Ann. Anim. Sci., Vol. 13, No. 1 (2013) 143–153, DOI: 10.2478/v10220-012-0066-7

VALIDATION OF A METHOD FOR DETERMINING CHOLESTEROL IN EGG YOLKS*

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Abstract

The aim of the study was to validate a gas chromatographic method for determining cholesterol in egg yolks according to the EN ISO/IEC 17025 standard. Of the two methods, with and without internal standard, the former was characterized by lower uncertainty, with a repeatability of 4% and within-laboratory reproducibility of 6%. The method's uncertainty (n = 2, P≤0.05), which included sample preparation errors and chromatographic measurement errors, was 10.6%. Mean recovery was 99.9% and limit of quantification was 0.16 mg/g. The coefficient of variation for repeatability, which is calculated during routine analyses, should not exceed the 8% limit of repeatability. The method is reliable, as confirmed by the results of validation, and the procedure is relatively rapid and simple.

Key words: cholesterol, egg yolk, validation, uncertainty, gas chromatography

The present study reflects the Central Laboratory's consistent quality policy to extend the range of analyses for animal-derived products such as egg yolks, meat and others. This research translates into better quality and safety of foodstuffs as well as health protection of the consumers of animal raw materials and products. The development (in accordance with EN ISO/IEC 17025 standard, 2005) and implementation of modern chromatographic procedures for cholesterol determination is associated with studies on the quality of eggs from conservation breeds (Cywa-Benko et al., 2000) and will help to extend the range of studies conducted at the National Research Institute of Animal Production on the nutritional value of products of animal origin (Barowicz and Pietras, 1999; Pietras et al., 2002; Brzóska, 2004 a, b; Połtowicz and Wężyk, 2005; Świątkiewicz and Koreleski, 2006).

*This study was financed from statutory activity of National Research Institute of Animal Production, project No. 2128.1.

The role of cholesterol in metabolic processes is significant and well known. Cholesterol is mainly used for synthesis of steroid hormones and bile acids, which play a major role in digestion of fats. It is also crucial in building cell walls and semipermeable membranes. Although the presence of cholesterol is required for normal functioning of human (and animal) organisms, its excess due to genetic and dietary factors is harmful and, in extreme cases, may be hazardous to health and life. Cholesterol exists in two major forms: that transported in blood as low-density lipoproteins (known as "bad" cholesterol), and that carried by high-density lipoproteins (known as "good" cholesterol). Attention has recently been drawn to the fact that oxidized forms of cholesterol (oxysterols) rather than its pure forms are more harmful. It is beyond question, however, that all forms of cholesterol, including the harmful ones, are reflected in the level of total cholesterol, the quantitative determination of which in both blood (using rapid diagnostic monitoring methods) and foods consumed by humans is very important and necessary. This has prompted the Central Laboratory to develop a method for determining cholesterol in egg yolks, among others.

Cholesterol was determined in many products using different procedures. Colorimetric (Rhee et al., 1982; Korzeniowski et al., 1992) and enzymatic-colorimetric methods (Hwang et al., 2003; Hanczakowski et al., 2004) that were once popular were replaced with liquid and gas chromatography methods that are most common and accurate today. In addition to the analytical technique employed to determine cholesterol, the mode of sample preparation is important. The most common methods used are based on preliminary clean-up of the sample by solid phase extraction columns (Russo et al., 2005) or saponification of the sample and extraction with organic solvent (Hwang et al., 2003). In addition, the procedure of sample preparation for analysis may, but need not, include the stage of pre-column derivatization with silylation reagent. The use of such reagent allows reducing the limit of quantification, but makes the procedure slightly more complicated and increases the cost of analysis. Among other methods the simple method using direct hydrolysis, extraction and HPLC cholesterol determination in materials of animal origin can also be used (Czauderna et al., 2009). In this study, derivatization was not used but the sample components were saponified and extracted with organic solvent.

In recent years, increasing emphasis has been placed, and rightly so, on the quality of analytical techniques, which should be appropriately tested and characterized for suitability in research. Such procedure is conducted in compliance with the principles of Good Laboratory Practice (GLP, 2003) and in accordance with the requirements of the accreditation standard EN ISO/IEC 17025 (2005). The present study made use of modern gas chromatography technique, and the developed methods were characterized for such parameters as repeatability, within-laboratory reproducibility, limit of repeatability, limit of quantification (LOQ), calibration curve parameters, uncertainty, and recovery (Arendarski, 2003; Dobecki, 2004; Gąsior and Pieszka, 2006; Gąsior et al., 2009; Gąsior and Szczypuła, 2010).

The aim of the study was to validate a gas chromatographic method for determining cholesterol in egg yolks according to the EN ISO/IEC 17025 standard. The developed method should be reliable and relatively simple and fast.

Material and methods

Cholesterol was determined by gas chromatography using FID detector with a column with 5% phenyl, 95% dimethylpolysiloxane phase, after saponification and hexane extraction.

Reagents and equipment

The following reagents (of at least pure for analysis grade) were used: double-distilled water, n-hexane (Merck, Darmstadt, Germany), KOH (POCH, Gliwice, Poland), NaCl (POCH, Gliwice, Poland), ethanol 96% (Chempur, Piekary Śląskie, Poland), cholesterol (5-Cholesten-3β-ol, Sigma-Aldrich, St. Louis, USA), 5α-cholestane (>97%, Sigma-Aldrich, St. Louis, USA), stigmasterol (95%, Sigma-Aldrich, St. Louis, USA). These reagents were used to make aqueous solutions of KOH (60 g/100 ml) and NaCl (1 g/100 ml) and standard hexane solutions of cholesterol (4 mg/ml, basic standard solution) and internal standards (IS): 5 α-cholestane (2 mg/ml) or stigmasterol (2 mg/ml). Solutions were evaporated dry under nitrogen.

In addition to basic laboratory equipment, use was made of a water bath, freeze-drier (Christ Beta, Germany), gas chromatograph (GC 2010, Shimadzu, Japan) with a flame-ionization detector and AOC-5000 autosampler. A GCMS-QP2010 Plus mass detector (Shimadzu, Japan) was used for preliminary identification of cholesterol.

Determination procedure

Fresh, freeze-dried or frozen (below –12ºC) yolks were collected for the analyses. The material was mixed and a representative sample was taken for analysis. To the tube with the sample (approx. 0.2–0.3 g of fresh or frozen yolk, or 0.17 g of freeze-dried yolk, weighed to the nearest 0.0001 g) was added 4 ml of ethyl alcohol followed by 0.5 ml of KOH solution (60 g/100 ml), after which the tube was tightly closed and thoroughly shaken. After hot maceration (75±3ºC, 1 h), cooling and salting out of the sample using 4 ml of NaCl solution (1 g/100 ml), cholesterol was double extracted in 8 ml of hexane by vortexing, and the extracts were combined in a glass vial and dry evaporated (40±3ºC) under inert gas (nitrogen). After adding each batch of hexane, 10 minutes were waited before phase separation (upper hexane layer). To the evaporation residue was added 1.5 ml of hexane and 0.5 ml of IS (total of V=2 ml), to eliminate most crucial errors of solvent evaporation before sample injection, and monitor a chromatographic accuracy. After thorough mixing, the sample solution was transferred to a chromatography vial and injected onto a chromatographic column. If necessary, the sample was diluted (f = 1 in the present study).

Five intermediate standard solutions were prepared by making consecutive solutions of the basic standard solution (4 mg/ml in hexane) to obtain solutions with concentrations of 4, 2, 1, 0.5 and 0.25 (mg/ml). Calibration standard solutions (3, 1.5, 0.75, 0.375 and 0.1875 mg/ml) were obtained by adding 0.5 ml hexane to 1.5 ml of each intermediate standard solution and injecting onto the chromatographic column. The above solutions were used for plotting the calibration curve.

Calculations

The amount of cholesterol CH (mg/g) was calculated using formula 1:

$$CH = \frac{c}{m} \times V \times \frac{100}{R} \times f \quad (1)$$

where:

c – concentration established from the calibration curve equation (mg/ml),
V (2 ml) – volume of hexane added to the sample after evaporation,
m – weight of sample (g),
R – recovery (%),
f – dilution factor.

The value of concentration c was calculated from the second-degree polynomial calibration curve showing relationship between cholesterol concentration and peak area.

Chromatographic analysis

Chromatographic separation was performed on a Zebron ZB-5 column (30 m × 0.25 mm, 0.50 µm, Phenomenex, Torrance, USA) housed in an oven at 265ºC. Carrier gas (helium) flow rate was 1.7 ml/min, sample volume was 5 µl. Injector and FID detector temperature was 300ºC, split ratio was 1:25, analysis time was 30 minutes.

Validation

Repeatability and within-laboratory reproducibility tests were performed with 33 samples based on a total of 224 analyses (78 analyses of fresh yolk, 72 analyses of frozen yolk, and 74 analyses of freeze-dried yolk). The analyses, for determination of repeatability and within-laboratory reproducibility, were performed with internal standard (16 analyses for repeatability and reproducibility each) and without internal standard (160 and 32 analyses for repeatability and the reproducibility, respectively). Percent repeatability was defined as being not less than the pooled coefficient of variation (CV_{ln}) for single determinations performed with the same method, using identical material, in the same laboratory, by the same laboratory assistant and during the same time period. Percent within-laboratory reproducibility was defined as being not less than the pooled coefficient of variation for single determinations performed using the same method and identical material, in the same laboratory, by two laboratory assistants at different times. CV_{ln} for l samples analysed in n replications was calculated from formula 2, where CV_{n2} is the coefficient of variation for determination of a given sample in duplicate (n = 2):

$$CV_{ln} = \sqrt{\frac{\sum_{l} CV_{n2}^{2}}{l}} \quad (2)$$

Double the coefficient of variation for repeatability was accepted as the criterion for repetition of the determinations (limit of repeatability). The recovery was determined with two methods, using a total of 87 analyses: the standard addition method (addition of 4 mg cholesterol, 11 analyses), the standard being added prior to the saponification, and by comparing the results of analyses performed by the Central Laboratory (76 analyses) with the reference values. These values were determined based on two reference materials (freeze-dried yolks) analysed by another accredited laboratory, with uncertainty of 11.3%. The limit of quantification was determined based on the calibration curve. The working range of the calibration curve was also determined.

The main components of method uncertainty (expressed in relative form, %) were determined, such as uncertainty of within-laboratory reproducibility (*u1%*), uncertainty of recovery (*u2%*), uncertainty of purchased standard purity (*u3%*) and uncertainty associated with lack of trueness of pipettes (*u4%*) and flasks (*u5%*). Before combining, uncertainties were expressed as standard uncertainties u_i% (68% confidence level, P≤0.32). The combined standard uncertainty of the u_c% method was calculated based on the law of propagation of uncertainty from formula 3:

$$u_c\% = \sqrt{u1\%^2 + u2\%^2 + u3\%^2 + u4\%^2 + u5\%^2} \tag{3}$$

The standard uncertainty of within-laboratory reproducibility (*u1*%), which comprises most errors, including sample preparation errors, was defined as within-laboratory reproducibility % (Rep%) divided by the root of n analyses of a given sample (formula 4):

$$u1\% = \frac{\text{Re}\,p\%}{\sqrt{n}} \tag{4}$$

The standard uncertainty of recovery was calculated as a coefficient of variation for the arithmetic mean of recovery values determined during the validation. The standard uncertainties concerning standard purity and the flasks and pipettes used (as regards lack of trueness but not precision) were calculated based on relative (%) values of limiting errors a_i. For flasks and pipettes, a_i values were estimated based on the calibration procedure accepted in the laboratory. For standard purity, ai values were estimated based on the manufacturer's specifications. Assuming a symmetric rectangular distribution of the values measured around the nominal value, u_i% uncertainties are calculated using the formula $u_i\% = a_i/\sqrt{3}$ (Ellison et al., 2000). The uncertainty factor associated with the lack of trueness of the pipettes and flasks was calculated by combining the individual components in accordance with the law of propagation. Method uncertainty U_c% (95% confidence level, P≤0.05) was computed by including the coverage factor $k = 2$ ($U_c\% = k \times u_c\%$) (Ellison et al., 2000). The u_c% and U_c% uncertainties were determined for n = 2 and n = 1.

The storage life of standard solutions and samples and the content of impurities in the blank sample (without weighing the material) was determined.

The validation (but not routine analyses) also included the identification of cholesterol in the analysed solutions using a mass spectrometer.

Results

The repeatability and within-laboratory reproducibility values for the analyses with and without standard differed significantly, but were similar independently of material analysed (fresh, freeze-dried or frozen). These values are presented, together with the limit of quantification and standard uncertainty of within-laboratory reproducibility in Table 1. The recovery, determined by adding a known standard amount to yolk samples was 95.1%, and the recovery for two reference freeze-dried yolks was 102.2%. The mean recovery was practically equal to 100% (99.9%). LOQ, which corresponds to cholesterol concentration that can be reliably measured within specified limits, calculated from the calibration curve, was 0.16 mg/g. The relationship between cholesterol content and peak area was described with a second-degree polynomial curve characterized by the determination coefficient r^2 not lower than 0.99. The working range of the calibration curve ranged from 0.25 mg/ml to 4 mg/ml. The uncertainty budget, which includes all the significant factors of uncertainty, combined standard uncertainty and combined expanded uncertainty (both values for n = 2 and n = 1) is grouped in Table 2. A sample chromatogram of freeze-dried yolk analysis using both internal standards is presented in Figure 1.

Table 1. Validation parameters of the method for determining egg yolk cholesterol

Method	Repeatability (%)	Repeatability limit (%)	Within-laboratory reproducibility (%)	Standard uncertainty of within-laboratory reproducibility (u1%), n=2/n=1*, (%)
With Internal Standard (5α -cholestane)	4.0	8.0	6.0	4.2/6.0
Without Internal Standard	6.0	12.0	14.0	9.9/14.0

* n – number of analyses of one sample.

Table 2. Standard uncertainty budget, combined standard uncertainty u_c% (68% confidence level) and combined expanded uncertainty U_c% (95% confidence level, k = 2)

Method	u1% * n=2/n=1	u2% *	u3% *	u4% *	u5% *	u_c % n=2/n=1	U_c % (k=2), n=2/n=1
With IS (5α -cholestane)	4.2/6.0	2.5	0.6	2.0	0.5	5.3/6.8	10.6/13.6
Without IS	9.9/14.0	2.5	0.6	2.0	0.5	10.4/14.4	20.8/28.8

* For explanations, see Validation in Material and Methods section.

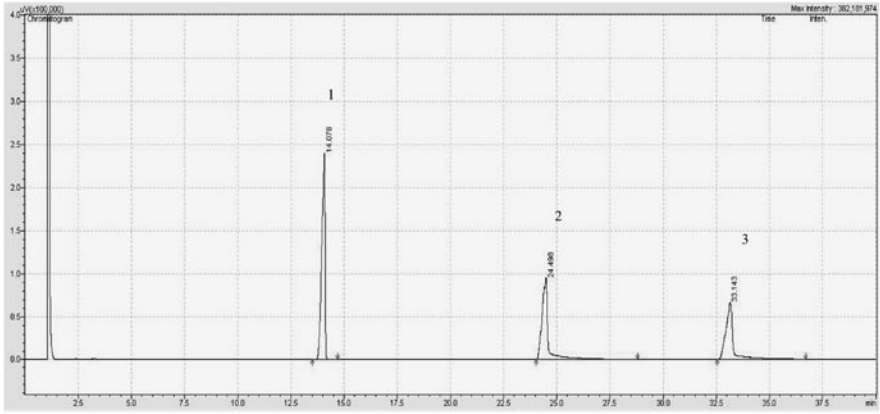

Figure 1. Sample chromatogram of freeze-dried yolk analysis. Peaks 1, 2 and 3 represent 5α–cholestane, cholesterol and stigmasterol, respectively

Discussion

The method used for determining egg yolk cholesterol does not include derivatization, which simplifies the analysis and reduces the costs. The use of sample preparation technique involving saponification and extraction instead of solid phase extraction is also beneficial and adequately cleans up the sample before chromatographic analysis. For many years, this inexpensive method of sample preparation has been used with success at the Central Laboratory of the National Research Institute of Animal Production for analysing animal and plant samples.

The present results show clear differences in uncertainty values (Table 2) depending on the sample preparation procedure: with or without the internal standard. The first method is characterized by about twice as low within-laboratory reproducibility uncertainty, which also had a similar effect on extended uncertainty (95% confidence level). Therefore, this method is recommended for routine analyses. Two internal standards were used in the study: 5α-cholestane and stigmasterol. They had similar effects on the above validation parameters (data not presented) and both can be successfully used for determining cholesterol, although 5α-cholestane is preferred for practical reasons (smaller retention time, sharper peak).

The method described and validated in the present paper is repeatable and reproducible. This is confirmed by the Horrat value H = 1.60 falling within the accepted values (0.5–2), calculated for the expected repeatability RSDr% = 2.50 (CIPAC 3807, Korol et al., 2011), according to the equation H = Repeatability % / RSDr% (4/2.50). RSDr% was calculated from the equation $0.67 \times 2C^{-0.1505}$, for the concentration C = 0.016 (the average cholesterol content in the samples was 16.0 mg/g, the content range was from about 6 mg/g to 34 mg/g). No reagent-derived impurities were found (blank sample). The working range of the calibration curve is rather narrow but enables obtaining more reliable results.

The lower limit values of analytes that can be determined, have been named and defined differently in various publications. As an example, Russo et al. (2005) estimated this value (0.8 mg/g) as 'effective' accompanied by a small standard deviation. While the LOQ values based on multifold standard deviation of blank sample (usually 10 × SD) are lower, and depending on analytical method and sample preparation, may range from 0.003 to 0.016 mg/g (Stroher et al., 2012), and even reach 88 ng/g (Mazalli et al., 2006), with the lower values for the GC methods with pre-column derivatization vs HPLC methods. In this paper the LOQ value of cholesterol determination (0.16 mg/g) concerns a weighed sample of 3 g and corresponds to the lowest point of the calibration curve, and this 'effective' value is associated with the accepted repeatability of the method. We can see that the cholesterol level, possible to determine is low enough, and the method presented here is very good for the determination of cholesterol content in cholesterol-rich yolks. This high content is good for method reliability because when even weighed amounts of test material are low, the concentrations of sample solutions obtained roughly correspond to the middle part of the calibration curve. What is more, the reliability of the method was confirmed by mass spectrometer.

The recovery obtained in this study was high and practically equal to 100%. Therefore, when cholesterol content is determined in egg yolks there is no need to adjust the raw data for recovery (R), but a general formula (1) that accounts for this parameter was proposed for the calculations.

The main factors of uncertainty that essentially determine uncertainty of the method are uncertainty of within-laboratory reproducibility, uncertainty of recovery and standard purity, and uncertainty associated with the lack of trueness (i.e. bias defined as the difference between the actual and nominal value) of pipettes and measuring flasks (trueness being defined in the ISO/IEC Guide (2007) and described in Hauck et al. (2008)). These components can be regarded as separate uncertainty factors that form the uncertainty budget. Other components, such as those related to weighing precision and precision of the pipettes and measuring flasks are not included in the uncertainty budget as separate factors (Gąsior et al., 2009). This is because they had been automatically accounted for in within-laboratory reproducibility, which is already found in this budget. This is consistent with the remark of Ellison et al. (2000) that double calculation of the uncertainty components should be avoided. It must be added that uncertainty of the calibration curve was not listed in the uncertainty budget either, because a separate curve was plotted for each series of analyses, and that meant that the associated errors were already included in the reproducibility. The uncertainty of within-laboratory reproducibility includes most sample preparation and chromatographic measurement errors. However, it is essential that these errors are automatically included in the uncertainty only when the results calculated from two replicates concern cholesterol determinations in two parallel weighed samples. If the sample was not weighed in duplicate and the solution for chromatographic analysis was analysed twice, then the uncertainty of within-laboratory reproducibility would be understated and would only include the chromatography assay error (Gąsior et al., 2005; Gąsior et al., 2007). These factors are the most important and, according to the Gaussian propagation law, they contribute the most to method uncertainty in the case

of analyses performed in one laboratory. Together with the result that is the mean of the measurements, method uncertainty ($P \leq 0.05$) is of practical significance during the interpretation of the result. It determines the tolerance interval in which the actual result should be determined with 95% probability. Uncertainty should be monitored during the analysis of every sample by checking, under repeatability conditions, the coefficient of variation for individual determinations, which should not exceed a specified limit of repeatability. It should be added that method uncertainty can be reduced by increasing the number of analyses performed on one sample ($n \geq 2$).

The observations made during validation of the method provided a basis for determining the storage life of the solutions. The standard solutions, which were stored in tightly closed flasks in a refrigerator (+2°C to +8°C) were stable for at least 2 months. For solutions of the samples stored in closed chromatography vials, the safe storage life was determined to be 2 days. Longer storage is possible but increases the risk that the solvent will evaporate and cholesterol concentration will change due to possible seal (septum) leaks in the chromatography vial. It is much better to store samples dry following evaporation under inert gas. In such a state, the samples can be stored at low temperature (+2°C to +8°C) for at least 1 month.

In conclusion, a sample preparation method and a chromatographic method for determining cholesterol in egg yolks was developed and validated according to the EN ISO/IEC 17025 standard and the principles of Good Laboratory Practice. The internal standard method was chosen as the preferred method for routine analyses due to its lower uncertainty. The validated method is relatively fast, simple and reliable, as confirmed by the validation results. Besides, the method used does not include the pre-column derivatization which can undoubtedly be regarded as a great advantage. The present study and its results form a significant part of the quality system implemented in 2004 at the Central Laboratory. The testing procedure, developed according to this system, will be used in routine analyses performed for the purposes of the National Research Institute of Animal Production and external entities.

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Accepted for printing 22 VI 2012

ROBERT GĄSIOR, MARIUSZ P. PIETRAS

Walidacja metody oznaczania cholesterolu w żółtkach jaj

STRESZCZENIE

Celem prowadzonych badań była walidacja metody oznaczania cholesterolu w żółtku jaj z wykorzystaniem techniki chromatografii gazowej, zgodnie z wymaganiami normy PN-EN ISO/IEC 17025. Spośród dwóch metod, z użyciem standardu wewnętrznego i bez standardu wewnętrznego, ta pierwsza cechowała się mniejszą niepewnością. Dla niej powtarzalność i odtwarzalność wynosiły 4 i 6 (%), a niepewność metody (n=2, P≤0,05) obejmująca błędy przygotowania próbki i samego pomiaru chromatograficznego wynosiła 10,6%. Średni odzysk wynosił 99,9%, a LOQ 0,16 mg/g. Sprawdzany podczas wykonywania rutynowych analiz współczynnik zmienności dla powtarzalności nie powinien przekraczać granicy powtarzalności wynoszącej 8%. Metoda jest miarodajna, co zostało potwierdzone wynikami walidacji, a procedura jest względnie szybka i prosta.