ELSEVIER

Food Chemistry 84 (2004) 149–157

Food Chemistry

www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods

Evaluation of GC and GC–MS methods for the analysis of cholesterol oxidation products

S.J.K.A. Ubhayasekera[a], T. Verleyen[b], P.C. Dutta[a,*]

[a]*Department of Food Science, Division of Food Chemistry, Swedish University of Agricultural Sciences, Box 7051, SE-750 07, Uppsala, Sweden*

[b]*Department of Organic Chemistry, Coupure Links 653, 9000 Gent, Belgium*

Received 1 October 2002; received in revised form 16 May 2003; accepted 16 May 2003

Abstract

Various methods are used to analyse cholesterol oxidation products (COP) due to the unavailability of a standard method. In order to select a suitable method for the enrichment of COP, three methods of saponification (A–C), and transesterification (D) of tallow with three levels (5, 10 and 20 μg) of spiked COP, were evaluated. Further enrichment of COP was done by solid phase extraction, quantified by GC, and confirmed by GC–MS. The in-house method A, and method D showed,the best results among the four methods evaluated. The recoveries at all levels of spiked COP were generally higher than 60% in method A. The recoveries of all spiked COP at 5 μg level were consistently lower in method D compared with method A. From the results of this study it can be concluded that method A may be more suitable for the analysis of very low levels of COP in foods.

Keywords: Analysis; Cholesterol oxidation products; COP; GC; GC–MS; Saponification; Tallow; Transesterification

1. Introduction

Cholesterol oxidation products (COP) commonly found in foods are cholest-5-en-3β,7α-diol (7α-HC), cholest-5-en-3β, 7β-diol (7β-HC), 5α,6α-epoxy- 5α-cholestan-3β-ol (α-CE), 5β,6β-epoxy- 5β-cholestan-3β-ol (β-CE), 5α-cholestan-3β,5,6β-triol (CT), cholest-5-en-3β,20α-diol (20α-HC), cholest-5-en-3β,25-diol (25-HC), 3β-hydroxycholest-5-en-7-one (7-KC) (Larkeson, Dutta, & Hansson, 2000). In the process of oxidation of cholesterol in foods, presence of unsaturated fatty acids, cholesterol level, heat, oxygen, light, UV light, γ-radiation, water activity, technologically related events, etc., influence the formation of COP in foods (Larkeson et al., 2000; Paniangvait, King, Jones, & German, 1995; Savage, Dutta, & Rodriguez-Estrada, 2002).

In both in vivo and in vitro studies the COP have been shown to have a variety of potentially atherogenic, cytotoxic, mutagenic and possibly carcinogenic effects (Schroepfer, 2000). Cholesterol-containing foods, when consumed fresh, contain low levels of COP and the levels go up during processing, storage and cooking. The consumption of pre-cooked foods of animal origin is becoming more popular in the world due to the low cooking time involved in preparing them at home. Therefore, the intake of foods containing COP has increased (Savage et al., 2002). Thus for health reasons, it is important that these products in food are identified and quantified accurately (Dutta & Savage, 2002).

Analysis of COP generally passes through four major steps; prior extraction of lipids from foods, saponification of extracted lipids, and subsequent enrichment of COP and quantification by GC or by HPLC. An alternative to saponification of extracted lipids is transesterification of total lipids and subsequent enrichment of COP. In both of these methods, total COP in the lipids are accounted for, i.e., COP originating from both free and esterified cholesterol in the lipids. Various saponification methods are in use, e.g., hot saponification or cold saponification using either ethanolic or methnolic potassium hydroxide (KOH). Details of the methods of saponification can be found in the literature (Park & Addis, 1992).

Literature reports on the levels of COP in same type of food show that a number of methods have been used

* Corresponding author. Tel.: +46-18-672068; fax: +46-18-672995.

E-mail address: paresh.dutta@lmv.slu.se (P.C. Dutta).

0308-8146/03/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/S0308-8146(03)00259-0

for each step in the analysis of COP, and thus the reported results are significantly different. This indicates the importance of the in-house method validation of COP analysis until a harmonised method is available (Dutta & Savage, 2002; McCluskey & Devery, 1993). The main objective of the present study is to evaluate and validate our in-house method by comparing it with a number of commonly used methods for analysis of COP. In order to achieve the objective, the methods of purification and enrichment of COP by various saponification methods and transesterification of lipids with spiked samples of standard COP in tallow were investigated.

2. Materials and methods

2.1. Materials

Crude tallow fat was donated by Baeten (Overmere, Belgium). Standard samples of cholesterol, 7α-HC cholester, 19-hydroxycholesterol (19-HC), 7β-HC, α-CE, β-CE, 20α-HC, CT, 25-HC and 7-KC were obtained from Steraloids (Wilton, NH, USA); 5α-cholestane was from Sigma-Aldrich (Stockholm, Sweden); Tri-Sil reagent was purchased from Pierce (Rockford, IL, USA). Ethanol and methanol were from Kemetyl (Haninge, Sweden). All other chemicals and solvents were of analytical grade and were purchased from Merck Eurolab AB (Stockholm, Sweden), unless otherwise stated.

2.2. Methodology

Prior to determine retention times (RT), relative retention times (RRT), linearity range (r) and response factors (RF), the purity (%) of the standard samples of COP and internal standards (IS) were checked by GC as their trimethylsilyl (TMS)-ether derivatives (methods are described below). To calculate the linearity response and RF, various amounts (1, 5,10, 15 and 20 μg) of standard samples of COPs were mixed with a fixed amount of 5α cholestane (3.06 μg) and 19-HC (2.69 μg) as the IS. RT were noted from single GC injection of COP standards at 10 μg level, and RRT were calculated from the same chromatogram by using both 5α-cholestane and 19-HC as internal standards. Linearity response (correlation coefficient *r*) and RF (linear regression) for COP were calculated using the area ratio versus weight ratio by constructing a multilevel calibration curve from single GC injection of the mixture of COP standards five concentration levels (Lee, Yang, & Bartle, 1984).

2.3. Analysis of COPs was done using methods A–D

(A) Cold saponification with 1 M KOH/95% EtOH: Tallow with or without spiking with standard samples of COP (5, 10, 20 μg) was saponified. Approximately 200 mg of tallow was dissloved in 3 ml of dichloromethane in a glass tube with stopper and mixed well by vortexing with 7 ml KOH (1 M) in 95% EtOH. This mixture was mixed well and left overnight (18–20 h) in a dark place in room temperature following previously published methods after some modifications (Larkeson et al., 2000; Park & Addis, 1986). Then 10 ml dichloromethane and 10 ml water were added and the mixture was shaken vigorously. Water phase was removed and the organic phase was washed once with 5 ml of 0.5 M KOH in water and few drops of saturated NaCl to facilitate phase separation. After that, the organic phase was washed repeatedly with water until the solution became clear. The solvent was dried under nitrogen, and the unsaponifiables were dissolved in 1 ml hexane/diethyl ether (75:25, v/v). The COP fraction was enriched further by solid phase extraction (SPE) as described below.
(B) Cold saponification with KOH/MeOH: According to a published procedure (Park & Addis, 1986). Mainly as procedure A without dichloromethane, and 95% EtOH was replaced by analytical grade methanol.
(C) Hot saponification with KOH/95% EtOH: As procedure A without dichloromethane, and the sample was incubated in a 60 °C water bath for 1 h. The reaction was stopped by cooling the sample tubes under running water. The method followed was in accordance with a published method, with minor modifications (Park, Guardiola, Park, & Addis, 1996).
(D) Transesterification of tallow was done in accordance with a published method (Schmarr, Gross & Shibamoto, 1996). For this purpose ca. 200 mg tallow was weighed. 2 ml of 10% sodium methylate in methanol, diluted with *tert*-butyl methyl ether (MTBE) (4:6, v/v) was added for transefterification. The mixture was vortexed and allowed to stand for 1 hour at room temperature with additional mixing after ca. 30 min. Two mililitres of water and 5 ml of chloroform were added and the vial was closed with a tight cap and shaken for 1 min to extract the organic material, followed by 5 min centrifugation at 2000 rpm to facilitate phase separation. The upper (aqueous) phase was removed. Neutralisation of excessive alkali was accomplished by adding 2 ml of 1% citric acid in water, shaking, centrifuging and again disposing of the aqueous phase. The residual chloroform extract was then evaporated under a stream of nitrogen. The COP fraction was enriched further by SPE as described below.

2.4. Enrichment of COP by solid phase extraction (SPE) for methods A–C

Enrichment of COP was done essentially following a published method (Larkeson et al., 2000) with slight

modifications in the procedures A, B and C. The unsaponifiable fraction was dissolved in 1 ml of *n*-hexane/diethyl ether (75:25, v/v) and applied to a silica cartridge (500 mg/6 ml; International Sorbent Technology Ltd., Mid Glamorgan, UK) pre-equilibrated with 3 ml of *n*-hexane. Then the cartridge was eluted with a solvent series of two. The first solvent mixture was 3 ml *n*-hexane/diethyl ether (75:25, v/v) and the second was 3 ml *n*-hexane/diethyl ether (60:40, v/v) and the eluents were discarded. For the elution of COP, 4 ml of acetone/methanol (60:40, v/v) was used. This fraction was dried under nitrogen and derivatised to TMS-ether prior to GC and GC–MS analyses as described below.

2.5. Enrichment of COP by SPE for method D

A small amount of anhydrous sodium sulfate was placed on top of a bonded amino-phase SPE cartridge (500 mg/6 ml; International Sorbent Technology Ltd., Mid Glamorgan, UK) for binding traces of moisture. The column was conditioned with 5 ml of hexane. Transesterified lipid was dissolved in 250 μl of chloroform and charged onto the SPE column and then washed again with 2×2.5 ml of hexane. Free cholesterol and other non-polar materials were then eluted with another 5 ml of hexane, 5 ml of hexane/MTBE (5:1, v/v), and 5 ml of hexane/MTBE (3:1, v/v). The polar fraction was eluted with 7 ml of acetone and collected in glass tubes (Schmarr et al., 1996). This fraction was dried under nitrogen and derivatised to TMS-ether prior to GC and GC–MS analyses as described below.

2.6. Analytical thin layer chromatography (TLC)

The unsaponifiables (methods A–C) and transesterified samples (method D) were analysed by analytical precoated TLC plates (silica gel 60, 20×20 cm, 0.25 mm thickness were used (Merck, Eurolab AB, Stockholm, Sweden) to check visually the completeness of hydrolysis of cholesterol esters. The unsaponifiables, and the transesterified samples along with a TLC-reference standard 18-4A (Nu-Chek-Prep Inc., Elysian, Minnesota, USA), were spotted onto a TLC plate and developed in the solvent system hexane:diethyl ether:acetic acid (85:15:1, v/v/v). The TLC plate was dried briefly in air and then sprayed with 10% phosphomolybdic acid in diethyl ether: ethanol (50:50, v/v). The plate was placed in an oven at 120°C for 15 min for colour development (Dutta & Appelqvist, 1997).

The enriched samples of COP after SPE from methods A–D were also checked by analytical TLC. For this purpose, high performance thin-layer chromatography plates (HPTLC), silica gel 60 F_{254}, 10×10 cm, 0.1 mm thickness (Merck, Eurolab AB, Stockholm, Sweden) were used. Along with the standard samples of COP, the enriched COP samples were spotted onto the HPTLC plate. The plate was developed in diethyl ether:cyclohexane (9:1, v/v) (13), up to the top of the plate. After a brief drying of the plate in the air, a reagent prepared by dissolving 1 g each of phosphomolybdic acid and cerium sulfate in 5.4 ml of sulfuric acid was sprayed and the plate was heated in an oven at 120°C for 15 min for colour development (Dutta & Appelqvist, 1997).

2.7. Preparation of trimethylsilyl ether (TMS-ether) derivatives of COP

Prior to GC and GC–MS analyses all samples were derivatised to TMS-ethers, as described previously (Dutta & Appelqvist, 1997). In order to calculate the absolute recovery percentages of the spiked COP, 5 μg of 5α-cholestane was added as internal standard in the samples used for quantification prior to silylationh. In brief, after drying the solvent under nitrogen gas, 100 μl of Tri-Sil reagent (Pierce, IL, USA) were added and the tubes were kept at 60°C for 45 min. The solvent was evaporated under a stream of nitrogen gas and the TMS-ether derivatives were dissolved in 100 μl hexane. The tubes were sonicated in an ultrasonic bath for 1 min and were centrifuged for 3 min and the tubes were stored at −20°C for subsequent analyses by GC and GC–MS within 1 week after derivatisation.

2.8. Gas chromatography

Analysis of COP as their TMS-ether derivatives was carried out by GC using a Varian 3700 instrument (Palo Alto, CA) fitted with falling needle injector and flame ionization detector. A capillary column DB-5MS 30 m×0.25 mm, 0.50 μm (J&W Scientific, Folsom, CA, USA) was used to separate the COP. The column temperature was set at 290°C for 20 min and raised to 300°C at 0.5°C/min. The detector was set at 320°C. Helium was used as the carrier gas at a pressure of 14 psig and nitrogen was used as the make-up gas at a flow rate of 30 ml/min. A HP 3396 integrator (Hewlett-Packard company, Avondale, PA) was used for recording chromatographic data. Identification and purity percentages were performed using TMS ether derivatives of COP standards. 5α-cholesatane was used as internal standard and for quantification. The response factors for each COP (Table 1), as calculated prior to analysis of spiked samples, were also used in quantification. The indigenous content of COP in the tallow was deducted from all spiked samples when calculating recovery percentages. The duplicate analyses were carried through all the steps in all samples for the quantification of COP.

2.9. GC–MS

For identification and purity checking purposes of COP peaks, a GC 8000 Top Series gas chromatograph (ThermoQuest Italia S.p.A., Rodano, Italy) coupled to a Voyager mass spectrometer with MassLab data system version 1.4V (Finnigan, Manchester, England) was used. The COPs were separated on the same column used in the GC analysis. Helium was used as carrier gas at an inlet pressure of 80 kPa. The injector temperature was 250°C and the samples were injected in a splitless mode of injection. Oven temperature was at 60°C for 0.5 min and then raised to 290°C at a rate of 50°C/min, and finally the temperature was raised to 300°C at a rate of 0.5°C/min. The mass spectra were recorded at an electron energy of 70 eV and the ion source temperature was at 200°C. The experimental design of the method performance study is shown in Fig. 1.

3. Results and discussion

Baseline separations among the standard samples of 5α-cholestane, 7α-HC, 19-HC, 7β-HC, β-CE, α-CE, 20α-HC, CT, 25-HC and 7-KC were achieved with the capillary column used in this investigation (Fig. 2). The resolution and elution pattern of COP are consistent in this column compared with published results where same column type, apart from higher film thickness, was used (Pie & Seillan, 1992; Regueiro & Maraschiello, 1997). In contrast, DB-1 type of column of same dimension used in this study has shown inconsistent elution patterns (Lai, Gray, & Zabik, 1995; Park & Addis, 1985).

Resolution among some COP was studied (Park & Addis, 1985) using a fused-silica capillary DB-1, 15 m×0.25 mm i.d.×0.10-μm column (temperature programming was from 180 to 250 °C at 3 °C/min). It was shown that the order of elution of the last three COP were CT, 7-KC and 25-HC, respectively. However, by using the same column (Lai et al., 1995) it was shown that the order of elution of these three peaks was 25-HC, 7-KC and CT. Thus, peaks for 25-HC and 7-KC are eluted in their respective order in DB-5 type of column, but in DB-1 type column those peaks are eluted in an inconsistent manner.

Separation among α-CE, β-CE and 7β-HC is also difficult in DB-1 (unpublished results), but DB-5 type column with similar dimensions used in this study gave a consistent resolution of these COP. Selectivity, i.e., the ability to resolve the analyte specifically from interfering substances by GC, is one of the basic criteria for a validated analytical method (McCluskey & Devery, 1993; Thompson, Ellison, & Wood, 2002). In this study, the peaks of each COP in the spiked samples were checked by GC–MS, and it was observed that no noticeable co-eluting contaminants were present.

Table 1
Retention times (RT), relative retention times (RRT), linearity response (r), and response factors (RF) of the TMS-ether derivatives of standard samples of cholesterol oxidation products (COP)

COP	RT (min)	RRT[a]	RRT[b]	r	RF
5α-cholestane	13.98	1.00	0.61	IS[c]	1.00
7α-HC	19.47	1.39	0.85	0.99	1.02
Cholesterol	21.74	1.56	0.95	ND[d]	ND
19-HC	22.91	1.64	1.00	ND	ND
7β-HC	24.49	1.75	1.07	0.99	0.99
β-CE	27.02	1.93	1.18	0.98	1.54
α-CE	27.84	1.99	1.22	0.97	1.27
20α-HC	28.56	2.04	1.25	0.98	0.82
CT	30.73	2.20	1.34	0.95	1.48
25-HC	33.51	2.40	1.46	0.97	1.35
7-KC	35.11	2.51	1.53	0.97	1.24

[a] Retention time relative to 5α-cholestane.
[b] Retention time relative to 19-HC.
[c] Internal standard.
[d] Not done. For details, see Section 2.

The RT, and RRT by using both 5α-cholestane and 19-HC as IS, r, and RF of each COP are presented in Table 1. Although we used 5α-cholestane as IS in the subsequent quantitative analysis, 19-HC was included in the initial qualitative GC analysis because the later is also comon in use as IS. From the results it can be seen that the retention time of unoxidised cholesterol is more than 2 min longer than for 7α-OH. Thus, even after enrichment of COP from unsaponifiables, which generally contain substantial amounts of cholesterol, 7α-OH is separated well from cholesterol. The separation of 7α-OH and cholesterol is also of reverse order in the DB-1 type of column and may be rather difficult to separate (Dutta & Savage, 1999). The total analysis time by GC is rather moderate, less than 40 min to elute all the common COP used in this study and under the analytical conditions.

The linearity of response (r) of all COP is almost 1.0. This shows that the signals generated for each COP was linear within the mass ranges (1–20 μg) in this study. This is another criteria for a good analytical method and shows that selective loss of COP in the system used in this study was minimal (Park & Addis, 1992).

The RF for different COP is another important parameter, and necessary to determine with the column and instrument used in a study (Park & Addis, 1992). The calculated RF values in this study are listed in Table 1, where generally the RRF values are higher than 1, except 20α-HC. The differences of the response factors may be influenced by the different functional groups in different COP, nature of internal standard, the nature of the column, GC conditions, etc. We were unable to compare our values with other researcherś results because there are no published data from similar experimental conditions. However, published reports

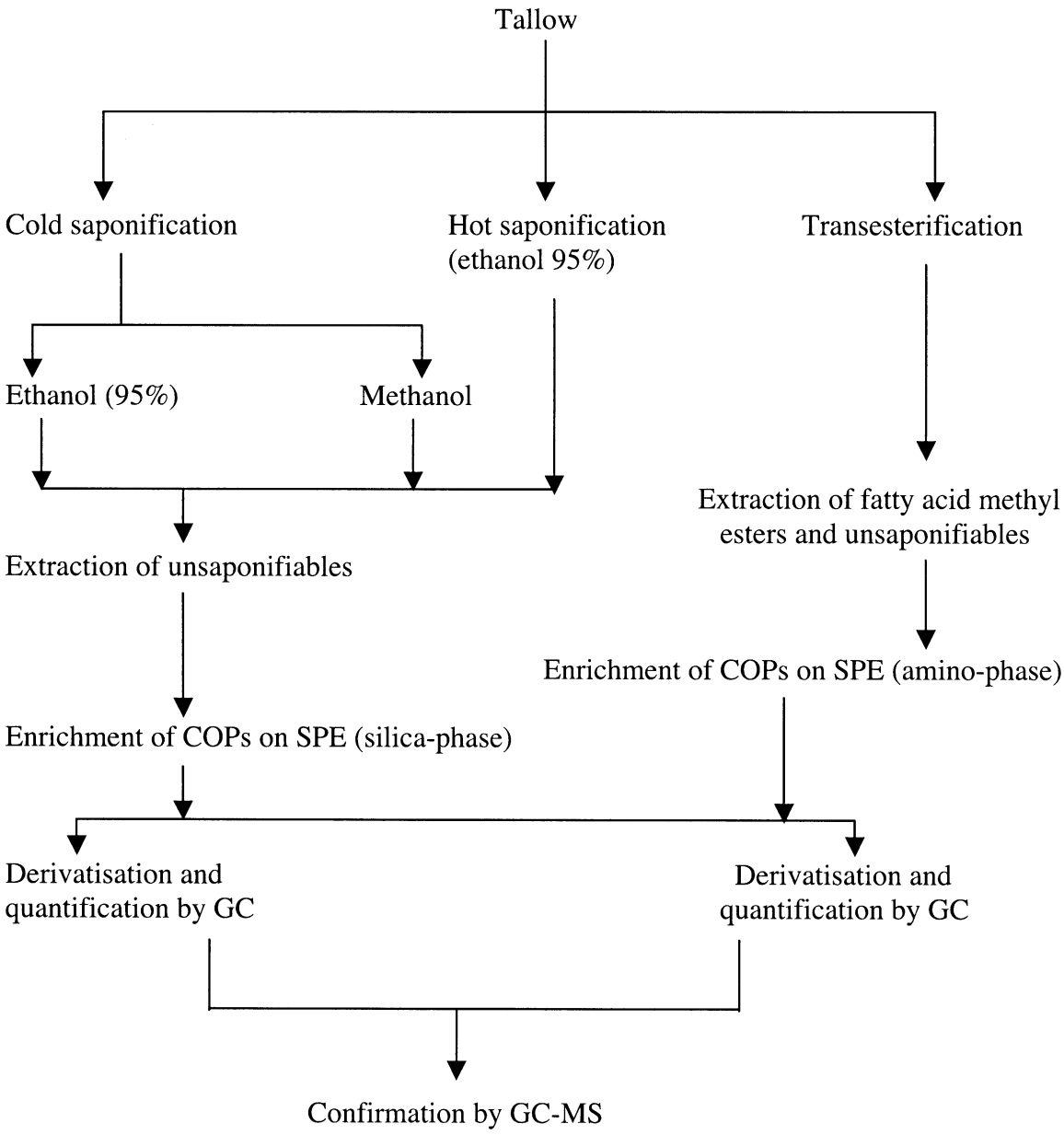

Fig. 1. Flow chart showing the experimental design of performance test of the four methods of analysis of cholesterol oxidation products (COP).

have shown that the response factors of different COPs were generally close to 1 in DB-1 type columns and 5α-cholestane as IS (Guardiola, Codony, Rafecas & Boatella, 1995; Park & Addis, 1985; Van de Bovenkamp, Kosmeijer-Schuil & Katan, 1988).

The completeness of hydrolysis of cholesterol esters was checked by analytical TLC in the three saponification procedures (A–C), and transesterification (D). It was observed on analytical TLC plates that no traces of cholesterol esters were present after saponification and transesterification. Also after SPE enrichment no co-eluting substances were observed above the cholesterol spot on TLC plate.

The results of the recovery percentages at a level of 5 μg of each COP determined by the four methods are shown in Fig. 3a. Generally all of the COP followed high recovery percentages in method A compared with the other methods (C and D). The recovery percentages ranged from 68 to 88%, except for 7α-OH and CT, which had comparatively lower recoveries of 58 and 57%, respectively. The recovery of CT was lowest (13%) in method B. The recovery of β-CE was low in methods C (18%) and D (16%), compared with the highest recovery in method A (69%). No comparable results on method A have been published previously, but relative recovery percentages of various COP at 5 μg level by method D were more than 86% for all COP by using an IS added in the beginning of work-up steps (Schmarr et al., 1996). Thus, the results of method D could not be compared with the published results

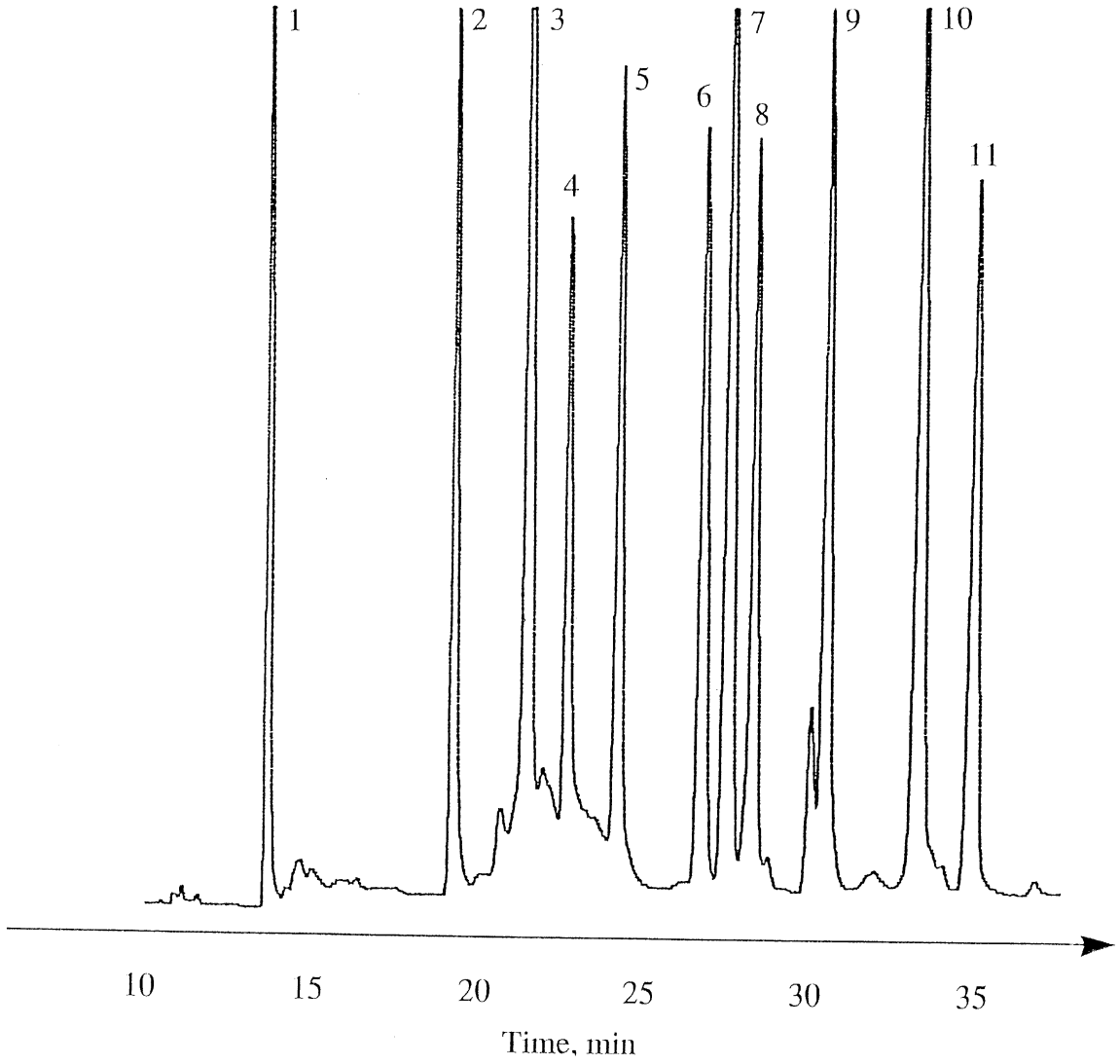

Fig. 2. Capillary column gas liquid chromatogram showing resolution of standard sample of cholesterol and some common cholesterol oxidation products. Column and GC conditions are given in Section 2. Peak identification: (1) 5α-cholestane, (2) Cholesterol (cholest-5-en-3β-ol), (3) 7α-HC (cholest-5-en-3β,7α-diol), (4) 19-HC (cholest-5-en-3β, 19- diol), (5) 7β-HC (cholest-5-en-3β, 7β-diol), (6) β-CE (5β, 6β-epoxy- 5β-cholestan-3β-ol), (7) α-CE (5α, 6α-epoxy-5α-cholestan-3β-ol), (8) 20α-HC (cholest-5-en-3β, 20α-diol), (9) CT (5α-cholestan-3β, 5, 6β-triol), (10) 25-HC (cholest-5-en-3β, 25-diol), (11) 7-KC (3β-hydroxycholest-5-en-7-one).

because the absolute recoveries of the COP were calculated in this study by using a different IS which was added prior to GC analysis.

From the spiking study it was observed that although some comparable results were obtained, method B did not show consistent and high recovery of the spiked COP in the samples compared with other methods (Fig. 3a–c). Methanol is widely used in extraction of lipids, often together with chloroform, which is considered harmful to our health and enviornment (Hara & Radin, 1978), and it may be wise to avoid using them in routine laboratory work, apart from when there is no other alternative.

In the hot saponification method (C) 95% ethanol was used. Similar to method B, this method (C) also showed mixed results of recovery. In addition, it is well known that 7-KC is degraded during hot saponification in methanolic KOH (Park et al., 1996). The results from this study concur with the previous observations from the consistently lower recovery of 7-KC in all the samples spiked with standard COP (Fig. 3a–c). Since methods B and C consistently showed lower recovery of COP (Fig. 2a–c), further discussion will mainly be concentrated on methods A and D.

The results of the recovery percentages at a level of 10 μg of each COP are shown in Fig. 3b. Method A produced the highest recovery percentages for β-CE (75%), α-CE (78%), 20α-HC (60%) and 7-KC (81%). Method D had the highest recoveries for 7α-HC (75%), 7β-HC (78%) and CT (55%). At this level, method A showed generally higher recoveries than method D, except slightly lower recoveries for 7α-OH, 7β-OH, and CT.

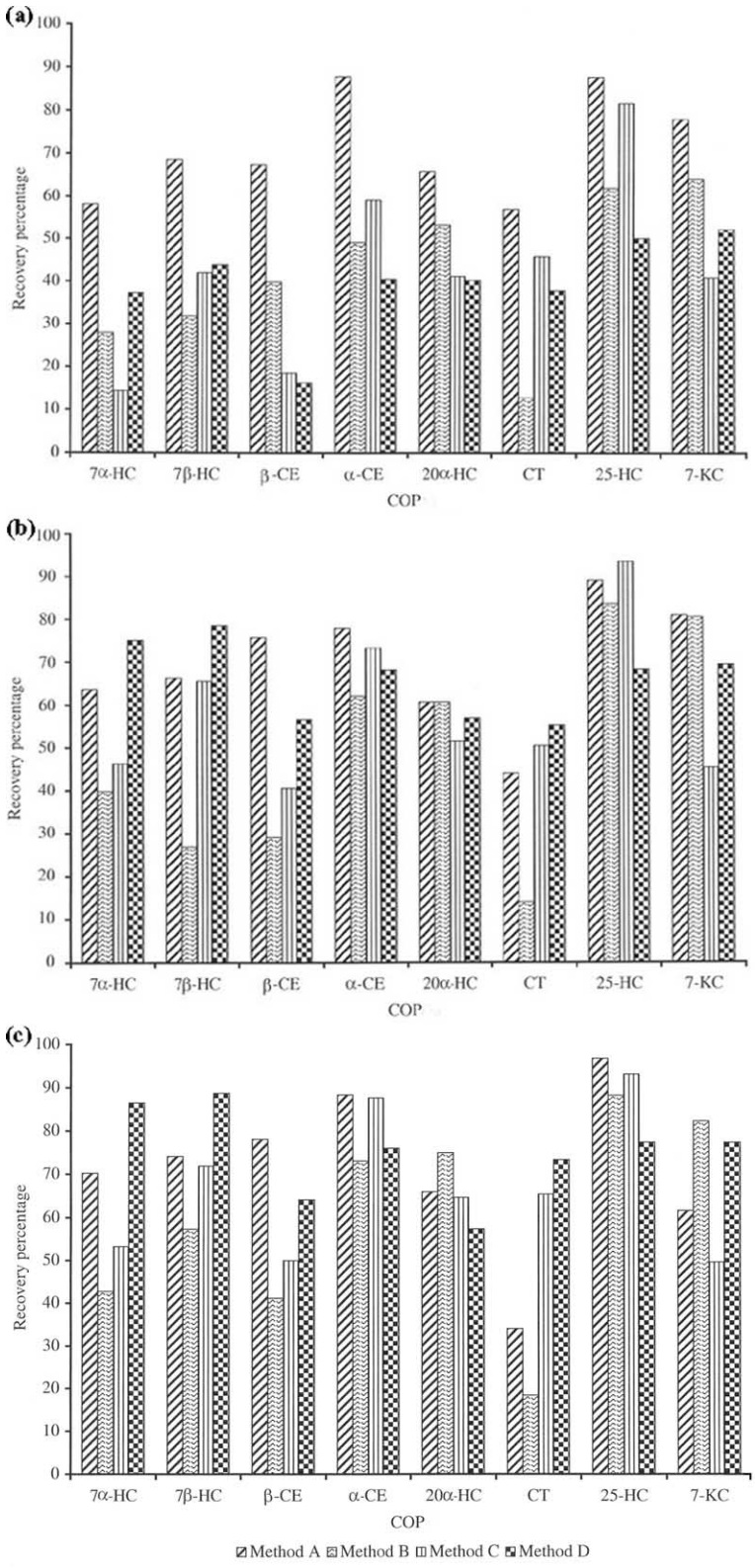

Fig. 3. (a) Recovery percentages of COP spiked with 5 μg standard samples of each component; method A—cold saponification with 1 M KOH in 95% ethanol in water; method B—cold saponification with 1 M KOH in methanol; method C—hot saponification with 1 M KOH in 95% ETOH in water; method D—transesterification. (b) Recovery percentages of COP spiked with 10 μg standard samples of each component; method A—cold saponification with 1 M KOH in 95% ethanol in water; method B—cold saponification with 1 M KOH in methanol; method C—hot saponification with 1 M KOH in 95% ETOH in water; method D—transesterification. (c) Recovery percentages of COP spiked with 20 μg standard samples of each component; method A—cold saponification with 1 M KOH in 95% ethanol in water; method B—cold saponification with 1 M KOH in methanol; method C—hot saponification with 1 M KOH in 95% ETOH in water; method D—transesterification.

The results of the recovery percentages at a level of 20 μg of each COP are shown in Fig. 3c. Method A had higher recovery percentages for β-CE (78%), α-CE (88%), 20α-OH (66%), and 25-HC (97%), compared with method D. Although the other COP showed higher recoveries with method D. However, only in the case of CT was the recovery considerably higher than in method A.

Comparative data on the results of recoveries of COP by methods A and D are presented in Table 2. The CV% of the recoveries of standard COP spiked at various levels in tallow showed consistently lower values given by method D compared with method A, although recovery percentages were generally higher in method A.

For 7α-HC, 7β-HC, β-CE and 7-KC in 5 μg spiked and cold saponified samples, the CV% were considerably higher in method A compared with method D, although in all these cases considerably higher recovery percentages were recorded in method A (Table 2).

Table 2
The recovery (%) of cholesterol oxidation products (COP) at different levels of spiking by methods of cold saponification (A), and transesterification (D)

COP	Amount spiked (μg)	Cold saponification (A)		Transesterification (D)	
		Mean % (±SEM)	CV%	Mean % (±SEM)	CV%
7α-HC	5	58.1 (21.0)	36.1	37.3 (2.5)	6.8
	10	63.6 (1.2)	1.8	75.1 (3.0)	3.9
	20	70.2 (3.2)	4.6	86.5 (7.3)	8.4
7β-HC	5	68.6 (11.7)	17.0	43.9 (1.3)	2.9
	10	66.1 (1.9)	2.9	78.4 (2.6)	3.6
	20	74.1 (0.4)	0.5	88.7 (5.9)	6.7
β-CE	5	67.3 (20.3)	30.2	16.2 (2.9)	18.1
	10	75.6 (5.9)	7.8	56.5 (3.0)	5.2
	20	78.1 (5.0)	6.4	64.1 (0.4)	0.7
α-CE	5	87.8 (1.2)	1.4	40.5 (0.2)	0.5
	10	77.7 (4.6)	6.0	67.9 (2.3)	3.4
	20	88.4 (2.9)	3.3	76.1 (3.1)	4.1
20α-HC	5	65.8 (1.4)	2.1	40.3 (5.2)	12.6
	10	60.5 (5.8)	9.7	56.7 (2.8)	4.9
	20	65.9 (9.3)	14.2	57.4 (8.7)	15.2
CT	5	56.8 (0.6)	1.1	37.8 (1.2)	3.2
	10	43.8 (5.1)	11.6	55.0 (3.7)	6.8
	20	34.1 (7.7)	22.6	73.4 (1.9)	4.97
25-HC	5	87.7 (1.8)	2.1	50.1 (0.4)	0.7
	10	88.7 (9.8)	11.0	67.9 (2.7)	4.0
	20	96.9 (8.3)	8.5	77.5 (1.2)	1.5
7-KC	5	78.0 (12.0)	15.3	52.2 (1.8)	3.5
	10	80.6 (8.7)	10.8	69.1 (2.8)	4.0
	20	61.6 (9.5)	15.4	77.6 (3.3)	4.2

Abbreviations: SEM, standard error of mean ($n = 2$); CV%, coefficient of variation.

Twenty-microgram spiked samples gave more than 10% of CV in 20α-HC, CT and 7-KC after cold saponification with ethanol. For 10 μg spiked samples, this value is less than 10% for all COP except CT, 25-HC and 7-KC. But in transesterification CV% is lower than 10% in all the samples except β-CE (5 μg spikes) and 20α-OH (5 and 20 μg spiked).

Among the three saponification methods (A–C), method A consistently showed higher recoveries of all the spiked COP. Some of the reasons of the differences in recoveries of COPs in these methods are dicsussed above. Additional reason could be, that dicholoromethane was used to dissolve the lipids only in method A which resulted the mixture a clear solution. In method B, homogeneous and clear mixture was formed initially after brief shaking the tube under running hot water, but this mixture became turbid as soon as the tubes became cold. This may contribute in the lower recoveries in method B during washing step.

It is worthy to mention that direct comparison between methods A and D would not be possible because of the fundamental differences in the principles of these two methods. In method A, the bulk lipids were separated from the unsaponifiables and the remaining amount is a small fraction, often less than 1% of the total lipids. Thus the risk of overloading the SPE silica column at the sample size used in this study is minimum. On the other hand, in method D the total amount of lipids after transesterification would remain almost same as the initial total lipids, that is ca. 200 mg. This amount may be quite high to be effectively separated in a 500 mg NH_2 column, and use of larger column can be of interest for further improvement of the recoveries of COP by this method.

Until now there is no standardised method available to analyse COP in foods and resulting uncomparable results in similar types of foods. To minimise the differences in analytical methods, two interlaboratory analytical studies on COP were conducted (Dutta & Savage, 2002). The CV percentages on the contents of 7α-HC, 7β-HC, 7-KC, α-CE, β-CE, and CT in egg powder in the second interlaboratory study were 36, 50, 51, 42, 30 and 38%, respectively, and the CV percentages for those COP in milk powder were even higher. It was concluded from those studies that the lower content of COP in samples contribute to more variable results. It was decided after the second study that further improvement in the in-house methods was necessary in order to get more acceptable CV% in the analytical results. The variation in the results of duplicate analyses achieved methods A and D in this study are generally quite low, even at the lowest level (5 μg) of the spiked samples (Table 2),

Comparing the results of the two methods (Table 2), it can be concluded that although method D showed consistently lower variation in duplicate analyses, it

generally had lower recovery percentages. This requires further study in order to utilise the advantage of shorter analysis time of this method compared with method A. It is further concluded that method A may be more suitable for analysis of samples containing lower levels of COP. The importance of carefully validating the in-house method is also emphasised, until a harmonised method is available for analysis of COP.

References

Dutta, P. C., & Appelqvist, L.-Å. (1997). Studies on phytosterol oxides I: Effect of storage on the content in potato chips prepared in different vegetable oils. *Journal of the American Oil Chemistś Society*, *74*, 647–657.

Dutta, P. C., & Savage, G. P. (2002). Harmonization of cholesterol oxidation product analysis. In F. Guardiola, P. C. Dutta, R. Codony, & G. P. Savage (Eds.), *Cholesterol and phytosterol oxidation products—analysis, occurrence, and biological effects* (pp. 114–123). Champaign, IL, USA: AOCS Press.

Guardiola, F., Codony, R., Rafecas, M., & Boatella, J. (1995). Comparison of three methods for the determination of oxysterols in spray-dried egg. *Journal of Chromatography A*, *705*, 289–304.

Hara, A., & Radin, N. S. (1978). Lipid extraction of tissues with a low-toxicity solvent. *Analtical Biochemistry*, *90*, 420–426.

Larkeson, B., Dutta, P. C., & Hansson, I. (2000). Effects of frying and storage on cholesterol oxidation in minced meat products. *Journal of the American Oil Chemistś Society*, *77*, 675–680.

Lai, S-M., Gray, J. I., & Zabik, M. E. (1995). Evaluation of solid phase extraction and gas chromatography for determination of cholesterol oxidation products in spray-dried whole egg. *Journal of Agriculture and Food Chemistry*, *43*, 1122–1126.

Lee, M. L., Yang, F. J., & Bartle, K. D. (1984). *Open tubular column gas chromatography—theory and practice*. New York: Wiley & Sons.

McCluskey, S., & Devery, R. (1993). Validation of chromatographic analysis of cholesterol oxides in dried foods. *Trends in Food Science and Technology*, *4*, 175–178.

Paniangvait, P., King, A. J., Jones, A. D., & German, B. G. (1995). Cholesterol oxides in foods of animal origin. *Journal of Food Science*, *60*, 1159–1174.

Park, S. W., & Addis, P. B. (1985). Capillary column gas-liquid chromatographic resolution of oxidized cholesterol derivatives. *Analytical Biochemistry*, *149*, 275–283.

Park, S. W., & Addis, P. B. (1986). Identification and quantitative estimation of oxidized cholesterol derivatives in heated tallow. *Journal of Agriculture and Food Chemistry*, *34*, 653–659.

Park, S. W., & Addis, P. B. (1992). Methods of analysis of cholesterol oxides. In S-K. Peng, & R. J. Morin (Eds.), *Biological effects of cholesterol oxides* (pp. 34–67). Boka Raton: CRC Press.

Park, P. W., Guardiola, F., Park, S. H., & Addis, P. B. (1996). Kinetic evaluation of 3β-hydroxycholest-5-en-7-one (7-ketocholesterol) stability during saponification. *Journal of the American Oil Chemistś Society*, *73*, 623–629.

Pie, J. E., & Seillan, C. (1992). Oxysterols in cultured bovine aoric smooth muscle cells and in the monocyte-like cell line U937. *Lipids*, *27*, 270–274.

Regueiro, J. A. G., & Maraschiello, C. (1997). Procedure for the determination of eight cholesterol oxides in poultry meat using on-column and solvent venting capillary gas chromatography. *Journal of Chromatography A*, *764*, 279–293.

Savage, G. P., Dutta, P. C., & Rodriguez-Estrada, M. T. (2002). Cholesterol oxides: Their occurrence and methods to prevent their generation in foods. *Asia Pacific Journal of Clinical Nutrition*, *11*(1), 72–78.

Schmarr, H-G., Gross, H. B., & Shibamoto, T. (1996). Analysis of polar cholesterol oxidation products: Evaluation of a new method involving transesterification, slid phase extraction, and gas chromatography. *Journal of Agriculture and Food Chemistry*, *44*, 512–517.

Schroepfer, G. J. Jr. (2000). Oxysterols: Modulators of cholesterol metabolism and other processes. *Physiological Reviews*, *80*, 361–554.

Thompson, M., Ellison, S. L. R., & Wood, A. R. (2002). Harmonized guidelines for single-laboratory validation of methods of analysis. *Pure and Applied Chemistry*, *74*, 835–855.

Van de Bovenkamp, P., Kosmeijer-Schuil, T. G., & Katan, M. B. (1988). Quantification of oxysterols in Dutch foods: Egg products and Mixed diets. *Lipids*, *23*, 1079–1085.