Determination of Cholesterol Oxidation Products in Milk Powders: Methods Comparison and Validation

F. Dionisi, P. A. Golay, J. M. Aeschlimann, and L. B. Fay*

Nestlé Research Centre, Nestec Ltd., Vers-chez-les-Blanc, P.O. Box 44, 1000 Lausanne 26, Switzerland

Many papers have been published during the past 15 years on suitable methodologies for the analysis of cholesterol oxidation products (COPs) in foodstuff. Regarding milk powders, data found in the literature are difficult to compare, if not contradictory. Therefore, the aim of the present study was to compare four of the most frequently used methods for COP analysis to identify the best. A method with direct saponification of the sample was compared with three methods involving preliminary fat extraction (Folch's, Radin's, and Maxwell's methods). After saponification all of the samples were enriched by solid-phase extraction (SPE) on aminopropyl cartridges and analyzed by GC-MS. [$^2H_6$]cholesterol was used to monitor artifact formation. The four methods were applied to the analysis of three milk powders containing different levels of COPs (low, medium, and high contents). The method offering the best compromise was found to be direct saponification of the milk powder, without preliminary fat extraction, followed by aminopropyl-SPE and GC-MS quantification.

Keywords: *Milk powder; cholesterol; oxidation; artifacts; GC-MS; validation*

INTRODUCTION

Cholesterol present in food can be oxidized leading to the formation of more than 30 compounds, generally called COPs (cholesterol oxidation products) (Paniangvait et al., 1995; Appelqvist, 1995). They can be present in both free and esterified forms.

COPs are present in many food preparations, especially those containing high levels of cholesterol (e.g. egg, milk, meat, fish products). COP formation is influenced by heat (especially for long exposure times), oxygen, light and UV, water activity, and the presence of unsaturated fatty acids. Technological treatments can increase cholesterol oxidation (e.g. frying, spray-drying). In addition, inadequate storage can drastically increase the COP content (Paniangvait et al., 1995; Rose-Sallin, 1996).

The health effects of COPs are an increasing concern for the scientific community. Several COPs have received much attention due to their biological effects such as cytotoxicity, atherogenicity, and changes in cellular membrane properties (Smith and Johnson, 1989; Peng et al., 1991; Guardiola et al., 1996). 5α-Cholestane-3β,5α,6β-triol and 25-hydroxycholesterol were demonstrated to be atherogenic, while α-epoxycholesterol is suspected to be carcinogenic (Imai et al., 1976; Taylor et al., 1979; Peng and Taylor, 1984, Sevanian and Peterson, 1984, 1986; Addis et al., 1993).

Despite extensive development and abundant literature on the analytical methods suitable for COPs quantification, there is still controversy about the reliability of the presented data (McCluskey and Devery, 1993). A large variety of methods has been described, but they are difficult to compare and lead to significantly different results. In addition, some treatments occurring during cleanup (e.g. saponification) may lead to formation of artifacts or disruption of COPs (e.g. 7-ketocholesterol and α-epoxycholesterol are sensitive to alkaline pH at high temperature (Park and Addis, 1992; Park et al., 1996)).

Many analytical methodologies have already been used for COP quantification in food products or biological fluids (GC-FID, HPLC-UV, TLC, GC/MS) (Addis et al., 1989; Zhang et al., 1991; Sallin et al., 1993; Guardiola et al., 1995; Penazzi et al., 1995; Rose-Sallin et al., 1995; Dzeletovic et al., 1995). GC/MS quantification working in selected ion monitoring mode (SIM) is generally accepted as the most selective and sensitive (Park and Addis, 1992; Rose-Sallin et al., 1995). However, the choice between different techniques of extraction and purification remains a problem.

Methods for COPs analysis can be divided into two categories: methods involving a preliminary fat extraction followed by a saponification (Sallin et al., 1993; Appelqvist, 1995, 1996; Addis et al., 1989; Zhang et al., 1991; Sander et al., 1989a,b; Guardiola et al., 1995) and methods which directly treat the sample (Rose-Sallin et al., 1995; Schmarr et al., 1996). Subsequently, the unsaponifiable matter is extracted and COPs are quantified by GC or HPLC. The first set of methods differs by the use of different solvent mixtures: dichloromethane/methanol for Maxwell's method (Maxwell et al., 1986), chloroform/methanol for Folch's method (Folch et al., 1957), and 2-propanol/hexane for Radin's method (Radin, 1981). The second set of methods uses direct saponification or transesterification of the sample. A round robin test organized between American, Asian, and European laboratories demonstrated that different methods can lead to different results and underlined the necessity for comparison of the most promising methods for choosing the best (Appelqvist, 1996).

* Author to whom correspondence should be addressed (telephone +41-21-785-86-09; fax +41-21-785-85-54; e-mail laurent.fay@chlsnr.nestrd.ch.

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Therefore, the aim of the present study was to optimize and validate the quantification of COPs in milk powders. Four methods (direct, Maxwell, Folch, and Radin) were statistically compared in terms of standard deviation (SD), coefficient of variation (CV), artifact formation, and accuracy.

EXPERIMENTAL PROCEDURES

Reagents and Chemicals. Acetone for chromatography, dichloromethane (analytical grade), methanol (analytical grade), water for chromatography, Celite 545, anhydrous sodium sulfate (analytical grade), calcium hydrogen phosphate dihydrate ($CaHPO_4 \cdot 2H_2O$), chloroform GR, sodium chloride, hexane for chromatography, 2-propanol GR, pyridine, and diethyl ether [deperoxidized 3 days before analysis by the addition of 50 g/L molecular sieve (Deperox from Fluka) according to Burfield (1982)] were purchased from Merck, Dietikon, Switzerland. *N*,*O*-bis(trimethysilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) were purchased from Fluka, Buchs, Switzerland.

5α-Cholestane, 19-hydroxycholesterol, 5-cholesten-3β-ol-7-one (7-ketocholesterol), cholestan-3β,5α,6β-triol (cholestanetriol), and 25-hydroxycholesterol were obtained from Sigma, Buchs, Switzerland. [2,2,3,4,4,6-$^2H_6$]Cholesterol (purified according to Rose-Sallin et al. (1995)) was purchased from Numelec SA, Geneva, Switzerland. 5-Cholesten-3β,7α-diol (7α-hydroxycholesterol) and 5-cholesten-3β,7β-diol (7β-hydroxycholesterol) were obtained from Steraloids, Chemie Brunschwig AG, Basel, Switzerland.

Sample Preparation. A sample of fresh milk powder was divided into three aliquots (called samples 1, 2, and 3, respectively) and oxidized in the laboratory under artificial light in the presence of air for three different times of exposure (2, 8, and 19 days) in order to form different levels of COPs. These samples were used to compare the four purification methods under evaluation. Before analysis, 100 μL of 19-hydroxycholesterol solution (0.02 mg/mL in acetone) and 1 mL of [$^2H_6$]cholesterol solution (1 mg/mL in acetone) were added to each sample.

A 1 g amount of sample 3, previously spiked with 20.4 μg of 19-hydroxycholesterol, 16.2 μg of 7α-hydroxycholesterol, 17.2 μg of 7β-hydroxycholesterol, 19.4 μg of 7-ketocholesterol, 18.9 μg of cholestanetriol, 19.3 μg of 25-hydroxycholesterol, and 1 mg of [$^2H_6$]cholesterol, was used to establish the accuracy of each method.

Direct Method. Exactly 1 g of milk powder, previously spiked with the required standards (see Sample Preparation), was directly weighed into a 40 mL tube with stopper. After addition of 10 mL of methanolic potassium hydroxide solution (1 mol/L in methanol) the sample was saponified under gentle shaking for 18 h at room temperature. The unsaponifiable matter was then extracted and analyzed according to the procedure used in common for all of the samples (see further).

Modified Maxwell Method. A wad of glass wool, 0.5 g of a mixture of 1 g of calcium hydrogen phosphate dihydrate and 9 g of Celite 545, and a second wad of glass wool were introduced in this order into a glass column (10 mm i.d., 300 mm length). Exactly 1 g of sample, previously spiked with the required standards (see Sample Preparation), was weighed into a mortar. A few drops of water, 2−3 g of anhydrous sodium sulfate, and 1 g of Celite 545 were then mixed to obtain a homogeneous free flowing slurry. The slurry was quantitatively transferred into the prepared chromatography column and the fat eluted with 25 mL of dichloromethane/methanol (9/1, v/v).

The eluate was evaporated to dryness at 40 °C, under a slight vacuum using a Rotavapor.

The extracted fat was transferred to a centrifuge tube for saponification using acetone.

Modified Folch Method. Exactly 1 g of sample previously spiked with the required standards (see Sample Preparation) was weighed into a 50−100 mL centrifuge tube and dissolved in 20 mL of chloroform and 20 mL of methanol. The mixture was homogenized using a Polytron at more than 10 000 rpm for 2 min and then centrifuged at 2000 rpm for 20 min. The liquid phase was filtered and transferred into a 100 mL separating funnel. The solid phase was homogenized again for 2 min with 25 mL of chloroform/methanol/water (10/10/1, v/v). After centrifugation, the liquid phase was filtered and transferred to a 100 mL separating funnel. A 20 mL aliquot of water was added and the solution vigorously shaken. The organic phase was transferred into a 100 mL preweighed round-bottomed flask, while the aqueous phase was extracted twice with respectively 5 mL and 10 mL of Folch solution (chloroform/methanol, 2/1, v/v) shaking vigorously. The organic phases were collected and evaporated to dryness under vacuum at 40 °C. After cooling at room temperature the residue was weighed.

The extracted fat was transferred to a centrifuge tube for saponification using acetone.

Modified Radin Method. Exactly 1 g of sample previously spiked with the required standards (see Sample Preparation) was weighed into a 80 mL centrifuge tube. A 10 mL aliquot of water, 10 mL of 2-propanol, and 15 mL of hexane were added, and the mixture was vigorously shaken for 2 min after each addition. After centrifugation at 2000 rpm for 5 min, the organic phase was separated and the solvent evaporated under vacuum at 40 °C maximum.

The extracted fat was transferred to a centrifuge tube for saponification using acetone.

Saponification. A 10 mL aliquot of potassium hydroxide methanolic solution (1 mol/L in methanol) was added directly to the milk powder samples or to the fat extracted according to the three methods described above (Maxwell, Folch, and Radin). The tube was wrapped in aluminum foil and saponified under gentle shaking for 18 h at room temperature.

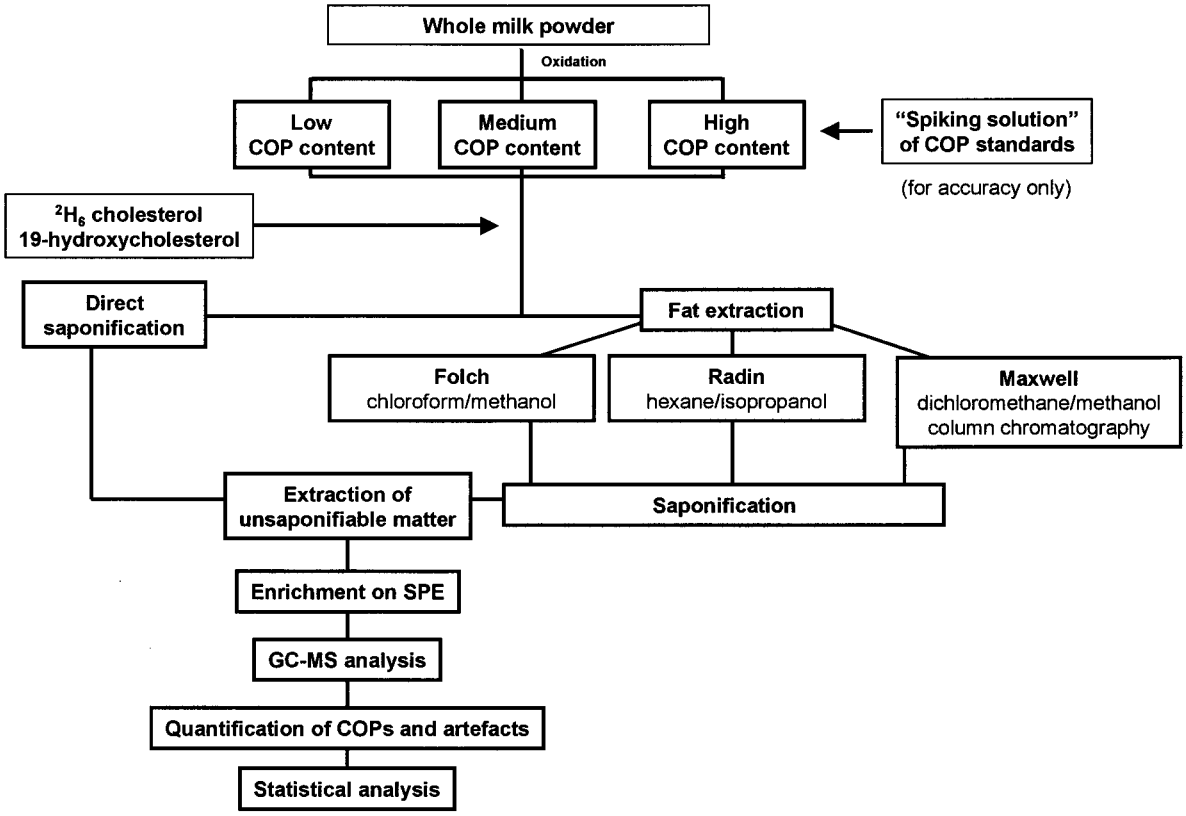
Extraction of Unsaponifiable Matter. The unsaponifiable matter was extracted twice with 10 mL of deperoxided diethyl ether after addition of 10 mL of water. A 15 mL aliquot of an aqueous solution of potassium hydroxide (0.5 mol/L) was added to the organic phase and washed twice using 15 mL of an aqueous solution of sodium sulfate (0.47 mol/L). After drying on anhydrous sodium sulfate, the unsaponifiable matter was then recovered from the organic phase by evaporation at room temperature and under vacuum using a Rotavapor.

Purification of Cholesterol Oxides on SPE Cartridge. COPs were separated from the unsaponifiable matter using a 3 mL aminopropyl-SPE cartridge activated with 3 mL of hexane. The SPE cartridge was first eluted with 6 mL of hexane/ethyl acetate (95/5, v/v) and then with 10 mL of hexane/ethyl acetate (90/10, v/v). COPs were recovered using 7.5 mL of acetone. The acetone was evaporated under nitrogen.

GC-MS Analysis. Before derivatization 2 μg of 5α-cholestane (external standard) was added to the previously purified COPs. The cholesterol oxides were converted to trimethylsilyl ethers by addition of 100 μL of BSTFA, 20 μL of dry pyridine, and 2 μL of TMCS. Silylation was performed overnight at room temperature. Then the extract was dried under a stream of nitrogen and dissolved in 100 μL of hexane/*N*,*O*-bis(trimethylsilyl)acetamide (9/1, v/v).

Quantification was carried out using an HP-5890 gas chromatograph equipped with an HP-7673 autosampler connected to a Finnigan MAT TSQ-700 mass spectrometer and an ICIS II data system (Finnigan MAT, Bremen, Germany). The GC-MS conditions were as follows: DB-1 J&W fused silica capillary column (30 m × 0.32 mm i.d.; film thickness, 0.25 μm); carrier gas, helium at 0.7 bar; on-column injection; oven temperature program, 60 °C (1 min), increasing at 30 °C/min to 200 °C, then 5 °C/min to 300 °C (5 min); transfer line temperature set at 280 °C. The mass spectrometer operated under electron ionization conditions with an electron energy of 70 eV and a source temperature of 180 °C. For the quantification, the ions of interest were acquired by selected ion monitoring at unit resolution (i.e. molecular ion for each compound, except for cholestanetriol and 19-hydroxycholesterol, for which the fragments $[M - H_2O]^+$ and $[M - (2 \times 90)]^+$, respectively, were monitored).

Scheme 1. Experimental Design of the Validation Trials



The response factor of each COP was measured using a multistandard solution containing 1.5 mg of 7α-hydroxycholesterol, 1.5 mg of 7β-hydroxycholesterol, 1.5 mg of 7-ketocholesterol, 1.5 mg of cholestanetriol, 1.5 mg of 25-hydroxycholesterol, and 1.5 mg of 19-hydroxycholesterol dissolved in 10 mL of acetone. The following response factors with respect to 19-hydroxycholesterol were obtained: 7α-hydroxycholesterol, 7.44; 7β-hydroxycholesterol, 5.49; 7-ketocholesterol, 0.88; cholestanetriol, 5.59; 25-hydroxycholesterol, 5.75.

Repeatability of the Direct Method. The repeatability of the direct method was calculated by repeating 8 times the complete analysis of a milk powder oxidized in the laboratory under artificial light for 19 days and then stored in the presence of air for 8 months and containing 67.8 μg/g of total COPs.

Statistical Analysis. Each powder sample was divided into two subsamples (duplicates) for measurement with each method. The duplicate values for each of the 3 concentrations (samples 1−3) provide a mean (M), a repeatability standard deviation (SD), and a variation coefficient (CV = SD/M, in percent).

To avoid the effects of potential outliers or of non-Gaussian distributions, robust estimates were also used: M was replaced by the median (MED) and SD by the "remedian S_n" of Rousseeuw, SD ≈ 1.1926S_n (Rousseeuw and Croux, 1993).

Due to the complexity of the results (five COPs each at three concentrations) the four methods were finally compared by scoring their performance. We chose four parameters: the repeatability SD, its related CV, artifact formation, and the recovery. We ranked the four methods for each of these parameters within each COP concentration, by giving a score between 1 (best) and 4 (worst). Globally, and by their definitions, CVs were very closely related to SDs. Thus, we used their average scores as a measure of precision.

RESULTS AND DISCUSSION

Three aliquots of a milk powder (24.7% of total fat and 807 mg/kg of total cholesterol) were oxidized in the laboratory under artificial light, in the presence of air for three different exposure times (2, 8, and 19 days) to form different levels of COPs. These three powders were analyzed in duplicate using four different methods: the direct method and three methods involving fat extraction (Maxwell's, Radin's, and Folch's methods) all followed by saponification, aminopropyl-SPE purification, and GC-MS/SIM quantification. Using the direct method the total COP contents were respectively 0.63 μg/g for powder 1 (low COP content), 9.5 μg/g for powder 2 (medium COP content), and 40 μg/g for powder 3 (high COP content). The experimental design of the proposed trials is shown in Scheme 1.

Overnight reaction at room temperature is known to allow complete saponification of the five COPs of interest: 7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol, 25-hydroxycholesterol, and 5α-cholestane-3β,5α,6β-triol (Rose-Sallin et al., 1995). As previously mentioned (Rose-Sallin et al., 1995), α/β-epoxycholesterol epimers (5α,6α- and 5β,6β-epoxy-5-cholestan-3β-ol) and 20α-hydroxycholesterol are not accurately silylated or purified by the used procedures here and were not investigated in this study.

COPs in the samples were quantified by monitoring the molecular ion of each compound, except for cholestanetriol and 19-hydroxycholesterol for which the fragments $[M - H_2O]^+$ and $[M - (2 \times 90)]^+$, respectively, were acquired. Cholesterol oxide artifacts were monitored using [$^2H_6$]cholesterol according to the procedure developed by Wasilchuk et al. (1992) and Rose-Sallin et al. (1995). For economical reasons, [$^2H_6$]cholesterol was used instead of [$^2H_7$]cholesterol as previously described (Rose-Sallin et al., 1995). Therefore, for each COP, the corresponding [$^2H_6$]cholesterol oxide artifact was measured, except for cholestanetriol for which [$^2H_5$]-cholestanetriol was monitored (Figure 1).

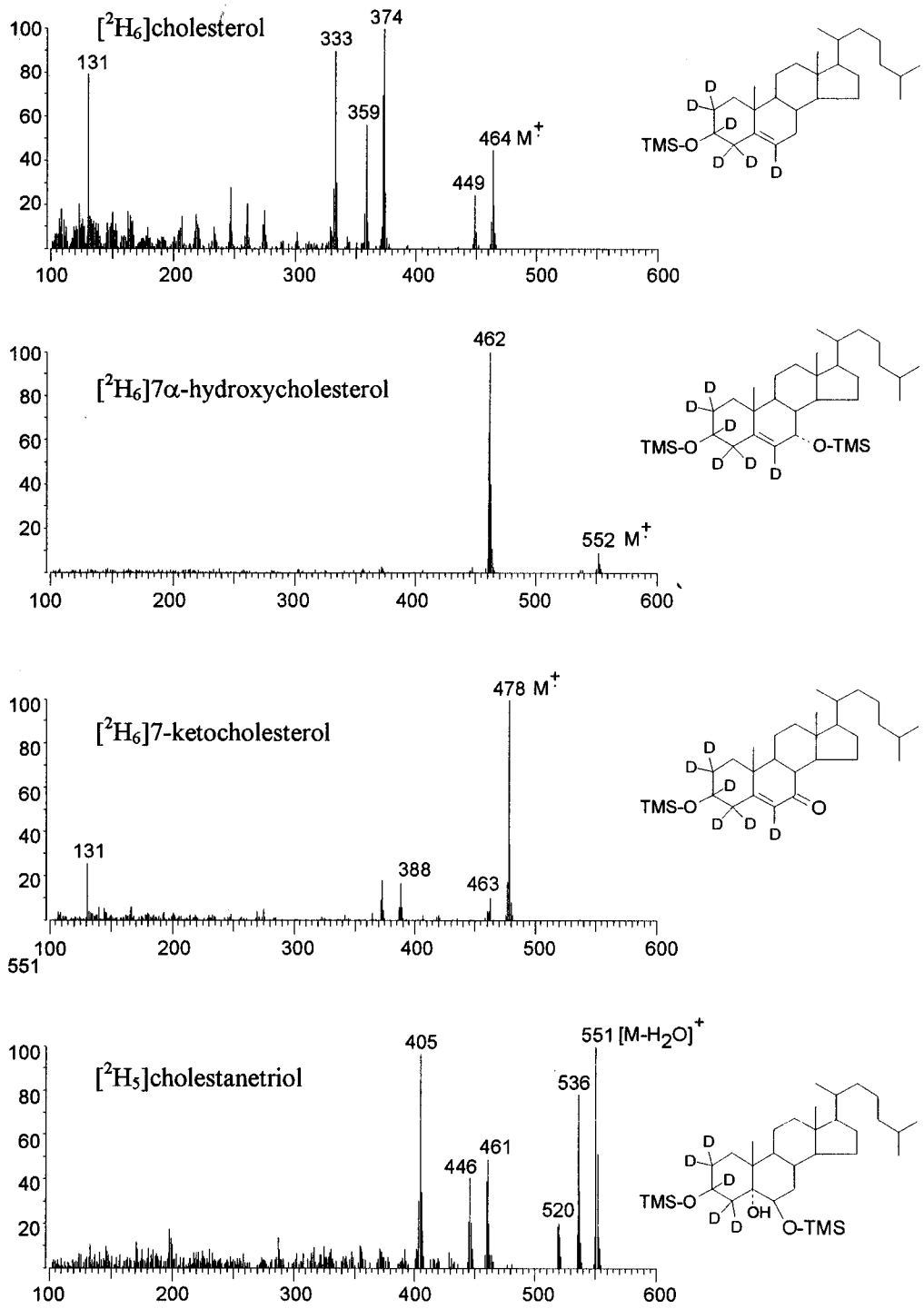


Figure 1. Mass spectra of the labeled COPs formed after oxidation of [$^2H_6$]cholesterol. For each COP, the corresponding [$^2H_6$]-cholesterol oxide is formed, except for cholestanetriol which forms [$^2H_5$]cholestanetriol.

Method Comparison. Table 1 summarizes the levels of COPs found in the three milk powders using the four methods. We see that SDs were rather similar for low and medium COP contents (samples 1 and 2), while SD of the high COP content powder was much higher. For CV the situation was opposite; i.e., CVs were much lower for medium and high COP content (samples 2 and 3).

It was decided to compare the four methods by scoring their performance. The results are reported in Table 2 for SD and CV and in Table 3 for artifacts and recovery and are summarized in Table 4. Radin's method gave the worst score (highest value) while the direct method had the best score (lowest value).

As a further comment we stress that direct, Maxwell's, and Folch's methods gave comparable results, whereas the values of COPs found using Radin's method were drastically low (see Table 1). The very low recovery of Radin's method is probably because of the quite apolar solvents used for the fat extraction. On the other hand, recovery after spiking using Radin's method was quite good (see Table 5): this confirms that spiked COPs are more easily recovered than COPs already present in the food matrix.

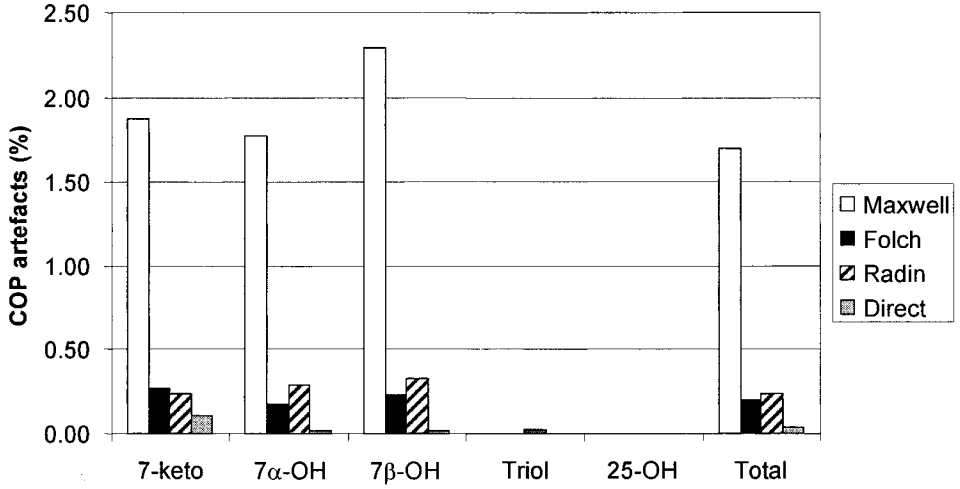


Figure 2. Cholesterol oxide artifact formation during the cleanup of milk powder using Maxwell's, Folch's, Radin's, or the direct method. The results are expressed as percentage of deuterated COPs of total amount of nondeuterated (deuterated COPs/nondeuterated CPOs × 100).

Table 1. COP Contents in Three Milk Powders at Different Levels of Oxidation (See Text) Quantified with Four Different Methods (Mean of Duplicate Analyses and Three GC-MS Injections; Values in Micrograms per Gram)[a]

		7-keto			7α-OH			7β-OH			triol			25-OH			total		
		mean	SD	CV (%)	mean	SD	CV (%)	mean	SD	CV (%)	mean	SD	CV (%)	mean	SD	CV (%)	mean	SD	CV (%)
direct	1	0.164	0.002	1.3	0.133	0.188	141.4	0.332	0.049	14.9	nd			nd			0.630	0.052	6.8
	2	1.968	0.061	3.1	3.429	0.036	1.1	4.107	0.062	1.5	nd			nd			9.505	0.128	1.3
	3	9.709	0.257	2.6	12.863	0.338	2.6	17.382	0.966	5.6	nd			nd			39.954	1.048	2.6
Maxwell	1	0.381	0.031	8.2	0.298	0.044	14.6	0.329	0.021	6.5	0.047	0.067	141.4	0.155	0.049	31.9	1.210	0.169	14.0
	2	4.105	0.176	4.3	2.949	0.053	1.8	2.996	0.057	1.9	nd			0.200	0.014	7.1	10.249	0.158	1.5
	3	17.332	0.720	4.2	8.819	0.528	6.0	10.484	0.730	7.0	nd			0.350	0.495	141.4	36.985	0.042	0.1
Folch	1	0.312	0.036	11.6	0.260	0.000	0.0	0.309	0.050	16.2	nd			0.025	0.035	141.4	0.905	0.022	2.4
	2	5.714	0.233	4.1	2.911	0.087	3.0	2.500	0.086	3.4	nd			0.125	0.021	17.0	11.250	0.428	3.8
	3	22.415	3.690	16.5	10.813	2.620	24.2	10.029	0.759	7.6	nd			0.375	0.106	28.3	43.632	7.175	16.4
Radin	1	0.117	0.041	35.4	0.075	0.039	51.4	0.086	0.021	25.0	0.007	0.010	141.4	0.005	0.007	141.4	0.291	0.119	40.9
	2	1.620	0.135	8.3	0.884	0.107	12.1	0.982	0.072	7.3	0.011	0.015	141.4	0.020	0.028	141.4	3.516	0.326	9.3
	3	4.471	0.062	1.4	1.815	0.000	0.0	2.014	0.014	0.7	0.054	0.005	9.4	0.075	0.007	9.4	8.430	0.078	0.9

[a] nd = not detectable.

Table 2. Scores for the Comparison of the Four Methods: SD and CV

		7-keto			7α-OH			7β-OH			triol			25-OH				
		SD	CV	mean	SD	CV	mean	SD	CV	mean	SD	CV	mean	SD	CV	mean	Σ
direct	1	1	1	1	4	4	4	3	2	2.5	1.5	1.5	1.5	1	1	1	10
	2	1	1	1	1	1	1	2	1	1.5	2	2	2	1	1	1	6.5
	3	2	2	2	2	2	2	4	2	3	2	2	2	1	1	1	10
	Σ			**4**			**7**			**7**			**5.5**			**3**	**26.5**
Maxwell	1	2	2	2	2	2	2	1.5	1	1.25	4	3.5	3.75	4	2	3	12
	2	3	3	3	2	2	2	1	2	1.5	2	2	2	2	2	2	10.5
	3	3	3	3	3	3	3	2	3	2.51	2	2	2	4	4	4	14.5
	Σ			**8**			**7**			**5.25**			**7.75**			**9**	**37**
Folch	1	3	3	3	1	1	1	4	3	3.5	1.5	1.5	1.5	3	3.5	3.25	12.3
	2	4	2	3	3	3	3	4	3	3.5	2	2	2	3	3	3	14.5
	3	4	4	4	4	4	4	3	4	3.5	2	2	2	3	3	3	16.5
	Σ			**10**			**8**			**10.5**			**5.5**			**9.25**	**43.3**
Radin	1	4	4	4	3	3	3	1.5	4	2.75	3	3.5	3.25	2	3.5	2.75	15.8
	2	2	4	3	4	4	4	3	4	3.5	4	4	4	4	4	4	18.5
	3	1	1	1	1	1	1	1	1	1	4	4	4	2	2	2	9
	Σ			**8**			**8**			**7.25**			**11.3**			**8.75**	**43.3**

Cholesterol oxide artifacts were monitored for each of the five COPs of interest. In Figure 2, cholesterol oxide artifacts are shown, expressed as percent of deuterated COPs of total amount of nondeuterated ([$^2H_{6\ or\ 5}$]-$COP_i/COP_i \times 100$). The figure shows that Maxwell extraction of fat leads to the highest artifact formation, while the direct method presents the lowest cholesterol autoxidation (total artifact < 0.05%). Table 3 shows the results obtained by scoring the four methods versus the amount of each cholesterol oxide artifact formed.

The accuracy of the four methods was compared after spiking sample 3 (containing high COPs levels) with a standard solution containing the five COPs under evaluation (see Sample Preparation). Table 5 summarizes the recoveries obtained with each method. All four methods show a good accuracy for 25-hydroxycholesterol, 7α-hydroxycholesterol, and 7β-hydroxycholesterol (>75%). The only exception was the Folch method with a recovery of 34 and 42% for 7α-hydroxycholesterol and 7β-hydroxycholesterol, respectively. Accuracies for 7-ketocholesterol and 5α-cholestane-3β,5α,6β-triol were less good (recovery below 50%).

The accuracy of the four methods (recovery) was scored following the described procedure, and results

Table 3. Scores for the Comparison of the Four Methods: Artifacts and Recovery

		7-keto	7α-OH	7β-OH	triol	25-OH	total
artifacts	direct	1.0	1.0	1.0	2.0	2.5	7.5
	Maxwell	4.0	4.0	4.0	2.0	2.5	16.5
	Folch	2.5	2.0	2.0	2.0	2.5	11.0
	Radin	2.5	3.0	3.0	4.0	2.5	15.0
recovery	direct	3.0	1.0	1.0	1.0	2.0	8.0
	Maxwell	1.0	2.5	2.5	2.0	2.0	10.0
	Folch	3.0	4.0	4.0	3.0	3.0	17.0
	Radin	3.0	2.5	2.5	4.0	3.0	15.0

Table 4. Scores for the Comparison of the Four Methods: Total Results

method	precision	artifact	recovery	Σ
direct	26.5	7.5	8.0	42.0
Maxwell	37.0	16.5	10.0	63.5
Folch	43.3	11.0	17.0	71.3
Radin	43.3	15.0	15.0	73.3

Table 5. Recovery of COPs after Spiking of Sample 3: Results Expressed as Percent[a]

	7-keto	7α-OH	7β-OH	triol	25-OH
direct	44.2	104.3	97.9	55.0	93.4
Maxwell	56.1	80.4	77.6	45.4	92.9
Folch	45.1	33.8	41.6	41.8	76.6
Radin	47.3	80.6	73.6	35.6	75.3
spiked (mg/g)	19.4	16.2	17.2	18.9	19.3

[a] The values are the mean of duplicate analyses and three GC-MS injections.

Table 6. Repeatability of Direct Method on a Milk Powder Sample[a]

	7-keto	7α-OH	7β-OH	triol	25-OH
n	8	8	8	8	8
mean	21.560	22.043	23.768	0.089	0.384
SD	1.03	0.88	1.14	0.03	0.04
CV (%)	4.8	4.0	4.8	35.2	9.9
MED	21.550	22.025	23.868	0.084	0.372
robust SD	1.36	1.05	1.51	0.03	0.04
robust % CV	6.3	4.8	6.3	36.1	9.8

[a] The values are the mean of eight repetions and three GC-MS injections.

are summarized in Table 3. Folch and Radin methods showed poorer recovery than the Maxwell and direct methods.

Considering that the recovery of cholestanetriol was poor, the analysis was repeated using a pure cholestanetriol standard. The obtained results confirmed a recovery of about 64% without any artifact formation or transformation into other COPs. Therefore cholestanetriol is lost due to its high polarity.

These data confirm that for an accurate quantification, the methods for determining COPs in milk powder must be optimized for each of the compounds to be quantified. Small differences in terms of polarity have a drastic influence on overall accuracy. The internal standard should also be carefully chosen in order to select a compound as chemically similar as possible to the COPs of interest.

The results of the scoring procedure clearly demonstrate that the direct method represents the best compromise for quantifying COPs in milk powders. The Folch and Radin methods were imprecise, and the Maxwell method should be avoided owing to the relatively high amount of artifacts formed.

Repeatability of the Direct Method. Table 6 summarizes the values obtained for mean, SD, CV, robust SD, and robust CV. Repeatability was good: robust CV was between 4.8 and 9.8% for 7-ketocholesterol, 7α- and 7β-hydroxycholesterol, and 25-hydroxycholesterol, while 5α-cholestane-3β,5α,6β-triol had a robust CV of 36%. This high value of CV can be explained by the very low amount of 5α-cholestane-3β,5α,6β-triol measured.

CONCLUSION

The direct method previously developed for the analysis of COPs in milk powder (Rose-Sallin et al., 1995) and consisting of a saponification followed by extraction of the unsaponifiables, COP purification by SPE, and quantification by GC-MS was compared to three methods involving preliminary fat extraction before saponification (Maxwell's, Radin's, and Folch's methods) followed by the same cleanup and quantification procedure. Each method's performance, in terms of standard deviation, coefficient of variation, artifact formation, and accuracy, was scored. The direct method was demonstrated to offer the best compromise among the four alternatives. It has a good repeatability and an acceptable accuracy, especially considering the low COP concentrations (μg/g) and the method's complexity. In addition, it leads to a minimal formation of artifacts (<0.05% of total cholesterol) and shows clear advantages of time and the quantity of solvent used.

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