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Validation of Analytical Method for Quantification of Egg Cholesterol Using Reversed Phase-High Performance Liquid Chromatography-Multiwavelength Detector

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ABSTRACT

In this research, analytical method of cholesterol content in eggs by Reversed Phase-High Performance Liquid Chromatography-Multiwavelength Detector (RP-HPLC-MWD) was validated. Our experiment validated the modified method of AOAC 994.10:2012 to get a more simple and efficient analytical method of cholesterol content. The sample was saponified using 10% KOH concentration for 15 min at 80 °C, then this analytical method was validated. RP-HPLC-MWD condition was at 100% MeOH as a mobile phase, flow rate of 1.0 mL/min, detection UV at 205 nm, cholesterol was detected at 10.38±0.13 min. As a result, the coefficients of determination for instrument and method linearities reached 0.9991 and 0.9912, respectively. The limits of detection and quantification of RP-HPLC-MWD instrument were found at 5 and 10 μg/mL, respectively, while the method-detection limit and quantification limit were 250 and 500 μg/g sample, respectively. Recovery values for the cholesterol analysis ranged from 98.62% to 112.26%, with a precision of 1.05%–3.90%. Additionally, intralab reproducibility was known to reach 3.27%. This validated method can be applied for the analysis of cholesterol in various eggs available in the market.

Keywords: cholesterol; chromatography criteria; eggs; method development; reversed phase

INTRODUCTION

Eggs are one of the richest sources of cholesterol. Specifically, chicken eggs are highly favored in the diet (up to 99.51%) compared to the other eggs, with a consumption rate of 0.122–2.119 kg/capita/week during 2007–2017 (Central Bureau of Statistics, 2017). Analysis of cholesterol in the food matrices in previous studies could use a spectrophotometer (Santi *et al*., 2015; Abdurrahman *et al*., 2016), but in general, the existing methods for assessing cholesterol content in food matrices are based on chromatographic technique. Gas chromatography (GC) was found to be more sensitive for determination of cholesterol in the food matrices (Ahn *et al*., 2012; Bavisetty & Narayan, 2015; Lioe *et al*., 2013; Park *et al*., 2013; Souza *et al*., 2017; Stroher *et al*., 2012). Compared to HPLC, GC-based analysis for cholesterol may be hindered by limitations, i.e., time-consuming sample preparation and costly, since derivatization of cholesterol compounds and reliability test using internal standard must be first performed before being used. In addition, GC instrument is operated at a higher temperature than HPLC (Albuquerque *et al*., 2016; Chen *et al*., 2015), which possibly induces the formation of cholesterol oxides (Cais-Sokolinska & Rudzińska, 2018; Min *et al*., 2015). Hence, this present work was designed to evaluate the applicability of HPLC for quantifying cholesterol level in foodstuffs.

Diode array detector (DAD) or UV seemed to be the most desirable detector for cholesterol quantification (Ahn *et al*., 2012; Albuquerque *et al*., 2016; Bavisetty & Narayan, 2015; Lioe *et al*., 2013; Stroher *et al*., 2012). MWD (Multi Wavelength Detector) constitutes one of the ultraviolet detectors capable of performing more sensitive and selective detection than fixed wavelength detector of UV-Vis and the other detectors such as ELSD and RI (Mariutti *et al*., 2008; Wolfender, 2009). Studies on the quantification of egg cholesterol using RP-HPLC-MWD have not been reported. Mobile phase—acetonitrile and methanol—is often applied in RP-HPLC at UV detection of 195–210 nm and flow rate of 1–1.5 mL/min (Ahn *et al*., 2012; Albuquerque *et al*., 2016; Bavisetty & Narayan, 2015; Stroher *et al*., 2012).

AOAC 994.10:2012 is a standard method for cholesterol analysis in foodstuffs by GC-FID instrument (AOAC, 2012a); however, the technique needs more samples, which in turn requires more chemicals and saponification time. A modified method for cholesterol analysis with less amount of sample can reduce saponification time and KOH concentration in the step of

sample preparation. In addition, the use of an available standard method for the other purposes needs an additional step, i.e. validation. Therefore, this present work aimed to validate the simple modified analytical method of eggs cholesterol using RP-HPLC-MWD.

MATERIALS AND METHODS

Chemicals and Reagents

Cholesterol standard with 95% purity (Sigma Chemical Inc, USA). Methanol; KOH; anhydrous Na_2SO_4; hexane and of pro-analytical grade from Merck (Darmstat, Germany) were used. Methanol of pro-analytical and liquid chromatography grade was obtained from Merck (Darmstat, Germany). Demineralized water, technical N_2 gas, and PVDF filter membrane 0.45 μm.

Preparation and Homogeneity Test of Sample

The materials used in this study were broiler chicken eggs *(Gallus sp.)* as food matrices for analytical validation, and the other commercial eggs such as red local chicken eggs, duck eggs, quail eggs, and low-cholesterol chicken eggs were used to applicate the validated method. All of the egg samples were obtained from the local market, in Bogor, West Java, Indonesia.

The mixed whole eggs of broiler chicken then were put into a small plastic bag and sealed. All samples were stored in a freezer at minus 18°C. Preparation of the other egg samples was carried out in the same way. Sample homogeneity was tested according to Sunanti *et al*. (2013). The tested parameter was moisture content, in duplicate. Moisture content analysis used the AOAC 925.30:2012 method (AOAC, 2012b).

Instrumental Performance for Cholesterol Analysis by RP-HPLC-MWD

Instrumental performance analysis used a serial cholesterol standard solution. The linearity of the instrument, the precision of peak area and retention time, peak symmetry of standard cholesterol and sample, the limit of detection and quantification of the instrument were evaluated. The linearity test of the instrument used a serial standard cholesterol solution at concentrations of 10, 25, 50, 75, 100, 125, 150, 200 μg/mL, in triplicate. This evaluation followed EURACHEM guidelines (2014).

Instrumentation and Condition of RP-HPLC-MWD

HPLC series Infinity Agilent Technologies 1200 (Agilent Technologies, Waldbronn, Germany), G1365D MWD (Multiwavelength Detector) series Agilent Technologies 1200 Infinity (Agilent Technologies, Waldbronn, Germany), reversed phase column ZORBAX Eclipse XDB-C_{18} (150 mm x id 4.6 mm, 5 μm) (Agilent Technologies, USA), 50 μL syringe (Agilent Technologies, Santa Clara, USA), 20 μL sample loop Rheodyne (IDEX Health & Science, Oak Harbor, USA). The mobile phase composition, flow rate, and wavelength were used according to the selected good chromatographic condition of RP-HPLC-MWD as a result of our experiment before (experiment data were not presented). Methanol (100%) was used as mobile phase at a flow rate of 1.0 mL/min in isocratic mode and total run time was 15 min at room temperature. Analytes were detected using multiwavelength detector (MWD) at a wavelength of 205 nm.

Preparation of Cholesterol Sample (Modification of AOAC 994.10, 2012a)

The initial step of sample preparation was saponification. In a 50 mL tube, ±1 g of whole chicken eggs were weighed. Ten milliliters of 10 % (w/v) methanolic KOH solution were added and thoroughly mixed for 20 s in a vortex, then the samples were blown under nitrogen for 10 s. The mixtures were heated in a water bath at 80°C for 15 min. After heating, the samples were cooled at room temperature for 15 min. Afterward, these mixtures were extracted three times with 10 mL of hexane, each addition of 10 mL hexane was mixed in a vortex thoroughly for 20 s. The upper phases (hexane phase) were transferred to the 25 mL tube, then the lower phases (water phase) were extracted again with hexane in the same way. Around 25 mL of cholesterol extracts were transferred to a 100 mL separator flask and the tube was rinsed with 2×1 mL hexane. Furthermore, the solution was rinsed using 3×10 mL of demineralized water. The last of residual rinse water was dripped with phenolphtalein 1% until the pH was neutral. Then the rinsed hexane phases were filtered with filter paper which was added 5 g of anhydrous Na_2SO_4. The cholesterol extracts were dried under nitrogen. Afterward, the dried cholesterol was redissolved in 0.5 mL of hexane then added with methanol (mobile phase HPLC) until the total volume of the solution was 5 mL. After that, this solution was filtered with a 0.45 μm PVDF membrane, then diluted 5 times. Finally, the aliquot (20 μL) was injected into HPLC-MWD.

Method of Validation

The linearity of the method, specificity, the precision of area and retention time to evaluate the behavior of chromatogram, accuracy and precision, method detection limit, and intralab reproducibility were evaluated according to EURACHEM (2014) and AOAC (2012c, 2016) guidelines. The linearity method was carried out by a serial spiked sample of chicken eggs at concentrations of 250, 500, 3011, 4014, and 5018 μg/g in triplicate. Method linearity was evaluated by linear regression analysis with plotting peak areas (mAU*s) versus spiked concentrations (μg/g sample), then the determination coefficient (R^2), slope, and intercept were evaluated. Specificity was conducted by comparing the chromatogram of pure standard cholesterol, unspiked egg samples, and spiked egg samples. Furthermore, the accuracy and precision of the method were determined after spiking eggs with cholesterol standard at concentrations of 500 μg/g and 5018 μg/g, each was assessed in seven replications. Mean, standard deviation (SD),

and relative standard deviation (RSD) for each spiked sample were calculated.

Furthermore, the method of detection limit (MDL) was determined from the method linearity curve of the relationship between the standard deviation values of three spiked samples at concentrations of 5018, 3011, and 500 μg/g, in triplicate. MDL curve linearity was evaluated by linear regression analysis, plotting SD of read cholesterol standard from each concentration (y axis) versus cholesterol spiked concentrations (x axis). The SD_0 value was intercepted, MDL value was calculated as $3\times SD_0$, then the values were verified. Intralab reproducibility of the method was determined by analyzing unspiked egg samples on three different weeks by preparing three samples each week. The intralab reproducibility of the method was good if RSD analysis (%) less than RSD Hortwitz (%).

Statistical Analysis

Cholesterol contents and descriptive statistics were calculated using the Microsoft Excel 2013 program. Statistical analysis was analyzed using IBM SPSS 22.0 (SPSS Inc, Chicago). Evaluation of cholesterol contents among weeks in intralab reproducibility test was analyzed by One way-ANOVA test, followed by Duncan test. P value < 0.05 was statistically significant.

RESULTS

Instrumental Performance of RP-HPLC-MWD for Cholesterol Analysis

Serial cholesterol standards were used in the instrumental-performance analysis. The calibration curve was determined with concentrations of 10–200 μg/mL, while the slope and intercept of linear regression obtained were 11.515±0.220 and 1.518±9.643, respectively. The relationship between standard concentrations and peak area displayed the desired linearity, with R^2 (0.9991) of > 0.99 (AOAC, 2012c). The LOD and LOQ instruments were 5 and 10 μg/mL. The concentration of cholesterol standard at 5 μg/mL could be detected by the instrument but had poor precision (Table 1). However, at 10 μg/mL standard cholesterol concentration, its precision and accuracy have fulfilled the acceptability range of AOAC 2016 (Table 1) and good behavior chromatographic, the precision of area and retention time < 2.00%, that was recorded at 1.89% and 0.39%, respectively (n=6). In addition, the peak symmetry of standard cholesterol and sample were 0.99 and 0.75, respectively.

Sampling

Based on the homogeneity test, resulting in F-test statistic (2.94) < F table (3.50). Therefore, it can be stated that broiler chicken eggs used in this study are considered as homogeneous.

Validation of Cholesterol Analytical Method

Method linearity. The method linearity was linear over the range of 250–5018 μg/g sample. The slope and intercept were obtained at 0.3734±0.0085 and 81.01±19.38, respectively. The linearity of the developed method seemed to be satisfied as was indicated by R^2 (0.9918) > 0.990 (AOAC, 2012c), indicating that it gave a proportional response to the increase in the concentration of cholesterol analytes in the samples.

Specifity. Method specificity is observed by comparing the peaks from pure cholesterol standard, unspiked egg samples, and spiked egg samples. Retention time achieved at 125 μg/mL cholesterol standard, unspiked and spiked samples (5018 μg/g) were 10.38±0.13 min, 9.49±0.01 min and 9.41±0.11 min, respectively. In Figure 1a–c, spiked egg samples (5018 μg/g sample) demonstrated higher peak and larger area than unspiked egg samples. In addition, the peak of pure cholesterol standard showed the lowest area and height. The higher concentration of cholesterol in sample accounted for higher and wider peaks as shown in the chromatogram output, but they were detected at the same time. This implies that our developed method demonstrates good specificity.

Method-detection limit (MDL) and method-quantification limit (MQL). The estimated method-detection limit was determined by using chicken egg samples spiked with standard cholesterol solutions at 500, 3011, and 5018 μg/g, in triplicate. Linearity and determination coefficient were presented as follows: y= 0.0262x + 70.438 and R^2= 0.8702. Based on the results, SD_0 and MDL reached 70.44 and 211.31 μg/g, respectively. This value was determined as a theoretical MDL, because samples spiked with cholesterol standard at a concentration of 250 μg/g (n= 3) possessed an unacceptable accuracy, but it had an acceptable precision (RSD < RSD Horwitz, 4.52%). The precision and accuracy of 250 μg/g spiked sample reached 4.44% and 194.71%, with the acceptable recovery of 85–110%. Therefore, MDL in this research was 250 μg/g. Then, the results showed that addition of cholesterol standard at the concentra-

Table 1. The limits of detection and quantification for instrument

Chromatograpic criteria	Cholesterol content (μg/mL)	RSD (%)	2/3 RSD Horwitz (%)	Recovery (%)	Acceptable recovery (%)[c]
LOD[a]	5	22.57	8.26	113.44	75-120
LOQ[b]	10	2.87	7.40	113.03	80-115

Note: [a]LOD (Limit of Detection), [b]LOQ (Limit of Quantification), [c]AOAC (2016).

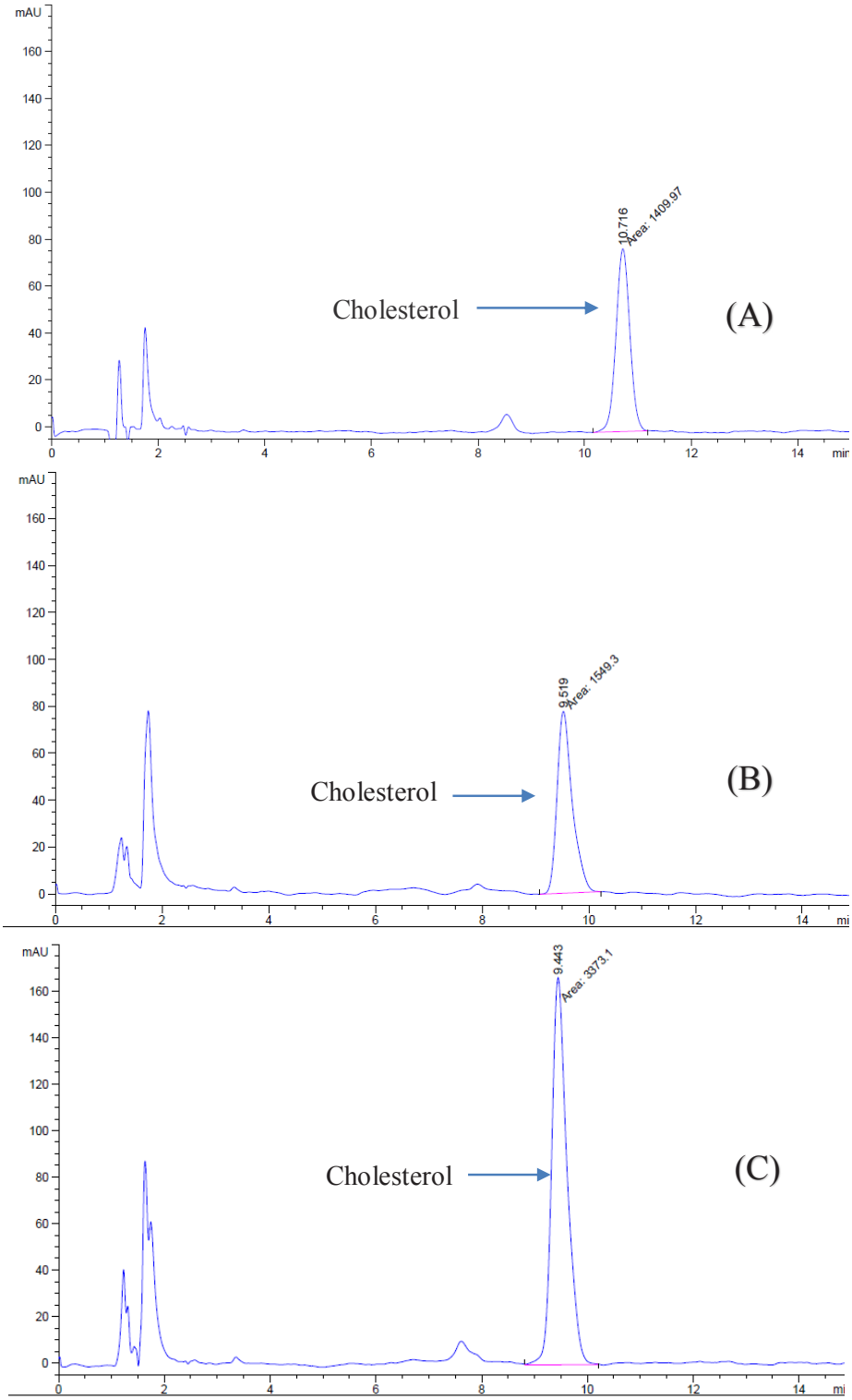

Figure 1. Chromatogram of cholesterol standard at 125 µg/mL (A), unspiked sample (B), spiked sample with 5018 µg/g sample (C)

tion of 500 µg/g (n=7) could still be detectable with precision (1.04 %) less than 2/3 RSD Horwitz (3.00 %) and accuracy of 112.26%. The concentration of spiked sample shows satisfied chromatographic behavior based on the precision of the area and retention time (RSD ≤ 2%), with the precision up to 0.99% and 1.29%, respectively. Furthermore, the average area and retention time reached 1760.20±17.58 mAU*s and 9.55±0.12 min (n=7). RSD analysis (< 2%) displayed a good suitability system of analysis method (JECFA, 2006). Therefore, the MQL was determined at 500 µg/g sample.

Accuracy. The accuracy of cholesterol analytical method was determined based on the recovery value of samples added with two levels of cholesterol standards at the initial step. The averages cholesterol found in 5018 µg/g and 500 µg/g of spiked samples were 4948.70 and 561.30 µg/g, respectively. The recovery values of two

spiked concentrations were entirely presented in Table 2. Spiked sample with 5018 μg/g cholesterol fulfilled the acceptability range of AOAC (2016), i.e., 90%–108%. Conversely, the recovery at 500 μg/g spiked sample was found to be out of the acceptability range of AOAC (2016), i.e., 85%–110%, even though the value was closed to the acceptability range.

Precision. This precision was evaluated by analyzing the spiked sample at 500 and 5018 μg/g sample. This procedure was repeated in a short period of time and injected on the one day for each group of concentration of spiked samples. Furthermore, RSD analysis and RSD Horwitz were compared, in which precision was expressed by RSD analysis. Table 2 shows that the precision values of both spiked samples are less than RSD Horwitz, which suggests that our cholesterol analytical method favorably affect precision.

Intralab reproducibility. In this study, the reproducibility tests used the same unspiked broiler chicken eggs as samples, instruments, operator at different times. As a result, average cholesterol value during 3-weeks experiment was 3920.18±128.23 μg/g. The RSD weeks (3.27%) was less than RSD Horwitz (4.06%), which confirmed that our modified method was sufficiently precise. Based on One way-ANOVA test results followed by Duncan's test, the egg cholesterol content at different weeks did not significantly differ ($p>0.05$). Hence, it can be stated that this method has a good reproducibility over a certain period of time.

Application of the Validated Method in Different Eggs from the Market

This validated method was applied for the determination of cholesterol in different eggs from the market. Cholesterol content of each egg was presented in Table 3, in which it differed according to the type of eggs as follows: red local chicken eggs > quail eggs > duck eggs > broiler chicken eggs > low cholesterol eggs.

DISCUSSION

Cholesterol analysis was conducted according to the standard protocol of AOAC 994.10:2012 with modifications, in which the improvement was carried out at saponification steps. Sample preparation is a critical step in cholesterol analysis using RP-HPLC. Saponification constitutes initial and crucial step to release cholesterol from the other components since saponification was reported capable of improving cholesterol concentration in egg yolk compared to sample without saponification (John *et al*., 2015). The use of KOH in saponification enables to hydrolyze disrupting components such as fatty acids and acyl glycerides (Ahn *et al*., 2012; Park *et al*., 2013), while its effect may depend on the concentration and heating time (Salimon *et al*., 2012; Souza *et al*., 2017). The modification in this procedure was made at the following conditions, including less amount of sample (1 g, from 6 g of standard method), KOH concentration, saponification time, and changing toluene with hexane for extraction. As reported by Albuquerque *et al*. (2016), the use of hexane for extracting complex food matrices such as eggs is more preferable than toluene. Toluene is reported to induce the formation of emulsion system; thus, the sample preparation time takes longer. This modified analytical cholesterol method has proved to reduce saponification time and KOH concentration of AOAC 994.10 method (AOAC, 2012a), from 70 ± 10 min to 15 min saponification time and from 50% to 10% of KOH concentration.

This HPLC-MWD instrument has proved good instrumental performance. In literatures, LOD of HPLC-diode array detector (DAD) for quantification of cholesterol were 1 and 3 μg/mL (Albuquerque *et al*., 2016; Bavisetty and Narayan, 2015), while LOQ was found at 11 μg/mL (Albuquerque *et al*., 2016). Meanwhile, LOD and LOQ of HPLC-fixed wavelength detectors were observed at 5 and 16 μg/mL, respectively (Stroher *et al*., 2012). In this case, the sensitivity of RP-HPLC-MWD instrument could be similar to that of HPLC-DAD and even higher than that of HPLC-fixed wavelength detector.

Our cholesterol analytical method tended to have a similar accuracy compared to several instruments operated with different detectors. Recovery of cholesterol analysis using HPLC-DAD (Bavisetty & Narayan, 2015; Albuquerque *et al*., 2016) ranging from 95.2%, 98%, to 111%–125%, respectively. The recovery of cholesterol analysis using HPLC-UV operated at fixed wavelength reached 84%–103%; 93.34%–102.34%; and 93.33±0.22%

Table 3. Cholesterol contents of various eggs from market (n=2)

Various eggs	Cholesterol contents (μg/g)	RSD (%)	*RSDH (%)
Quail	4351.63±172.49	3.96	4.53
Duck	4295.91±13.73	0.32	4.54
Red local chicken	4692.51±74.64	1.59	4.48
Low cholesterol	3146.83±68.69	2.18	4.76
Broiler chicken	3918.20±58.87	1.50	4.61

Note: *RSDH = RSD Horwitz.

Table 2. Accuracy and precision of validated method for determination of cholesterol in eggs using RP-HPLC-MWD (n=7)

Spiked concentration (μg/g sample)	Analyzed-spiked concentration (μg/g sample)		Precision (%)	Accuracy (%)	
	Range	Mean		Range	Mean
500	517.37-620.33	561.30	1.05	103.47-124.07	112.26
5018	4236.62-5321.35	4948.70	3.90	84.43-105.87	98.62

Note: Accuracy expressed by recovery; Precision expressed by RSD

(Ahn *et al*., 2012; Mariutti *et al*., 2008; Stroher *et al*., 2012), while Lioe *et al*. (2013) found that the recovery value in HPLC-ELSD was 108%–122.13%.

Previously, the validation of cholesterol analytical methods on diverse foodstuffs (sour cream, whole eggs, egg yolks, and some fish species) using HPLC-DAD demonstrated RSD analysis of 0.69%, 0.54%, 1.92%, 3.05%, respectively (Albuquerque *et al*., 2016; Bavisetty & Narayan, 2015). In our present results, the precision value tended to be similar, even lower than that obtained from HPLC-DAD experiment, but what we achieved in this present study seemed to have better precision compared to that obtained from HPLC-ELSD experiment. The precisions were reported to reach 5.26%, 4.29%, and 10.11% (Lioe *et al*., 2013). The reproducibility of cholesterol analytical method using HPLC-ELSD and HPLC-DAD in previous studies reached 2.25% and 1.75%, respectively (Albuquerque *et al*., 2016; Lioe *et al*., 2013). Even though this reproducibility intra-lab value was larger than previous studies, this value still fulfilled the acceptability requirement.

Red chicken eggs were recognized as local egg, containing DHA and omega 3. Some studies reported lower cholesterol of DHA- and omega 3-enriched eggs compared to control eggs (Khan *et al*., 2017; Mattioli *et al*., 2016). On the other hand, Faitarone *et al*. (2013) reported that the addition of DHA and omega-3 in egg feed could not reduce the cholesterol concentration of eggs. In this study, the cholesterol content of quail and duck eggs seemed to be smaller than that reported by USDA (2018a, 2018b), i.e. 8440 and 8840 μg/g, respectively. The cholesterol content could be higher due to the other factors, such as the age of the bird (Faitarone *et al*., 2013).

The cholesterol content of broiler chicken egg in the study was smaller than the previous report by Albuquerque *et al*. (2016), which reached up to 4390.00 μg/g. However, it was greater than the cholesterol concentration in whole chicken eggs, i.e. 3046.00–3062.00, 2368.73, 562.68 μg/g, as described by numerous preceding works (Lioe *et al*., 2013; Naviglio *et al*., 2012; Park *et al*., 2013). Cholesterol content of chicken eggs in this study was 3720 μg/g, which was closed to that reported by USDA (2016) and Exler *et al*. (2013).

CONCLUSION

The validated cholesterol analytical method using RP-HPLC-MWD in sample preparation step could reduce the use of chemicals, saponification, and analysis time. It is more simple and efficient than standard method-AOAC 994.10:2012. This method can be applied to the other various eggs from the market.

CONFLICT OF INTEREST

Authors declare that they have no conflict of interest with Laboratory of Food Science and Technology, Faculty of Agricultural Technology, IPB University, Bogor, Indonesia that funded this research.

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