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Comparison of three methods for the determination of oxysterols in spray-dried egg

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Abstract

Three methods for the GC determination of oxysterols (OSs) in spray-dried egg, which combine different steps of purification, are compared. In addition, the efficiency of silica cartridges in the purification of OSs using four different systems of elution with increasing polarities is studied. The absence of cholesterol oxidation during the application of the analytical procedures is checked, and the linearity of the response and the chromatographic limits of detection and quantification are established. The methods are characterized by the calculation of precision and recovery for the different OSs. The method based on saponification alone is rejected, since it shows much lower precision. The method that includes saponification and silica cartridge purification offers higher reliability than the method based on cartridge purification alone, because it shows a higher precision and larger samples can be processed, which improves the limits of detection and quantification.

1. Introduction

The analysis of oxysterols (OSs) is quite a new field and several aspects of the methodology are still controversial. As a consequence, many systems have been proposed, and many of these still need improvement and standardization. The purification of the OS fraction, which involves saponification and/or chromatographic techniques, is the most critical step of the analytical procedure, since the high structural similarity of the various OSs and the complexity of the lipid fraction of many foods demand high selectivity. These initial steps depend on the technique finally used in the OS determination, which is usually GC or HPLC. Moreover, the possibility

of cholesterol oxidation during the analytical procedure is another factor that must be borne in mind, but which can be avoided through the optimization of the operating conditions.

Various solvent mixtures have been used for fat extraction in egg samples, but the mixture chloroform–methanol (2:1, v/v) provides the best extraction of the total fat and polar compounds, such as cholesterol and OSs [1,2].

Purification is a critical step in the method, since it improves the selectivity of the determination, and it involves one or two of the following steps: saponification of lipids, and column or TLC fractionation. Saponification is usually applied to the lipid fraction, although some authors attempt to saponify the whole food sample directly [1,3]. However, most authors report better results with the previous extraction of

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lipids. Saponification temperature is also controversial. Cold saponification with methanolic KOH has been widely accepted in recent years, instead of hot saponification [1], since the latter increases the possibility of cholesterol oxidation [3,4]. It also increases the instability of some OSs, such as 7-ketocholesterol (7-KC) and cholesterol-5 α ,6 α -epoxide (α -CE). The instability of 7-KC in hot alkaline media has been known since the 1940s, when Bergström and Wintersteiner [5] suggested that cholesta-3,5-dien-7-one could be one of its major decomposition products. Later, several authors supported this observation [4,6–8]. Other authors have reported that hot saponification induced the decomposition by hydrolysis of α -CE to cholestantriol and Tsai et al. [9] reported only 25% recovery of α -CE from hot saponification. The stability of 7-KC and α -CE is well preserved by cold saponification [10–12] and the oxidation of cholesterol is not produced [12,13]. However, contrasting results were reported by Van de Bovenkamp et al. [14] and Bascoul et al. [15], who suggested, respectively, that 7-KC is not stable during cold saponification and that cholesterol is not oxidized in hot alkaline media. In spite of the results reported by Van de Bovenkamp [14] for cold saponification, the methods of OS determination in foods that follow cold saponification give a good recovery of 7-KC and its main decomposition product, cholesta-3,5-dien-7-one, is not detected. This decomposition product is usually detected when hot saponification is used for OS determination in foods [15–17].

Some authors proposed a column fractionation step to purify OSs. This purification can be applied to the total lipids extracted from foods or to the non-saponifiable fraction. Silica is usually the adsorption stationary phase and increasing polarity solvent mixtures are applied to the column first to remove the interference compounds and then to isolate the OS fraction. In recent years, most studies [1,9,18–23] have used short columns or cartridges to purify the OS fraction. Other techniques have found less application, such as gel filtration chromatography [1,21,22,24], argentation chromatography (Florisisil-AgNO₃ columns) [1,8] or preparative

TLC applied to the total lipid or non-saponifiable extract [1,12,13,25].

The lack of comparative studies dealing with analytical methods for the determination of cholesterol oxides led the authors to design the present study, with the aim of comparing three alternative methods. Two of these were chosen from among the most usually reported in recent references. The third was proposed by the authors and resulted from the fusion of the other two methods. In addition, an attempt was made to improve and characterize the three methods and finally select the one that gives the best reliability.

In order to improve these methods, the efficiency of silica cartridges in the separation of a purified OS fraction, in the presence of the main interference attributable to a much higher cholesterol concentration in some samples (e.g. egg products), was studied. Four different systems of elution, chosen from those reported in the reference list and differing in the polarity of the final solvent used for the elution of OSs, were compared. The respective recoveries of each system for OSs and cholesterol, as well as the precision of these recoveries were used to characterize the system of elution, and finally to select the most efficient one. The recovery of phytosterols was studied using the system finally selected in order to apply it in the determination of OSs in samples of plant or mixed origin.

2. Experimental

2.1. Samples

All experiments were performed using the same dried egg sample, obtained by spray drying.

2.2. Reagents and standards

The solvents used were of the following origin and quality: chloroform, methanol, diethyl ether and acetone (all ACS grade) and hexane (for analysis) were from Panreac (Barcelona, Spain), and the ethyl acetate (ACS grade) and the dried

pyridine (max. 0.01% water, for analysis) were from Merck (Darmstadt, Germany).

Other reagents used were sodium hydroxide and anhydrous sodium sulphate (both for analysis) supplied by Panreac and the Sylon BTZ [N,O-bis(trimethylsilyl)acetamide–trimethylchlorosilane–N-trimethylsilylimidazole, 3:2:3, for research], in 0.1-ml glass ampoules, from Supelco, Bellefonte, PA, USA. Silica Sep-Pak cartridges were supplied by Waters, Millipore Division (Milford, MA, USA).

The cholesterol standard was supplied by Merck (>99%, by GC) and 5 α -cholestane (99%, by GC) by Supelco. All other standards were from Sigma (St. Louis, MO, USA): cholesterol-5 α ,6 α -epoxide (99%, by TLC), 7 β -hydroxycholesterol (7 β -HC) (99%, by TLC), cholestanetriol (CT) (>97%, by GC), 7-ketocholesterol (>99%, by HPLC), 25-hydroxycholesterol (25-HC) (>98%, by TLC), β -sitosterol (95%, by GC), stigmasterol (96%, by GC), 11 α -hydroxyprogesterone (>98%, by GC), 11 β ,17 α -dihydroxyprogesterone (99%, by HPLC), 19-hydroxycholesterol (19-HC) (>99%, by TLC) and 6-ketocholestanol (>99%, by GC). All these standards were weighed with an accuracy of 0.01 mg, and were made up as ethyl acetate solutions. The purity of these standards was checked by GC.

The choice of a suitable internal standard (I.S.) was made according to the following criteria: (1) the internal standard 5 α -cholestane was not recovered through the silica cartridge and had to be added to the final OS extract; (2) recently several authors [12,13,19–21,25,26] used one of several I.S.s (6-ketocholestanol, 7-ketopregnenolone, 5 α -androstan-3 β -ol-17-one acetate and 19-HC), which were well recovered together with OSs through silica columns and plates; (3) four standards were assayed, 11 α -hydroxyprogesterone, 11 β ,17 α -dihydroxyprogesterone, 6-ketocholestanol and 19-HC, in order to find an I.S. well recovered through silica cartridge, and the first two standards were rejected, since they showed unknown impurities when they were injected in the chromatograph; (4) the 6-ketocholestanol was rejected since the chromatographic retention time was too close to that

corresponding to the cholestanetriol, working under the conditions of temperature programme II. So, 19-HC was chosen as the most suitable I.S., after checking that it was well recovered through the silica cartridges.

2.3. Silica cartridge fractionation

Elution systems compared

Four different solvent elution sequences were compared, as shown in Fig. 1. Elution sequence I is similar to that used by Tsai and Hudson [23] and Tsai et al. [9] for the determination of epoxycholesterols in egg products. Sequence II was proposed by Morgan and Armstrong [18] for the determination of the same OSs in dried egg yolk. Sequence III is similar to that used by Morgan and Armstrong [19,20] for the determination of several OSs in dried egg yolk. Sequence IV was a modification of sequence III, which aimed to increase the polarity of the last solvent used for the OS elution. Thus, four sequences of increasing polarity from I to IV were compared. Solvents were applied to the cartridge at 7 ml/min using a syringe.

Method

In all cases, the cartridge was equilibrated with 5 ml of hexane and then the solution (in 5 ml of hexane) containing the OSs and cholesterol or phytosterols standards was applied to the cartridge. The elution was then carried out applying, first, a further 5 ml of hexane and then solvent mixtures of increasing polarity. The four sequences assayed differed mainly in the composition of the last solvent, by which the OSs were eluted from the cartridge. This OS solution was recovered in a round-bottomed flask and concentrated to approx. 1 ml, by using a vacuum rotatory evaporator. This concentrate was quantitatively transferred with diethyl ether to a glass microtube (75 \times 10 mm), containing 25 μ g of 5 α -cholestane as I.S. The evaporation of solvent was then completed by a slight nitrogen stream, at 25°C, and by keeping the tube in a vacuum desiccator at 10 mmHg (1 mmHg = 133.322 Pa) for 1 h. The residue of OSs was silanized and determined by GC in a Perkin-Elmer chromato-

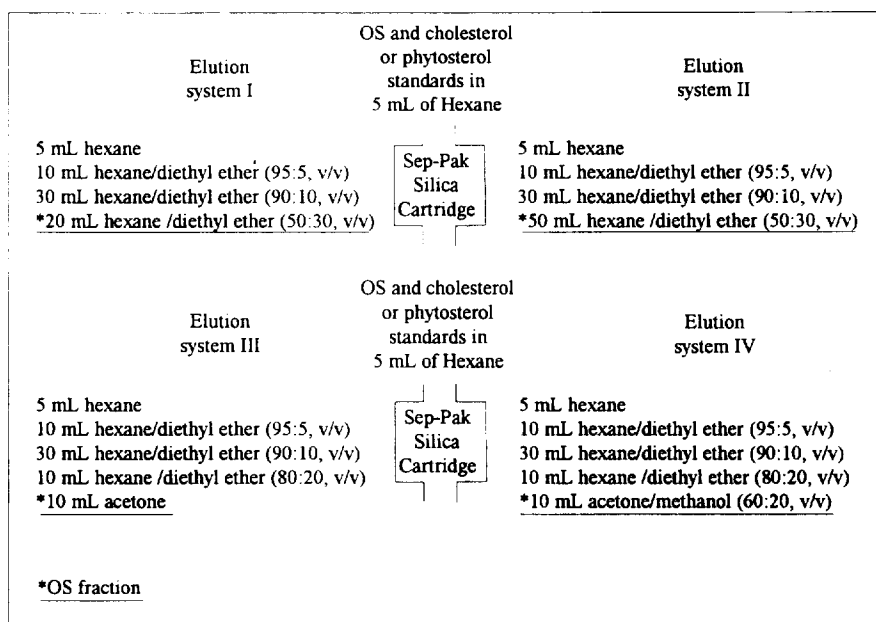


Fig. 1. Diagram of the elution systems compared.

graph, Model Sigma 2000, as described below in the section on GC conditions.

Amounts of standards fractionated: experiment I

The fractionation of three different solutions containing a constant amount of cholesterol (30 μg) and three increasing amounts of five OSs (2.5, 5 and 10 μg of α -CE, CT, 7-KC and 25-HC; and 5, 10 and 20 μg of 7 β -HC) was assayed. This concentration range was chosen considering that samples for the analysis were dried egg and that one of the analytical problems was to separate the OS fraction from a high concentration of cholesterol.

Fifteen replicates of the four different elutions were performed, five corresponding to each of the three concentration levels. For the 60 OS extracts obtained, the recoveries of cholesterol, α -CE, 7 β -HC, CT, 7-KC and 25-HC were calculated.

Experiment II

The phytosterols were a clear interference, when the determination of OSs was carried out in samples of plant or mixed origin. This led to

the study of the recovery of these compounds using the elution system finally chosen. The fractionation of three different solutions containing the amounts of the five OSs, described above; and 2.5, 5 and 10 μg of stigmasterol and β -sitosterol was assayed. Five replicates of each of the three concentration levels were performed and the recoveries for the different standards were calculated.

2.4. Linearity of response

The linearity of the response of the cholesterol and the OS standards up to 55 μg was checked by calculation of calibration curves. These curves were determined on the two Perkin-Elmer gas chromatographs (Sigma 2000 and Autosystem), using two internal standards, 5 α -cholestane and 19-HC. The chromatographic conditions were those described below. The formula of these calibrate curves resulted from the regression of the variable y (A_{xs}/A_{1s}) on the variable x (W_{xs}/W_{1s}) and its correlation coefficient (r^2) expresses the linearity of the response. From the curve, the relative response factors (RRFs) were also calculated, according to the formula: $\text{RRF} =$

$(W_{xs}A_{I.S.})/(A_{xs}W_{I.S.})$ (where W_{xs} = standard mass; $W_{I.S.}$ = internal standard mass; A_{xs} = standard area; $A_{I.S.}$ = internal standard area).

Three of these curves were calculated. The first two with 5α -cholestane as I.S., using the Sigma 2000 and Autosystem. The third curve was developed using the Autosystem, with 19-HC as I.S. The amounts of standards used were: 0.5, 1, 2.5, 5, 10, 20 and 30 μg , for the first curve; 0.6, 1.2, 2.5, 5, 10 and 20 μg , for the second; and 2.5, 5, 10, and 20 μg for the third. All masses of standards were dissolved in 50 μl of pyridine and silanized with 50 μl of Sylon BTZ, and then injected in duplicate. Five replicates of each level of concentration were performed for the first two curves and four replicates for the third.

2.5. Chromatographic limits of detection and quantification

These limits in relation only to the chromatographic method were determined using the definition and calculation procedure proposed by Knoll [27]. For the calculation of these limits 10 blank solutions and 15 standard solutions (three different concentrations, 2.5, 5 and 10 $\mu\text{g}/\mu\text{l}$) were injected into each chromatograph described below.

2.6. Procedure of the three methods for the determination of OSs compared

Two of the three methods for the determination of OSs that were compared were chosen from among the most reliable methods proposed in the reference list (methods I and II). Method III was a combination of the other two.

Method I

This method consists of a lipid extraction by agitation with chloroform–methanol (2:1, v/v), a further 1 M KOH in methanol cold saponification, the extraction of the unsaponifiable material with diethyl ether, and silanization before GC determination. This method was first introduced by Park and Addis [11] to determine OSs in heated fats and it has been applied by several authors to different food and biological samples

[28–34]. Some authors complete the method by TLC fractionation of the non-saponifiable components [12,13,25].

Method II

This method consists of the same lipid extraction as in method I; further purification by adsorption on a silica cartridge and selective elution of the OS fraction and silanization before the GC determination. This method has been used for the determination of OSs in egg yolk powder by Morgan and Armstrong [19,20].

Method III

This is a result of the fusion of methods I and II and consists of the same lipid extraction; the cold saponification and the extraction of unsaponifiable material with diethyl ether; the purification of the OS fraction by selective elution from a silica cartridge and silanization before the GC determination.

Fig. 2 shows a diagram of the three methods compared. All these methods have in common the conditions of attenuated light during all the procedure.

2.7. Lipid extraction (common to the three methods)

Lipids were extracted according to the method proposed by Folch et al. [35], with the following modifications. A 0.65-g amount of powder egg was accurately weighed in a 25-ml conical flask, and 25 μg of 19-HC¹ and 15 ml of chloroform–methanol (2:1, v/v) were added. The mixture was stirred magnetically for 30 min. Then the liquid phase obtained was decanted through a paper filter and recovered in a tube (100 \times 26 mm) with a screw cap. The solid residue in the conical flask was reextracted for 10 min with 10 ml of chloroform–methanol (2:1, v/v) and rinsed in 5 ml of the same mixture. The liquid phases obtained were filtered and recovered in the same tube. A 5-ml volume of distilled water was added to the tube, which was agitated and then cen-

¹ When 19-HC is used as I.S., it is added to the sample, and when 5α -cholestane is used, it is added to the final extract.

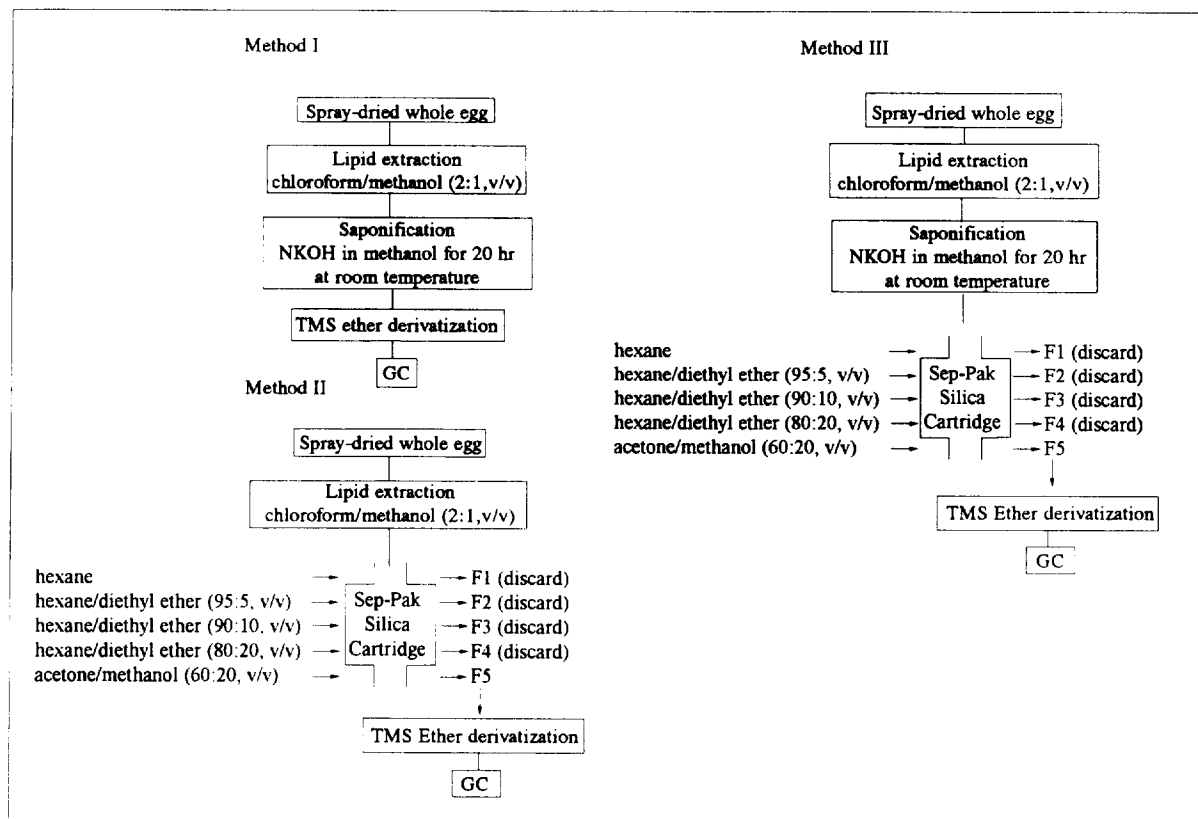


Fig. 2. Diagram of the three methods compared.

trifuged at 2200 rpm for 20 min. The chloroformic extract was transferred to a round-bottomed flask (in method II this extract was previously filtered through anhydrous sodium sulphate), and was concentrated to 1 ml in a vacuum rotatory evaporator, at 35°C. Removal of solvent was completed in a slight nitrogen stream and by keeping the flask in a vacuum desiccator at 10 mmHg for 30 min (in methods I and III) or 1 h (in method II).

2.8. Cold saponification (only in method I and III)

A 10-ml volume of 1 M KOH in methanol was added to the flask containing the lipid extract, with gentle agitation to obtain a homogeneous phase. Then the mixture was kept at room temperature for 20 h to complete the saponifica-

tion. The blend was then quantitatively transferred to a tube (100 × 26 mm) using 10 ml of diethyl ether and 10 ml of distilled water. The sealed tube was then shaken and the organic phase was transferred to a separating funnel. The aqueous phase in the tube was again re-extracted twice with two portions of 10 ml of diethyl ether and the new organic phases obtained were also transferred to the same funnel. The whole organic extract was then washed in the funnel, first in 5 ml of 0.5 M aqueous KOH solution, and then in two portions of 5 ml of distilled water. The washed organic extract was filtered through anhydrous sodium sulphate and recovered in a round-bottomed flask, from which the solvent was evaporated to 1 ml, using a rotatory vacuum evaporator at 30°C. From this point, different procedures for methods I and III were followed. In method I, the concentrated

extract was transferred quantitatively with diethyl ether to a glass tube (75 × 10 mm), containing 25 µg of 5α-cholestane (see footnote 1). Glass tubes were used to avoid variability of results, since we have found that plastic materials showed different adsorption of OSs and 5α-cholestane [36]. The non-saponifiable residue was obtained by removal of solvent, using a slight nitrogen stream, at 25°C, and finally in a vacuum desiccator at 10 mmHg for 1 h. Afterwards, the silanization and GC determination were carried out. For method III, the non-saponifiable residue was obtained by removing the remaining solvent by a slight nitrogen stream, at 25°C, and by keeping the flask in a vacuum desiccator at 10 mmHg for 30 min.

2.9. Silica cartridge purification (only in methods II and III)

The lipid extract (method II) or the non-saponifiable extract (method III) was redissolved in 5 ml of hexane and applied to a silica cartridge, previously equilibrated with 5 ml of hexane. The cartridge was then eluted with solvent sequence IV, as described above. The obtained OS fraction plus 25 µg of 5α-cholestane (see footnote 1) was silanized and determined by GC, as described below.

2.10. GC conditions (common to the three methods)

The final residue obtained in any of the three procedures described was redissolved in 50 µl of anhydrous pyridine and 50 µl of Sylon BTZ were then added, and the mixture was kept at room temperature for 20 min to complete the silanization reaction, before injection to the column. The silyl derivatives are stable for several days at -20°C [37]. All injections were performed in duplicate.

GC was performed using two Perkin-Elmer chromatographs, Models Sigma 2000 and Autosystem, equipped with a flame ionization detector and fused-silica capillary column (25 m × 0.25 mm I.D.), with a film thickness of 0.13 µm stationary phase of 100% methylsilicone

(Chrompack, Middelburg, Netherlands). Helium was used as carrier gas and the chromatographic conditions were as follows. Oven temperature programmes: (I) from 210 to 240°C at 6°C/min, from 240 to 270°C at 4°C/min, from 270 to 290°C at 2°C/min and 5 or 82 min at 290°C (used in the Sigma 2000); (II) from 210 to 264°C at 2°C/min, from 264 to 290°C at 5°C/min and 2 or 80 min at 290°C (used in the Autosystem). Injector temperature 290°C. Detector temperature 350°C. Split ratio 1:40 for the Sigma 2000 and 1:30 for the Autosystem. Inlet pressure 15 p.s.i. (1 p.s.i. = 6894.76 Pa). Sample volume injected is 2 µl.

2.11. Identification of OSs

Co-chromatographic identification

Firstly, the identification was accomplished by comparison of the relative retention times of the silanized standards with the times corresponding to the peaks obtained for a silanized purified extract from a dried egg sample. After that, the identification was confirmed by addition of silanized OS standards to the same extract (co-chromatography). For major peaks in the sample, like those corresponding to 7β-HC and 7-KC, a previous dilution with pyridine was carried out to obtain a similar size to that obtained with the injection of the standard solution (1.2 µg/100 µl). The dilution was made in order to give lower peaks, which facilitated asymmetry observation. In all OS peaks, the amount of standard added was calculated to increase the area between 50 and 100% in order to facilitate the detection of asymmetrical peaks. All standard-sample mixtures were injected several times and using the two former temperature programmes, as well as the following: (III) from 210 to 290°C at 6°C/min and 10 or 87 min at 290°C; (IV) from 210 to 240°C at 6°C/min, from 240 to 290°C at 4°C/min and 7 or 84 min at 290°C; (V) from 210 to 240°C at 6°C/min, from 240 to 290°C at 2°C/min and 2 or 80 min at 290°C. The last temperature programme did not separate α-CE from 7β-CE and was not used for their identification. Identification was only confirmed when the re-

sulting peak in the spiked sample was symmetrical at all chromatographic temperatures assayed.

Mass spectrometric identification

A further confirmation of the peak identified in egg samples was provided by GC–MS. The system used was a Hewlett-Packard 5988A mass spectrometer coupled to a Hewlett-Packard 5890 gas chromatograph, equipped with a capillary fused-silica column (25 m × 0.32 mm I.D.), with a film thickness of 0.52 μm stationary phase of 100% methylsilicone (Hewlett-Packard, Geneva, Switzerland). Helium was used as carrier gas and chromatographic conditions were as follows. Oven temperature programme: from 210 to 264°C at 2°C/min, from 264 to 290°C at 5°C/min and 2 or 80 min at 290°C. Injector temperature: 290°C. Split ratio: 1:50. Inlet pressure: 15 p.s.i. Sample volume injected: 5 μl. MS conditions were: interphase temperature 280°C; ion source temperature 200°C; electron energy 70 eV. Cholesterol oxides were identified in the mass range (m/z) from 100 to 650. The SIM (selected ion monitoring) technique was also applied, in which the ions of m/z 120, 129, 131, 321, 367, 382, 384, 403, 456, 472, 474 and 546 were selected as the most characteristic of the OSs determined.

2.12. Absence of cholesterol oxidation during the procedures

A 15-mg amount of cholesterol standard (amount present in approximately 0.95 g of dried egg or in 3.8 g of fresh egg), in which absence of OSs was checked by GC, was subjected to the whole procedure of method III and then injected to the column. This test was carried out in quadruplicate.

3. Results and discussion

3.1. Identification of OSs in the egg sample

Fig. 3 shows the peaks identified according to the procedure described above in the co-chro-

matographic identification section, which correspond to 7β-HC, α-CE, CT, 7-KC and 25-HC.

Comparing the mass spectra of peaks from the egg sample analyzed with those corresponding to standards injected in the same conditions and with spectra reported by several authors [11,22,24,33,37–40] the identity of the five OSs previously pre-identified was confirmed.

3.2. Comparison of the elution systems

The results of experiment I are given in Table 1. Elution systems I and II did not recover the CT. The recoveries obtained can be explained by the polarity of the compounds analyzed, which increased in the following order: cholesterol < cholesterol-5,6-epoxides < 7-KC < 7-hydroxy-cholesterols < CT [1,24,41,42]. So, systems I and II, having lower polarities, recovered lower amounts of highly polar compounds and did not recover CT. However, 7-KC was more recovered than α-CE in systems I and II, in spite of the polarity scale defined above.

Analysis of variance (ANOVA) was applied in order to ascertain whether the recovery percentage for every elution system and every OS depended on the concentration level. Results of this analysis showed that recoveries did not differ significantly, for any system of elution, according to the concentration. So, a global recovery value was considered for every OS. Then, ANOVA was applied in order to discover whether there were significant differences in the recoveries of cholesterol, α-CE, 7β-HC, 7-KC and 25-HC, according to the elution system. Results of this analysis show statistically significant differences for the five compounds ($P < 0.0001$). For the compounds that showed significant differences the Scheffé's test for "a posteriori" contrasts ($\alpha = 0.05$) was applied, in order to determine which elution systems differ in recovery values. For the four OSs, mean recoveries of elution system III and system IV were significantly higher than II and I, while significantly lower values were found for cholesterol in systems III and IV. In contrast, there were no differences between mean recoveries of systems III and IV. Lower values of cholesterol recovery are inter-

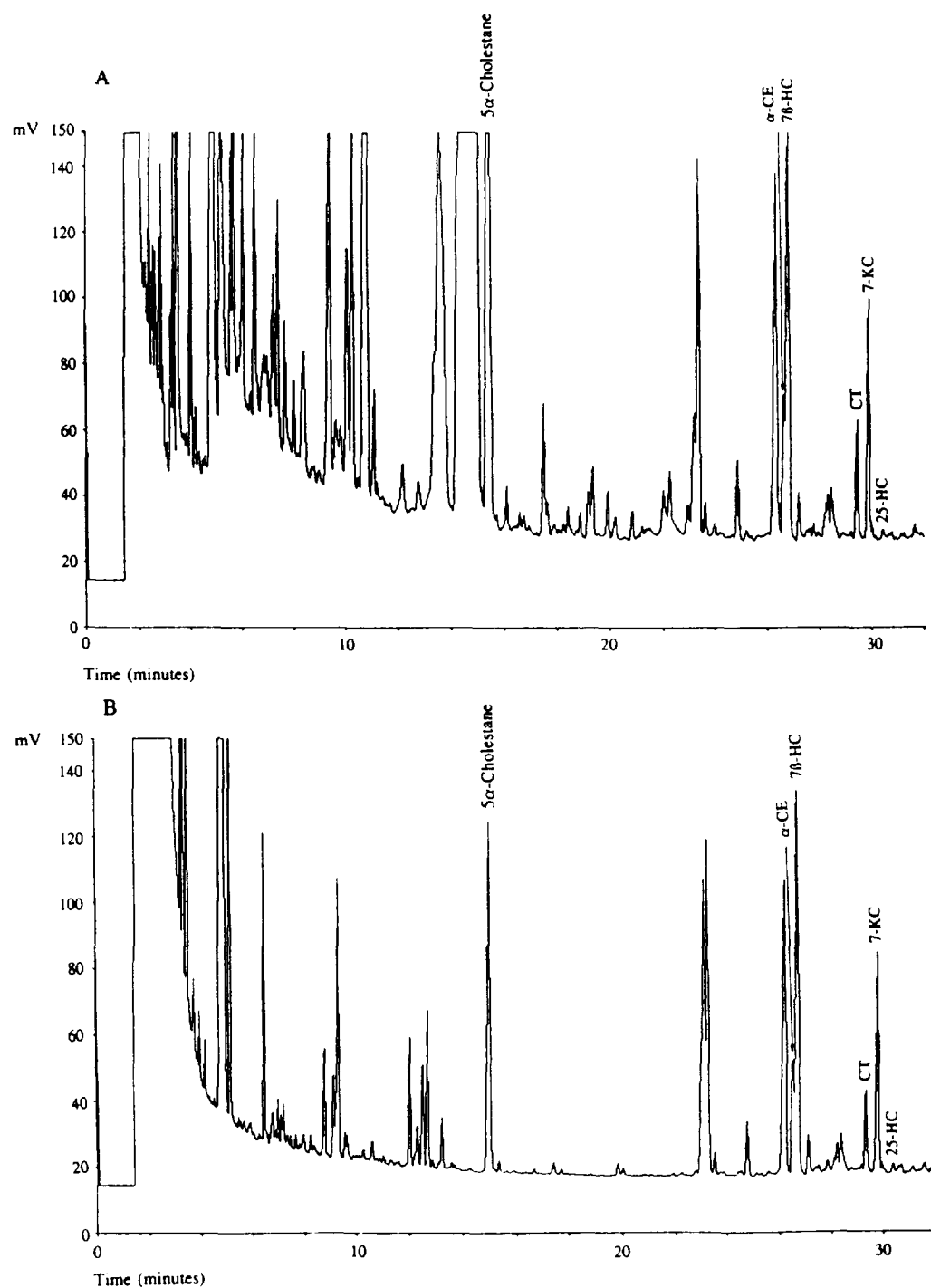


Fig. 3. Chromatograms of silanized extracts obtained from a dried egg sample (temperature programme II). (A) Method II. (B) Method III.

Table 1
Standard recoveries for the different systems of elution

Standard	Amount (μg)	Recovery (%)			
		System I	System II	System III	System IV
Cholesterol	30	7.4 ^a (39.6) ^b	9.5 (47.9)	3.4 (26.7)	3.0 (24.8)
α -CE	2.5	33.2 (16.5)	65.5 (14.9)	97.6 (4.0)	98.9 (1.9)
	5	33.8 (12.3)	65.9 (10.3)	98.4 (3.8)	98.9 (1.0)
	10	34.6 (7.6)	65.4 (11.7)	98.2 (3.3)	98.8 (0.9)
	Global recovery	33.9 (11.7)	65.6 (11.5)	98.1 (3.5)	98.9 (1.2)
7 β -HC	5	2.7 (117.3)	6.4 (89.2)	96.7 (2.9)	98.6 (1.9)
	10	2.6 (91.8)	6.2 (70.8)	97.3 (2.0)	98.0 (1.3)
	20	2.8 (28.9)	6.5 (39.9)	97.0 (1.3)	98.9 (1.1)
	Global recovery	2.8 (80.1)	6.4 (64.3)	97.0 (2.0)	98.5 (1.4)
CT	2.5	NR	NR	98.7 (3.9)	100.0 (2.1)
	5	NR	NR	98.6 (2.1)	101.2 (1.4)
	10	NR	NR	98.8 (1.6)	101.3 (1.3)
	Global recovery			98.7 (2.5)	100.9 (1.6)
7-KC	2.5	65.4 (6.7)	90.6 (5.2)	100.5 (2.8)	101.3 (2.2)
	5	65.1 (5.6)	90.3 (4.5)	100.5 (2.4)	101.9 (2.0)
	10	65.3 (4.3)	90.5 (3.9)	100.7 (2.0)	102.0 (1.6)
	Global recovery	65.6 (5.2)	90.5 (4.2)	100.5 (2.2)	101.7 (1.9)
25-HC	2.5	48.1 (7.3)	76.5 (5.3)	98.9 (1.5)	100.8 (0.8)
	5	48.2 (7.1)	77.2 (4.6)	98.8 (1.4)	100.4 (0.5)
	10	47.5 (6.5)	76.6 (3.3)	99.0 (1.0)	100.7 (0.3)
	Global recovery	47.9 (6.5)	76.8 (4.2)	98.9 (1.2)	100.7 (0.6)

NR = Not recovered.

^aMean value ($n = 5$ for amounts 2.5, 5, 10 and 20, and $n = 15$ for amount 30 and global recovery).

^bR.S.D. (%).

esting because this compound can interfere in the determination of 7 α -hydroxycholesterol since this OS elutes close to cholesterol in the gas chromatogram when a 100% methylsilicone capillary column is used [21,26,37]. In addition, for the four OSs but not for cholesterol, elution system II showed significantly higher recoveries than system I (Table 2). For CT, only the recoveries for systems III and IV could be compared, and the application of the Student-Fisher's t test showed that a significantly higher CT recovery was found using system IV ($P < 0.0009$). According to these results, it can be concluded that elution systems III and IV give significant advantages, due to their lower chole-

sterol recovery and better recoveries of the different OSs. The significantly higher recovery of CT and the better precision obtained ($n = 15$) in all OS recoveries, using elution system IV, led to the choice of this method for future assays.

For elution system IV similar recovery results were obtained in experiment II for the five OSs. Stigmasterol and β -sitosterol presented low recoveries, a finding of interest, given the desirability of avoiding the interfering effect of these compounds. ANOVA was applied in order to determine whether the recovery for every standard depended on the concentration level. Results of this analysis showed that recoveries do not vary significantly with concentration. There-

Table 2

Significance level and confidence interval of the "a posteriori" contrasts for cholesterol, α -CE, 7 β -HC, 7-KC and 25-HC (Scheffé's test, $\alpha = 0.05$)

Standard	S ^a	System II	System III	System IV
Cholesterol	I	NS ^b	$P = 0.0026$ (1.1–7.0) ^c	$P = 0.0007$ (1.6–7.4)
	II		$P < 0.0001$ (3.2–9.1)	$P < 0.0001$ (3.7–9.5)
	III			NS
α -CE	I	$P < 0.0001$ (26.8–36.6)	$P < 0.0001$ (59.3–69.1)	$P < 0.0001$ (60.1–69.9)
	II		$P < 0.0001$ (27.6–37.3)	$P < 0.0001$ (28.4–38.1)
	III			NS
7 β -HC	I	$P = 0.0053$ (0.8–6.4)	$P < 0.0001$ (91.5–97.0)	$P < 0.0001$ (92.9–98.5)
	II		$P < 0.0001$ (87.9–93.4)	$P < 0.0001$ (89.3–94.9)
	III			NS
7-KC	I	$P < 0.0001$ (22.1–28.3)	$P < 0.0001$ (32.2–38.3)	$P < 0.0001$ (33.3–39.5)
	II		$P < 0.0001$ (6.9–13.1)	$P < 0.0001$ (8.1–14.3)
	III			NS
25-HC	I	$P < 0.0001$ (26.4–31.3)	$P < 0.0001$ (48.5–53.5)	$P < 0.0001$ (50.3–55.2)
	II		$P < 0.0001$ (19.7–24.6)	$P < 0.0001$ (21.4–26.4)
	III			NS

^aS = System of elution.

^bFor this standard the difference between the mean recoveries (%) using these two systems of elution is not statistically significant (NS = not statistically significant).

^cP = Significance level of the contrast; the confidence interval is stated in parentheses.

fore, a global recovery value (mean value, $n = 15$) was considered for every standard [relative standard deviation (R.S.D., %) in parentheses]: α -CE, 100.6 (2.1); 7 β -HC, 100.2 (2.1); CT, 99.5 (1.6); 7-KC, 101.4 (1.8); 25-HC, 98.5 (1.5); stigmasterol, 3.2 (24.9); and β -sitosterol, 4.1 (22.2).

Finally, for the five OSs, these results were grouped with the former for the system IV and the mean value ($n = 30$) was calculated. Mean recoveries [R.S.D. (%) in parentheses] were: α -CE, 99.7 (1.9); 7 β -HC, 99.4 (1.9); CT, 100.2 (2.1); 7-KC, 101.5 (1.8); and 25-HC, 99.6 (1.5).

3.3. Linearity of response

Table 3 gives values obtained with the three curves for the linearity (r^2) and the RRF of each compound analyzed. As can be seen in the table, the linearity of response was good for all standards. RRFs show some differences according to

the chromatograph used, which could be related to split discrimination. Values from curve I agree with those reported by different authors [1,12,14,37] suggesting that the RRFs of the more common OSs are close to 1.0 except those of 7-KC and α -CE.

3.4. Limits of detection (DLs) and quantification (QLs)

The DLs and QLs in relation only to the chromatographic method are given in Table 4. To make an approximation to the DL and QL values relative to the whole method, the values corresponding to the chromatographic determination were referred to the mass of sample (0.65 g of dried egg sample was extracted, the extract purified and the final residue was completely silanized). The limits calculated with respect to the sample are given in Table 5.

Table 3
Linearity of response for OSs

Curve	Standard	<i>n</i>	<i>a</i>	<i>b</i>	<i>r</i> ²	RRF (R.S.D.) ^a
C _I	Cholesterol	35	0.022	1.022	0.9968	0.876 (6.2)
	α-CE	35	0.008	0.943	0.9973	1.030 (4.1)
	7β-HC	35	0.046	1.007	0.9985	0.979 (3.3)
	CT	35	0.010	0.984	0.9977	0.985 (6.0)
	7-KC	35	0.001	0.801	0.9929	1.242 (6.1)
	25-HC	35	0.015	0.994	0.9974	0.941 (5.5)
	C _{II}	Cholesterol	24	0.012	1.021	0.9988
α-CE		24	0.003	1.065	0.9990	0.899 (6.0)
7β-HC		24	0.003	0.811	0.9999	1.192 (3.0)
CT		24	-0.006	1.457	0.9978	0.690 (6.1)
7-KC		24	-0.015	1.387	0.9950	0.762 (6.0)
25-HC		24	-0.009	1.338	0.9957	0.764 (5.9)
C _{III}		Cholesterol	20	0.061	0.997	0.9915
	α-CE	20	-0.059	0.871	0.9935	1.181 (5.7)
	7β-HC	20	0.051	0.753	0.9968	1.299 (6.7)
	CT	20	-0.129	1.268	0.9915	0.822 (6.1)
	7-KC	20	-0.108	1.122	0.9950	0.993 (6.3)
	25-HC	20	-0.031	1.274	0.9961	0.801 (6.2)

The regression of *y* (Area standard/Area internal standard) on *x* (Weight standard/Weight internal standard) presents the equation $y = a + bx$, where *a* is the intercept and *b* the slope; *r*² = Determination coefficient.

^aRelative response factors, their R.S.D.s (%) are given in parentheses.

Table 4
Detection (DL) and quantification (QL) limits relative to the chromatographic method for each chromatograph

Chromatograph	Parameter	α-CE	7β-HC	CT	7-KC	25-HC
Sigma 2000	DL	1.65	2.34	2.28	2.94	2.16
	QL	4.80	6.82	6.66	8.59	6.31
Autosystem	DL	1.78	1.61	1.40	1.47	1.16
	QL	5.18	4.68	4.09	4.30	3.38

Results expressed as ppm in silanized extract.

Table 5
Detection (DL) and quantification (QL) limits relative to the whole method

Chromatograph	Parameter	α-CE	7β-HC	CT	7-KC	25-HC
Sigma 2000	DL	0.25	0.36	0.35	0.45	0.33
	QL	0.74	1.05	1.03	1.32	0.97
Autosystem	DL	0.27	0.25	0.22	0.23	0.18
	QL	0.80	0.72	0.63	0.66	0.52

Results expressed as ppm in sample.

3.5. Absence of cholesterol oxidation during the procedures

No trace peak corresponding to OSs was detected in the assay described above. Method III is the fusion of methods I and II, and for this reason, it can be concluded that no OS formation should be expected following any of the three methods, paying attention to the following operation conditions: attenuated light; evaporation of solvents until 1 ml, and completing to dryness by using a slight nitrogen stream and by keeping in a vacuum desiccator at 10 mmHg. Moreover, temperature must be lower than 35°C, since traces of some OS (7 β -HC and 7-KC) were found when the evaporation was carried to dryness at 45°C.

3.6. Comparison of the three methods for the determination of OSs

Comparison of the precision

In the application of the three methods, the 5 α -cholestane was added as I.S. and the same mass of sample (0.65 g of dried egg) and the same chromatograph (Perkin-Elmer Sigma 2000 plus HP 3396A) were used. Ten determinations of the five OSs were performed on the same sample, on successive days, and using the same materials and reagents. The results obtained using method I corresponding to the mean contents of OSs in ppm ($n = 10$), with their R.S.D.s (%) in parentheses, are: α -CE, 8.6 (10.3); 7 β -HC, 78.3 (9.2); CT, 12.5 (9.6); 7-KC, 20.2 (9.1); and 25-HC, 1.6 (21.0). For method II: α -CE, 10.8 (9.5); 7 β -HC, 91.5 (6.0); CT, 12.3 (7.7); 7-KC, 27.0 (8.2); and 25-HC, 1.6 (19.7). For method III: α -CE, 9.0 (8.0); 7 β -HC, 74.6 (3.4); CT, 11.7 (7.5); 7-KC, 21.6 (7.2); and 25-HC, 1.6 (16.6).

As the R.S.D. shows the precision increased from method I to method III. This could be explained by the increasing degree of purification, which improves the selectivity, as the comparative chromatograms show (Fig. 3).

Comparison of the recovery

The recovery was also determined in the three

cases on the same chromatograph (Perkin-Elmer Sigma 2000 plus HP 3396A) and using the same I.S. (5 α -cholestane). Three levels of the OS standards (5, 10 and 20 μ g) were added to 0.65-g aliquots of the same egg dried sample. Five replicas of each level were performed. Table 6 shows the results of the mean recoveries obtained for each OS, at the three levels of addition (\bar{X}_5 , \bar{X}_{10} and \bar{X}_{20}). The application of ANOVA to the recovery values indicates that the differences in the recovery of each method between the three levels of addition were not statistically significant for any OS. For this reason, the global mean values of the recovery (\bar{X}_G) are also given in the table.

This table shows that the variability of the recovery increases when the level of addition decreases. Furthermore, the mean recovery values are higher for method II than for method I and method III, which shows the lowest values. The variability of recovery increases from method III to method I. The recoveries of CT were clearly lower for methods I and III than for method II, which could be due to the poor recovery induced by the saponification step (common to methods I and III). Moreover, α -CE recovery was higher than 100% for all three methods, which may be explained by a defect in the integration of the peak, due to its overlap with the 7 β -HC peak. Another interesting conclusion of this study is the high recovery obtained for the α -CE and the 7-KC for the methods (I and III) using a saponification step. This demonstrates that these two OSs are stable during cold saponification, which agrees with the data reported by many authors [10–12], although Van de Bovenkamp et al. [14] reported low recovery for the 7-KC with this method.

From all these results of precision and recovery, method III is the most reliable, since it gives a higher precision in the determination and recovery. The other two methods, although they show higher recoveries, have lower precision and in consequence give less reliable results. However, method III should be improved in some aspects, especially the variability. For this reason, improvement was attempted using a new chromatograph, a Perkin-Elmer Autosystem,

Table 6
Mean recoveries of OSs at different levels of addition for the three methods

Method		Oxysterols				
		α -CE	7 β -HC	CT	7-KC	25-HC
I	\bar{X}_5^a	103.6	88.3	61.0	85.4	81.3
	R.S.D. (%)	12.0	14.6	11.8	12.3	8.9
	\bar{X}_{10}^b	99.5	86.4	60.5	83.8	77.3
	R.S.D. (%)	9.2	9.3	7.9	9.7	9.2
	\bar{X}_{20}^c	100.6	86.9	61.7	86.5	79.5
	R.S.D. (%)	6.9	5.8	5.2	5.5	5.5
II	\bar{X}_G^d	101.2	87.2	61.1	85.2	79.4
	R.S.D. (%)	9.1	9.9	8.1	9.0	7.8
	\bar{X}_5	111.2	95.1	83.6	86.8	94.9
	R.S.D. (%)	9.8	12.4	10.5	12.1	8.4
	\bar{X}_{10}	110.4	97.5	84.7	87.5	95.8
	R.S.D. (%)	7.4	9.9	7.4	8.6	6.5
III	\bar{X}_{20}	110.4	95.6	86.8	89.3	97.3
	R.S.D. (%)	6.1	8.8	5.6	6.0	3.6
	\bar{X}_G	110.7	97.0	85.0	87.9	96.0
	R.S.D. (%)	7.4	9.8	7.6	8.6	6.0
	\bar{X}_5	105.9	80.4	56.2	83.5	70.0
	R.S.D. (%)	5.7	12.7	8.8	8.6	5.4
III	\bar{X}_{10}	109.3	78.6	51.5	80.3	74.3
	R.S.D. (%)	4.5	8.0	4.7	8.9	4.5
	\bar{X}_{20}	107.6	76.2	54.0	77.1	72.1
	R.S.D. (%)	2.7	4.2	5.3	7.4	4.1
	\bar{X}_G	107.6	78.4	53.9	80.3	72.1
	R.S.D. (%)	4.3	8.8	7.2	8.4	5.0

^aMean recovery at level of addition of 5 μ g ($n = 5$).

^bMean recovery at level of addition of 10 μ g ($n = 5$).

^cMean recovery at level of addition of 20 μ g ($n = 5$).

^dGlobal mean value of recovery ($n = 15$).

equipped with a personal integrator PE Nelson 1020, more reliable than the HP 3396A. The temperature programme of the oven was also modified to give better resolution. The conditions finally used are those described in the Experimental section.

Thus, the following study was carried out to select the most reliable method. Method I was ruled out, since it does not offer any significant advantage, and the comparative study was only focused on methods II and III. In order to improve the precision, 19-HC was used as the I.S., because it could be recovered through the

silica cartridge and so could be added to the sample at the beginning of the analytical procedure. These modifications, in addition to the use of a new chromatographic system (PE Autosystem plus PE Nelson 1020 integrator) were applied to methods II and III. The results of precision for method II corresponding to the mean contents of OSs in ppm ($n = 10$), with their R.S.D. (%) in parentheses, are: α -CE, 9.4 (5.1); 7 β -HC, 81.1 (5.0); CT, 13.1 (5.8); 7-KC, 25.9 (4.6); and 25-HC, 1.8 (10.5). For method III: α -CE, 9.3 (3.9); 7 β -HC, 83.4 (3.8); CT, 14.1 (3.9); 7-KC, 22.2 (3.6); and 25-HC, 1.7

Table 7
Mean recoveries of OSs at different levels of addition for the methods II and III

Method		Oxysterols				
		α -CE	7 β -HC	CT	7-KC	25-HC
II	\bar{X}_5^a	98.6	95.9	92.5	91.2	100.4
	R.S.D. (%)	6.6	14.2	7.7	12.3	7.7
	\bar{X}_{10}^b	99.3	94.4	91.8	90.5	98.1
	R.S.D. (%)	5.6	7.7	4.7	6.9	6.1
	\bar{X}_{20}^c	97.4	95.3	93.0	92.5	98.0
	R.S.D. (%)	4.2	4.8	3.5	3.4	2.5
	\bar{X}_G^d	98.4	95.2	92.4	91.4	98.8
	R.S.D. (%)	5.2	9.1	5.2	7.8	5.6
III	\bar{X}_5	96.2	100.3	93.3	93.5	98.1
	R.S.D. (%)	6.4	10.6	6.2	8.3	6.3
	\bar{X}_{10}	96.4	99.9	95.0	93.6	97.3
	R.S.D. (%)	4.3	5.8	5.1	5.9	6.7
	\bar{X}_{20}	96.0	98.8	95.4	95.0	97.3
	R.S.D. (%)	3.6	3.8	3.0	3.2	2.0
	\bar{X}_G	96.2	99.7	94.6	94.0	97.5
	R.S.D. (%)	4.5	6.8	4.7	5.7	5.1

^aMean recovery at level of addition of 5 μ g ($n = 5$).

^bMean recovery at level of addition of 10 μ g ($n = 5$).

^cMean recovery at level of addition of 20 μ g ($n = 5$).

^dGlobal mean value of recovery ($n = 15$).

(6.2). The R.S.D. showed that method III gives better precision.

Results of the determination of the recoveries of OSs at three levels of addition (5, 10 and 20 μ g) are given in Table 7. The recovery of the I.S. (19-HC), which was 80.96% for method II and 72.98% for method III, was taken into account for the calculations of precision and recovery of the methods. The 19-HC recovery was calculated using 5 α -cholestane as the I.S. Applying ANOVA to the results of Table 7, no significant difference in the recovery was found between the three levels of addition. For this reason, the global mean values of these recoveries were calculated, which are also given in the table.

The mean global recoveries of 7 β -HC, CT, 7-KC and 25-HC were higher for method III than for II, and the recovery of α -CE was higher for method II. The application of the Student-Fisher's t test showed that these differences are not statistically significant.

From all these data it can be concluded that method III is the most reliable for the determination of OSs in the dried egg, since it shows a higher precision. In addition, this method allows the analysis of a greater mass of sample, since it supplies better purification, and consequently the limit of quantification decreases.

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