of $m/z=$ 81, 95, and 195 were used for multiple reaction monitoring analysis.

Keywords. Fragmentation pattern; High pressure liquid chromatography; Mass spectrometry; Oxides.

Introduction

Cholesterol oxidation products (*COP*s) are formed by oxidation of cholesterol *via* either a photosensitive or an autooxidative pathway. So far as many as 80 different species have been identified [1]. In view of their toxicological relevance this study focused on the following seven *COP*s: cholestanetriol (cholestane-3β,5α,6β-triol), 7-ketocholesterol (cholest-5-en-3β-ol-7-one), 7α-hydroxycholesterol (cholest-5-en-3β,7α-diol), 7β-hydroxycholesterol (cholest-5-en-3β,7β-diol), 25-hydroxycholesterol (cholest-5-en-3β,25-diol), α-epoxycholesterol (cholestane-5α,6α-epoxy-3β-ol), β-epoxycholesterol (cholestane-5β,6β-epoxy-3β-ol). All these compounds have been implicated in the context of atherosclerosis and some are reported to be potential carcinogens [2].

*COP*s are found in raw and processed food of animal origin like meat, fish, milk and eggs [3] and have been shown to be absorbed in the human gastrointestinal tract [4, 5].

The determination of cholesterol oxidation products is difficult since there is a broad variety of food matrices. To date, many analytical methods have been developed essentially based on thin-layer chromatography (TLC) [6], gas chromatography (GC) [7] and high performance liquid chromatography (HPLC) [8]. Mass spectrometry has proven to be rather practicable for GC [9] and especially for HPLC [10]. *A priori* UV detection is less suitable since the toxicologically most interesting epoxides show no UV absorption [11]. They can only be detected after derivatization *e.g.* benzoylation [12]. Mass spectrometry based on atmospheric pressure chemical ionization (APCI) can be used for all *COP*s without further derivatization steps [10].

The use of tandem mass spectrometry improves the selectivity and therefore diminishes matrix interference which is always critical in food analysis. So far HPLC-MSMS based on APCI has been applied for determination of chemically related steroid hormones and their metabolites [13–17] and the

* Corresponding author. E-mail: wolfgang.luf@vu-wien.ac.at

endogenous cholesterol precursor lanosterol [18]. In addition, tandem mass spectrometry based on (nano) electrospray ionization (ESI) has been successfully conducted with cholesterol [19], cholesterol and dehydrocholesterol [20] and sterol epoxides [21]. Occasionally, ESI ionization of the rather lipophilic sterols has been reported as problematic [22] therefore it was not the method of choice for this work. Derivatization of oxysterols into *Girard* P hydrazones has been shown to function reasonably well for proper electrospray ionization [22].

Purpose of this study was to show whether and in which chemical pathway APCI-MSMS of the seven *COP*s of interest would generate specific daughter ions that could be utilized for multiple reaction monitoring (MRM) analysis.

Results and Discussions

Daughter Ion Spectra

To characterize the MSMS-transitions of the eight oxysterols, spectra of pure standards were acquired. The results are summarized in Table 1.

As parent ions the dominant ions formed in MS1 were chosen. These have also been successfully applied by *Razzazi-Fazeli et al.* in their LC-APCI-MS method [10].

Conforming m/z values clearly show that *COP*s are fragmented in the collision cell in a comparable way. Two main fragments of the sterol molecule are formed: one with $m/z = 159$ and one with $m/z = 95$ or 81.

Table 1. Overview of the daughter ions with the highest intensity

COP	Molecular mass / Da	Parent ion mass (m/z) [formation]	Daughter ions with the highest intensity (m/z)	
Triol	420.6	367.4 [MH-3H_2O]	159.0	95.0
		385.4 [MH-2H_2O]	158.9	95.0
7-OH	402.6	367.4 [MH-2H_2O]	158.9	81.1
25-OH	402.6	367.4 [MH-2H_2O]	81.1	95.0
7-Keto	400.6	383.4 [MH-H_2O]	81.1	95.0
		401.4 $[MH]^+$	95.1	81.1
5,6-Ep	402.6	367.4 [MH-2H_2O]	158.9	95.0
		385.4 [MH-H_2O]	159.0	95.0
19-OH	402.6	367.4 [MH-2H_2O]	95.0	159.0
		355.4 $[MH\text{-}12]^+$	147.0	95.0

Triol cholestanetriol, *7-OH* 7-hydroxycholesterol, *25-OH* 25-hydroxycholesterol, *7-Keto* 7-ketocholesterol, *5,6-Ep* 5,6-epoxycholesterol, *19-OH* 19-hydroxycholesterol

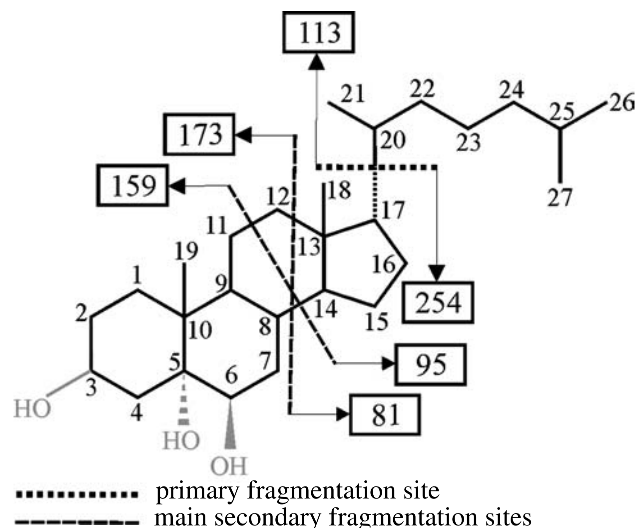

Fig. 1. Main fragments of sterols. The fragmentation process is demonstrated here by using cholestanetriol as a representative for the other *COP*s

For the asymmetric structure of the sterol carbon skeleton it is possible to identify the fragmentation points of the molecule as shown in Fig. 1.

The calculations of the fragment masses are based on the mass of a completely or partially dehydrated and single protonated parent ion as usually formed in MS1. The formation of these parent ions is also indicated in Table 1.

We assume the first step during the fragmentation process is disconnection of the aliphatic side chain and the sterol ring system which would result in two primary fragments with $m/z = 113$ and 254.

Because the conditions in the collision cell are rather rough further fragmentations occur. The incidence of these processes is so high that the side chain fragment and the main sterol fragment are hardly detectable.

The side chain fragment appears to further break into very small parts that are below the detected mass range. It is the sterol ring fragment that causes the typical MSMS spectra found.

Considering the masses of the daughter ions, two main fragmentation sites can be identified, *i.e.* one is positioned between the carbon atoms with the IUPAC numbers 11–12 and 8–14, the other between 12–13 and 8–14.

*COP*s differ from each other only in the position of their OH-, epoxy- or keto-groups. In the collision cell they are all split off by dehydrogenation and subsequently replaced by double bounds. The result-

ing molecular structure is therefore the same for cholestanetriol, 7-hydroxycholesterol, 7-ketocholesterol and 5,6-epoxycholesterol and of equal mass distribution for 19-hydroxycholesterol. Their daughter ion spectra show the same fragment combinations and only differ in the intensities of the masses found (see below).

The stereoisomeric compounds 7α- and 7β-hydroxycholesterol as well as 5α,6α- and 5β,6β-epoxycholesterol result in the same MS1 spectra and the MS–MS transitions are equal too. Neither the ionization nor the fragmentation seems to be influenced by the steric orientation of the OH- or epoxy-group.

For 25-hydroxycholesterol the situation is slightly different. The alcoholic group is positioned at the carbon atom with the number 25 in the side chain. OH-, keto-, and epoxy-groups are split off in MS1 through dehydration. Since there are two of these groups in the sterol ring system of all other *COP*s two dehydrations take place. As the sterol ring system of 25-hydroxycholesterol includes merely one OH-group dehydration takes place only once, which leads to a shift of the fragment masses from the usual $m/z = 159$ to 161.

Figures 2–7 include the scans of the parent and daughter ions based on the precursor ions with the highest intensities.

The data have been processed as follows: spectrum combine (Cm), smoothed (Sm, method: *Savitzky Golay, SG*), and centroid (Cn).

In addition to the main fragments, a pattern consisting of about 20 bands of masses appears in the daughter ion spectra. These can be interpreted as the minor fragmentation sites. The carbon skeleton that contains 20 carbon atoms in the sterol ring system appears to break at each possible position which is indicated by the 14 Da (CH_2) mass differences of the bands.

To demonstrate that these results are equally valid for other sterols, additional MS–MS analyses of cholesterol, sitosterol and stigmasterol were conducted. The daughter ion spectra (data not shown) indicated the same fragmentation pattern as for the eight *COP*s. The main fragments were represented by the masses $m/z = 161$ and 95 that again resulted from the breaking site between the carbon atoms 11–12 and 8–14.

Our data are in agreement with the findings of *Trösken et al.* in whose study on lanosterin d_6-cho-

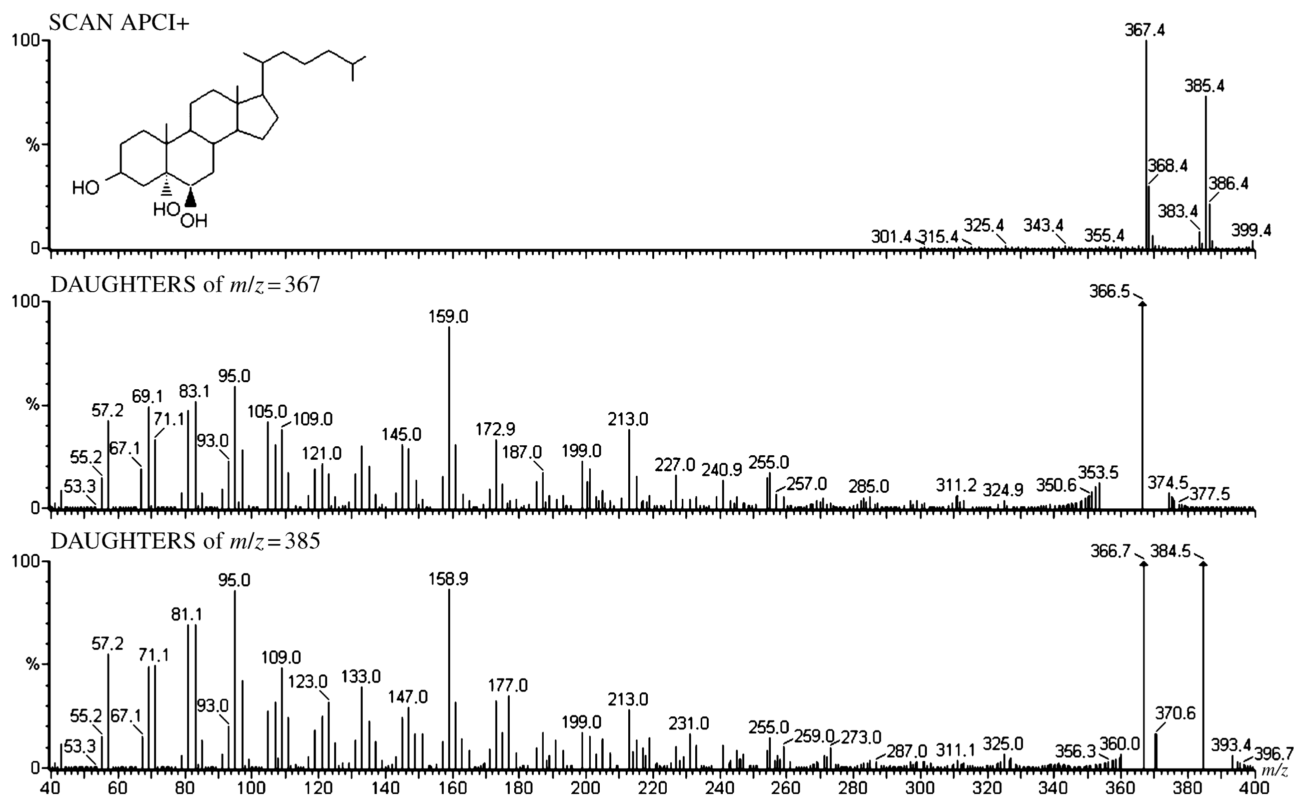

Fig. 2. Daughter ions of cholestanetriol. c(Triol): 100 μg/cm^3, scan APCI+: 300–500 m/z (V_{inj}: 50 mm^3), daughter ion scan: 40–400 m/z

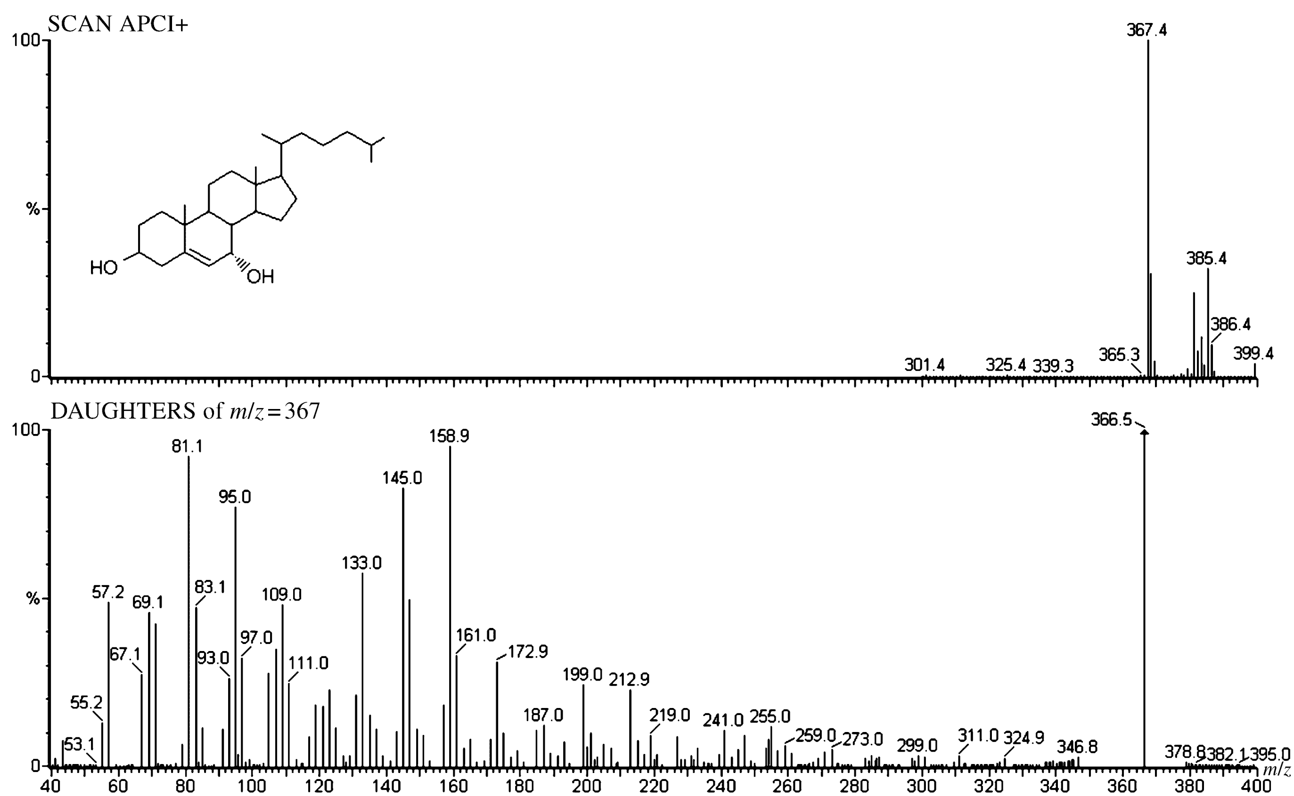

Fig. 3. Daughter ions of 7α-hydroxycholesterol. c(7α-OH): 100 μg/cm^3, scan APCI+: 300–500 m/z (V_{inj}: 50 mm^3), daughter ion scan: 40–400 m/z

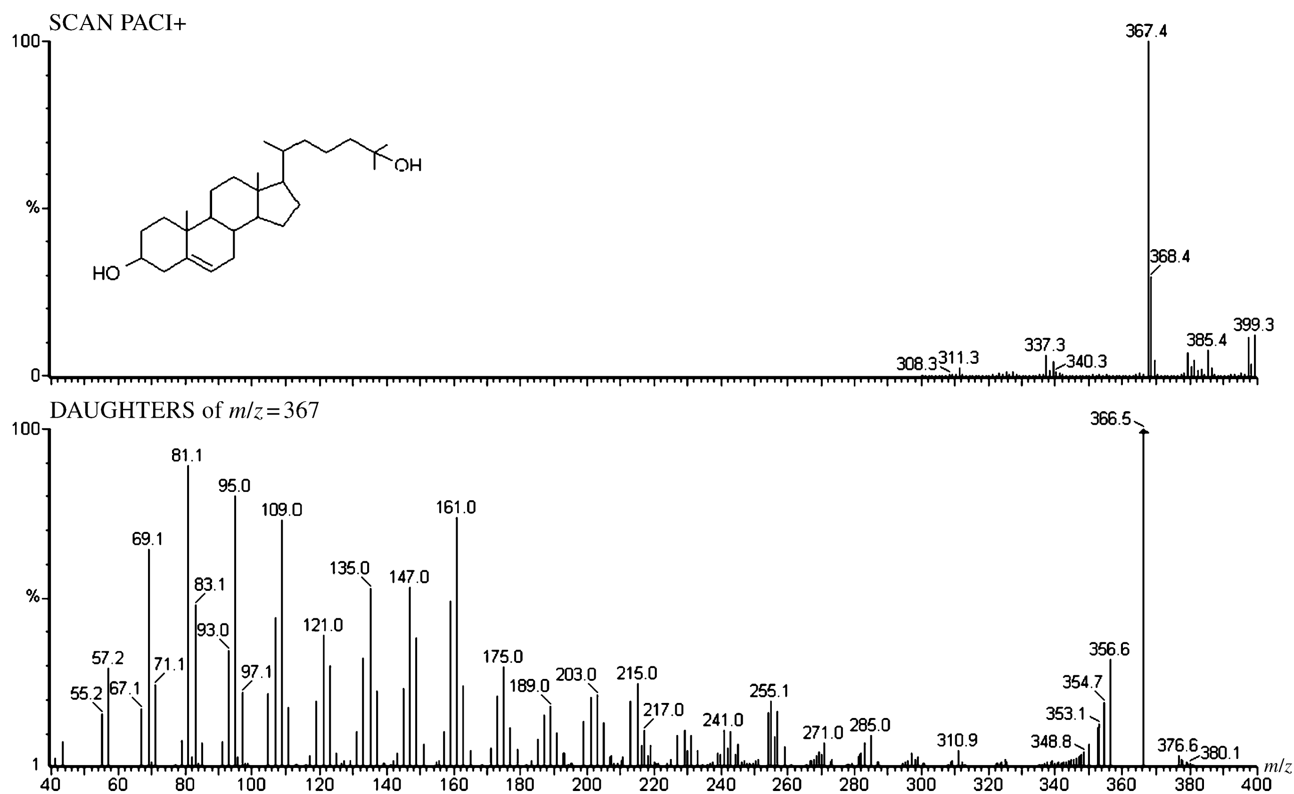

Fig. 4. Daughter ions of 25-hydroxycholesterol. c(25-OH): 100 μg/cm^3, scan APCI+: 300–500 m/z (V_{inj}: 50 mm^3), daughter ion scan: 40–400 m/z

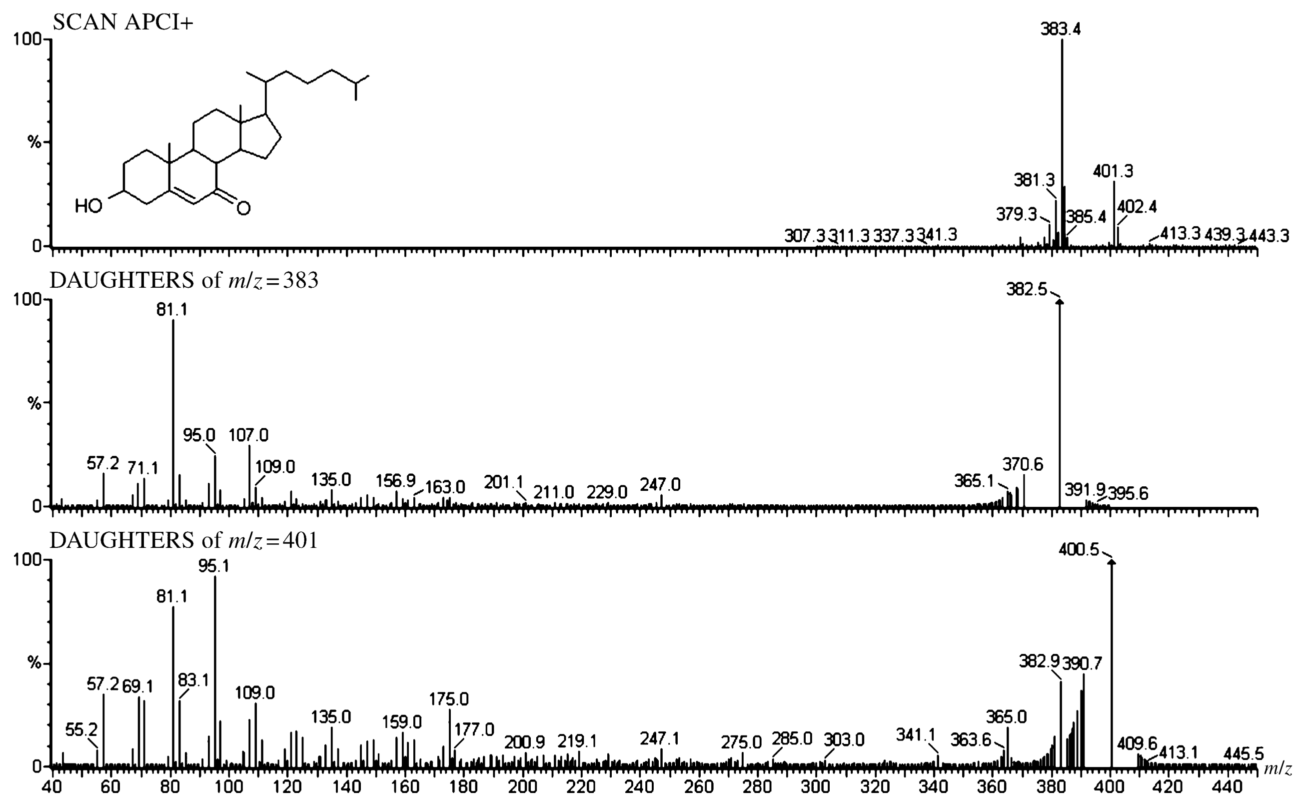

Fig. 5. Daughter ions of 7-ketocholesterol. c(7-Keto): 100 μg/cm^3, scan APCI+: 300–500 m/z (V_{inj}: 50 mm^3), daughter ion scan: 40–450 m/z

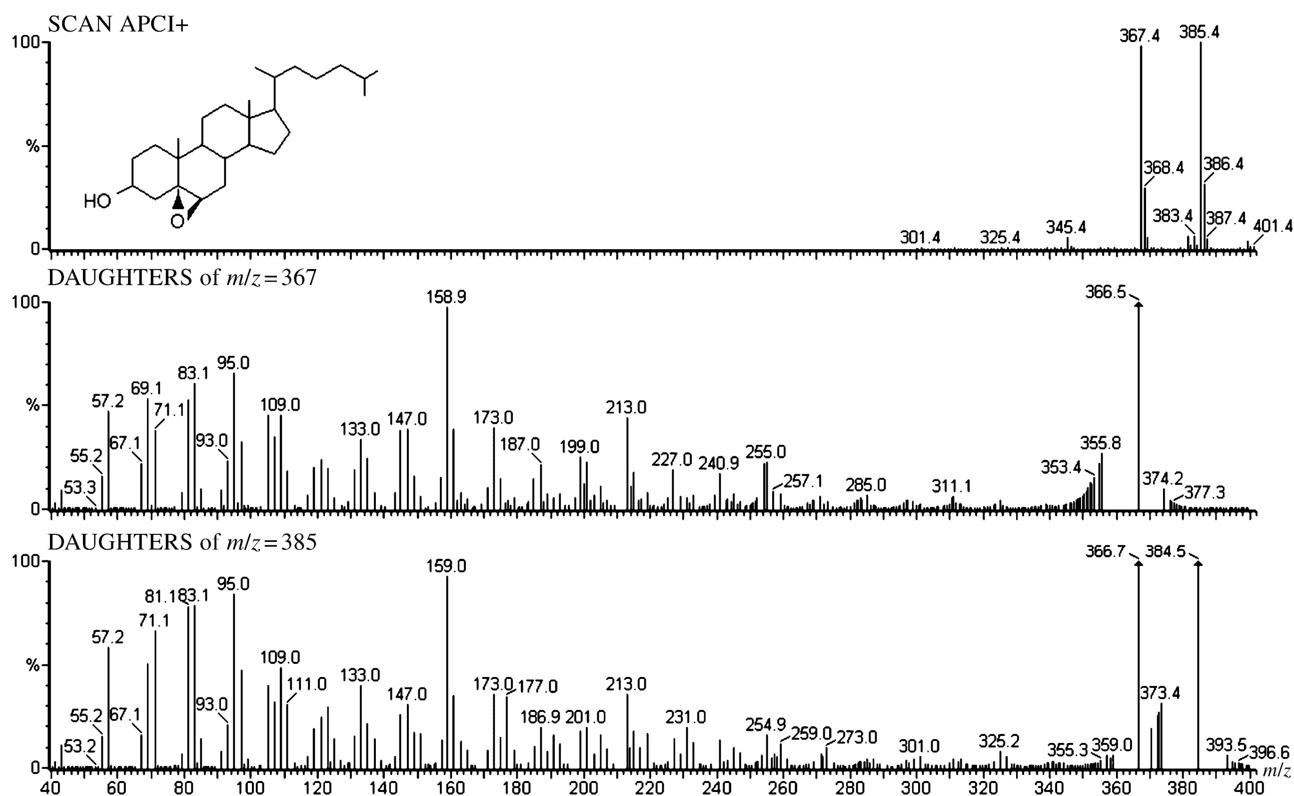

Fig. 6. Daughter ions of 5β,6β-epoxycholesterol. c(5β,6β-Ep): 100 μg/cm^3, scan APCI+: 300–500 m/z (V_{inj}: 50 mm^3), daughter ion scan: 40–400 m/z

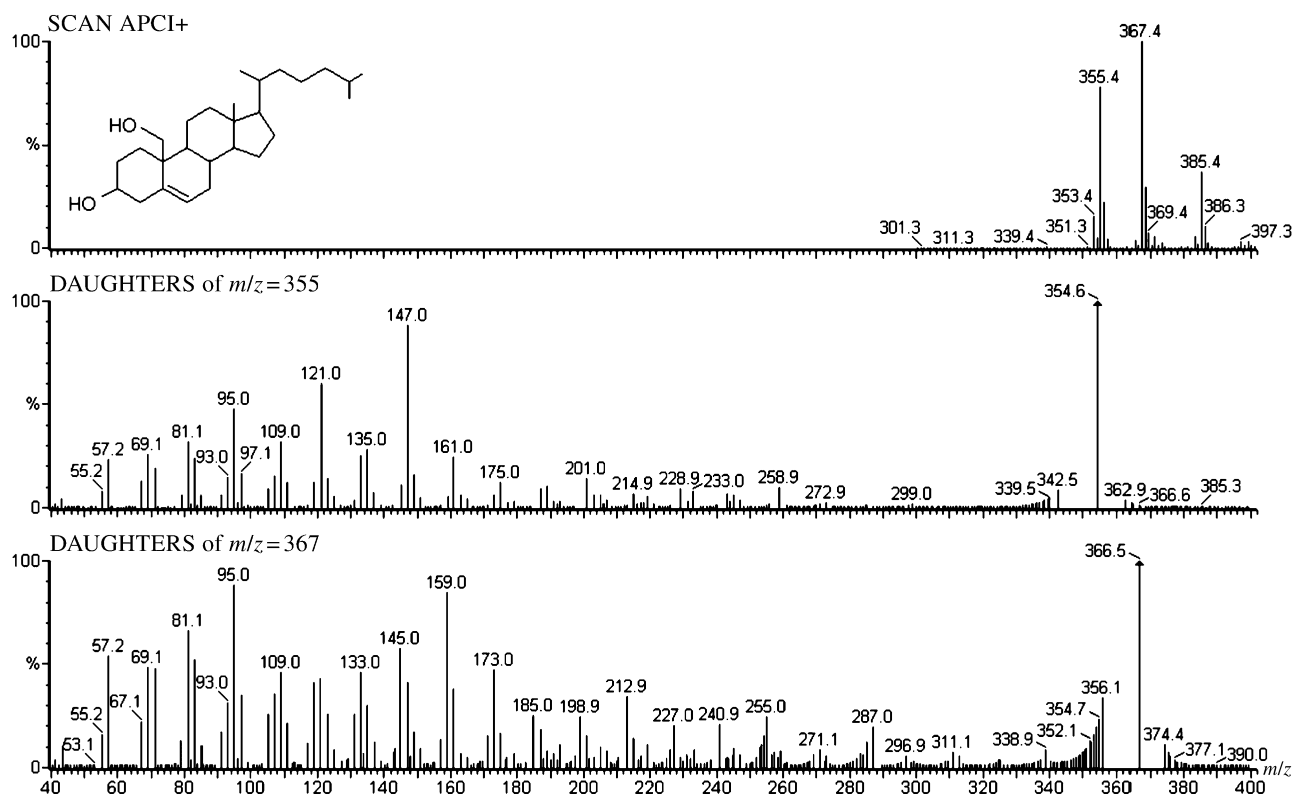

Fig. 7. Daughter ions of 19-hydroxycholesterol. c(19-OH): 100 μg/cm^3, scan APCI+: 300–500 m/z (V_{inj}: 50 mm^3), daughter ion scan: 40–400 m/z

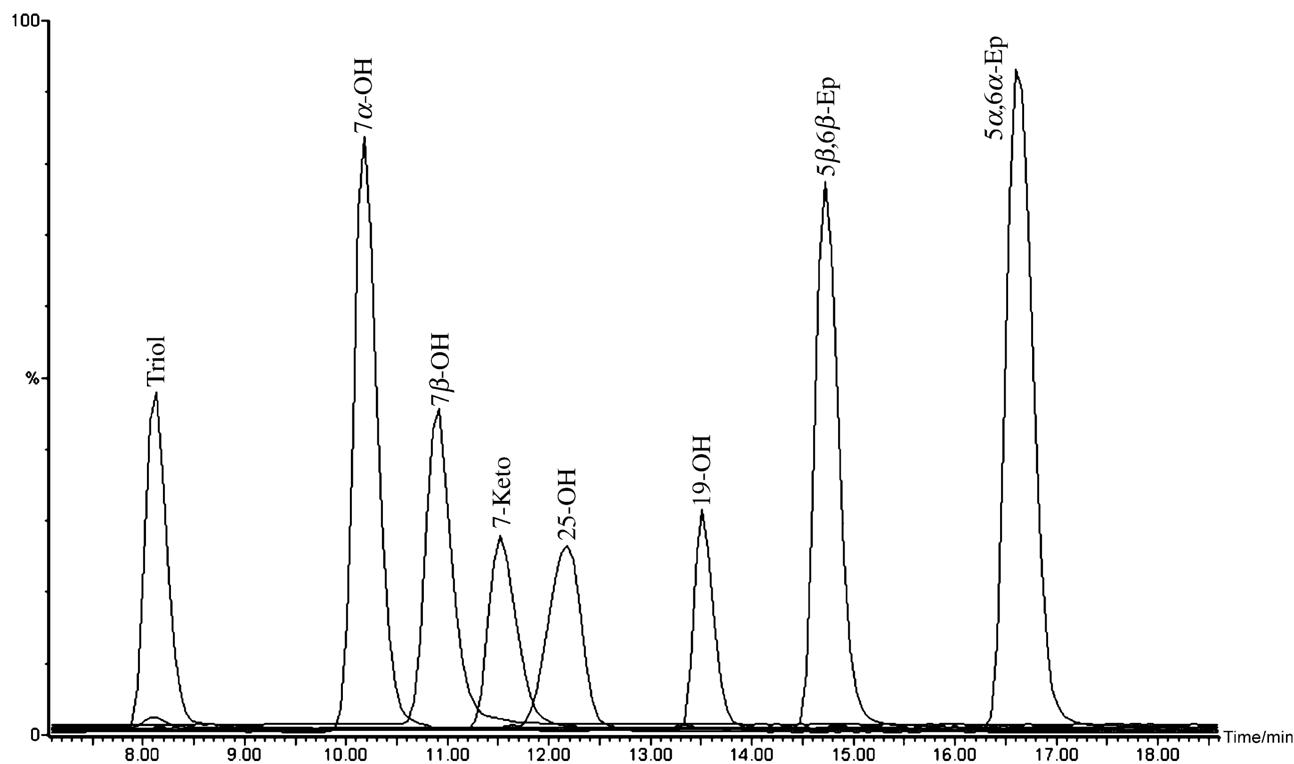

Fig. 8. MRM chromatograms of 8 single standards (overlaid). c(standard): 5000 ng/cm^3, V_{inj}: 50 mm^3

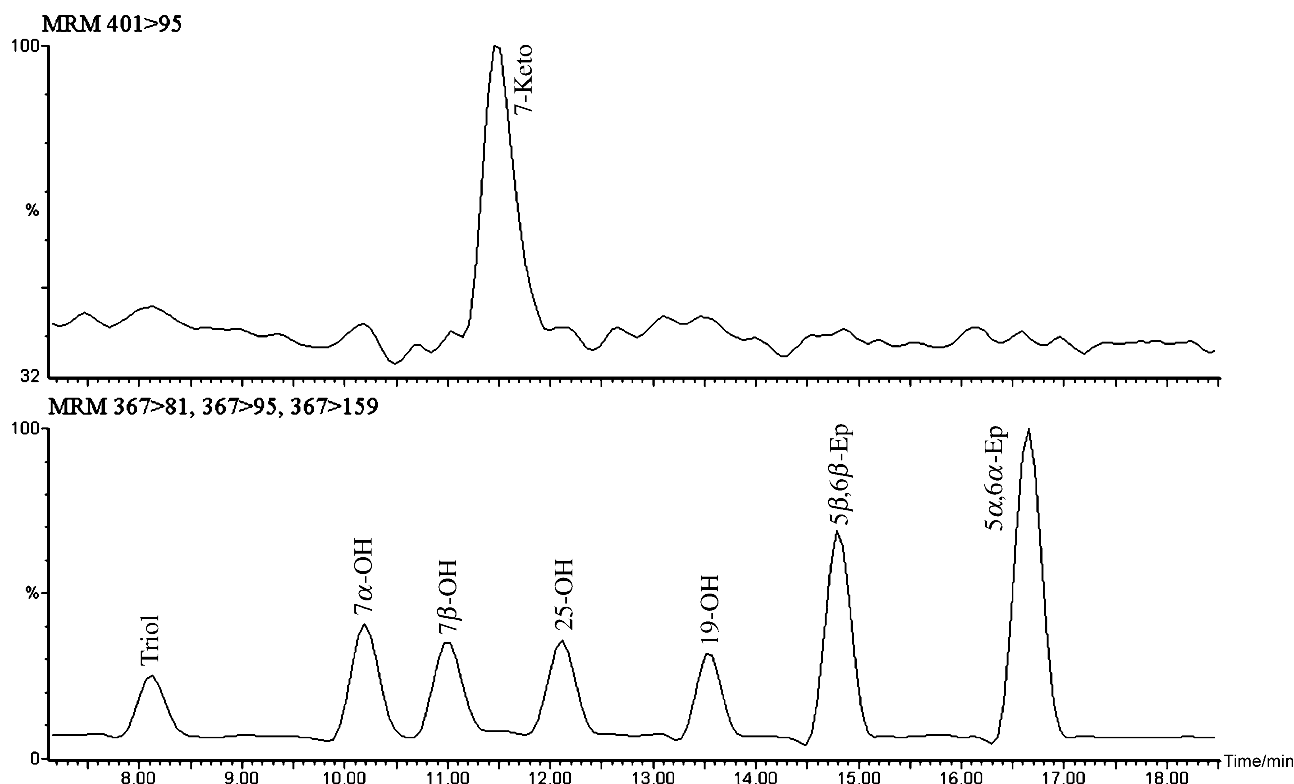

Fig. 9. MRM chromatogram of a standard mixture (7 *COP*s and 19-hydroxycholesterol the prospective internal standard). c(standard): $5000\,\mathrm{ng/cm^3}$, V_{inj}: $50\,\mathrm{mm^3}$

lesterol was used as an internal standard. In their report analogue breaking points were presented and the daughter ion spectra showed similar band structures [18].

Another study on nortestosteron indicates that the fragmentation pattern for the steroid hormone may be somewhat different, which appears to be caused by the other positions of the double bounds [16].

Surprisingly, the application of a different collision gas *i.e.* nitrogen used by *Trösken et al.* [18] opposed to argon used in our study had no effect on the resulting fragmentation patterns.

HPLC-MRM of a Standard Mixture

Based on the HPLC-APCI-MS method of *Razzazi-Fazeli et al.* [10] a HPLC-MRM method was developed.

MRM analysis was performed with eight single standard injections as well as with a mixture of the seven *COP*s and the internal standard 19-hydroxycholesterol. Figure 8 includes the overlaid chromatograms of single injections. It should be noticed that all analytes, including 7-ketocholesterol that is detected by a different MSMS transition, are additionally separated on the HPLC column.

Figure 9 includes the analysis of the mixture. The peaks were identified by comparing their retention times with pure standard injections and by MS1 scans (data not shown).

The *COP*s in the mixture all had the same concentration ($5000\,\mathrm{ng/cm^3}$), whereas their intensities differ in the MRM chromatogram. The daughter ions of the epoxides are formed about twice as much as that of the other *COP*s. (Notice: The heights of the peaks in Figs. 8 and 9 are not comparable since the chromatograms are displayed differently.)

For ionization is a rather critical process the use of an internal standard is inevitable for this system.

Conclusions

Cholesterol oxidation products share a common fragmentation pattern during tandem mass spectrometry. The statistically dominant breaking sites of the molecule are situated in the sterol ring system between the carbon atoms with numbers 11–12, 12–13, and 8–14 according to IUPAC nomenclature.

The characteristic fragments can be used to develop a MRM based method for *COP*s analyses that through its superior selectivity should prove a higher resistance against matrix interferences.

Unfortunately the sensitivity is rather low. This is mainly caused by technical parameters of the mass spectrometer such as the collision cell and the use of an APCI ion source. Consequently we have to date been unable to detect *COP*s in unspiked food samples.

To increase the sensitivity of our analytical approach up to a level were food analysis would be possible, one therefore needs to consider the use of an APPI ion source.

Experimental

Oxysterol Standards

The pure standards cholestane-3β,5α,6β-triol, cholest-5-en-3β-ol-7-one, cholest-5-en-3β,25-diol, cholestane-5α,6α-epoxy-3β-ol, cholestane-5β,6β-epoxy-3β-ol, cholest-5-en-3β,19-diol were purchased from Sigma-Aldrich (St. Louis, USA), cholest-5-en-3β,7α-diol and cholest-5-en-3β,7β-diol from Steraloids (Wilton, USA).

Mass Spectrometry

Tandem mass spectrometry was performed on a quadrupol mass spectrometer equipped with APCI+ (FISONS INSTRUMENT VG QUATTRO II, Waters, Milford, USA). Nitrogen was taken as sheath, nebulizing and drying gas and supplied by a ultrapure N_2 generator (Model 75–72, Parker Balston, Haverhill, USA). Ar was used as collision gas (Ar 5.0, Linde, Stadl-Paura, Austria).

Probe temperature was 500°C, source temperature 110°C, dwell time 0.30 sec, collision energy 70 eV and cone voltage 20 V. All parameters were optimized according to the protocol of the manufacturer.

For the daughter ion spectra the standard solutions were applicated with a syringe pump (HAVARD Apparatus 22, Havard Apparatus, Hollystone, USA). Flow rates were approximately 500 mm^3/min.

For MRM analysis the following MSMS transitions were chosen 367.4 > 81.1, 367.4 > 95.0, 367.4 > 159.0, 369.4 > 95.0, 369.4 > 161.0, 383.4 > 81.1, 385.4 > 95.0, 385.4 > 159.0 and 401.4 > 95.1.

High Performance Liquid Chromatography

A Waters HPLC system was used that consisted of WATERS In-Line Degasser ILD, WATERS 600S Controller, WATERS 626 Pump, WATERS 717 plus Autosampler (Waters, Milford, USA) combined with a column oven W.O. electronics (Jetstream, Langenzersdorf, Austria).

The column used for separation of the *COP*s mixture was Synergi Hydro-RP 4 μ Analytical Column pore size 80 Å, 150 × 4.6 mm (Phenomenex, Torrance, USA).

Elution was performed with a gradient: 0.0–8.0 min: 15% *Me*OH 85% ACN; 8.1–25.9 min: 85% *Me*OH 15% ACN; 26.0–32.0 min: 15% *Me*OH 85% *ACN*. Acetonitrile (*ACN*) and methanol were of gradient grade (Merck, Darmstadt, Germany).

HPLC-analyses were done with injection volumes (V_{inj}) of 50 mm^3 and flow rates of 1 cm^3/min.

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