COMPARATIVE SENSITIVITIES OF CHOLESTEROL ANALYSIS USING GC, HPLC AND SPECTROPHOTOMETRIC METHODS

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Abstrak : Kolesterol telah diekstrak dengan menggunakan kaedah Bohac (1984), Beyer & Jensen (1989) dan Queensland Health Science Institute (1995) dan prestasi ketiga-tiga kaedah ini telah diuji untuk mengekstrak kolesterol dari kuning telur. Kandungan kolesterol setiap sampel telah di analisis dengan menggunakan kaedah spektrofotometri serapan UV-VIS, HPLC dan kromatografi gas. Data yang diperolehi telah diuji dari segi kelinearan, ketepatan, peratus perolehan dan kesan saiz sampel. Dalam kajian ini analisis HPLC telah memberikan keputusan yang paling memuaskan bagi penentuan kolesterol di mana had pengesanannya adalah 0.08 mg/mL, kepersisan 97.5% dan pekali variasi 4.94%. Dalam ekstraksi beberapa kepekatan kolesterol piawai, kaedah Bohac adalah yang paling cekap, dengan memberikan peratus perolehan sebanyak 96.53%, kelinearan $r^2 > 0.99$ dan julat pengesanan 0.00 – 0.75 mg/mL. Berdasarkan keputusan yang diperolehi analisis kolesterol yang baik dapat dilakukan dengan menggunakan ekstraksi kaedah Bohac dan dikuti dengan analisis HPLC.

Abstract : Three methods of extraction namely Bohac (1984), Beyer & Jensen (1989) and Queensland Health Science Institute, Australia (QSE-CAM-004) (1995) were compared for the extraction of cholesterol from egg yolk. Each of the extracted samples was then analyzed by using Spectrophotometer, HPLC and GC respectively. The results were then evaluated for their analytical linearity, accuracy, precision, percentage of recovery and the effects of sample sizes. In this study, the results showed that HPLC system was found to be more suitable in determination of cholesterol with the limit of detection of 0.08 μg cholesterol/mL, precision of 97.5% and coefficient variation of 4.94%. By using cholesterol standard in the comparison of methods, Bohac extraction method was found to be the most effective, with the percentage of recovery of 96.53%, linearity of $r^2>0.99$ and effective range of 0.0 - 0.75mg/mL. The Bohac extraction method and HPLC were the best combination for extraction and analysis of cholesterol.

Keyword: Cholesterol analysis, methods of extraction, methods of analaysis, LOD, LOQ

Introduction

Cholesterol is a structure containing 27 carbon, commonly found as the component in cell membrane. Biologically it is an important precursor for bile acid, provitamin D3 and several steroidal hormones. Accurate determination of cholesterol is important due to its close correlation to the occurrence of coronary heart disease. Earlier methods of cholesterol analysis rely heavily on spectroscopic and gravimetric procedures[1] . Methods that were based on enzymatic, coupled with spectrophotometric for analysis of cholesterol from blood were unsuitable for food applications [2, 3]. Chromatographic methods were more reliable, selective and accurate [4, 5] because interference from other sterols can be easily resolved [6,7]. However, the data from various chromatographic techniques are very dependent on the extraction procedures and intensity of the saponification steps [8, 9, 10, 11, 12]. Furthermore, previous investigators limit their investigation to either comparison of extraction methods or on the types of analytical equipment used [13, 14, 15]. This investigation will attempt to compare the efficiency of three published extraction procedures and their contributions in affecting the sensitivity of cholesterol analysis.

Material and Methods

a. Standard cholesterol

Standard cholesterol (5-α-cholestan-3-β-ol, chromatography grade; Sigma Chemical Inc, USA) solution was prepared by dilution from a stock solution (3 mg/mL). The stock solution used to prepare working solution containing 0.03, 0.15, 0.30 and 0.60 mg/mL cholesterol. The standard solutions were extracted to determine the limit of detection (LOD), limit of quantification (LOQ) and linearity of each method. Recovery of each extraction procedure was tested by dissolving the diluted standards in oil matrix (pure palm oil)

c. Analysis according to Bohac [13]

The working cholesterol standards were saponified using 10mL ethanolic 2% KOH and 0.3mL pyrogallol solution. The mixture was incubated at 80^{o}C for 15 minutes. Upon cooling, 5 mL distilled water was added. The unsaponified matter was extracted with 2x10mL hexane. The final extract had the hexane removed by heating in a water bath (45^{o}C).

d. Analysis according to Beyer & Jensen [5]

The working solutions were saponified using 2% alcoholic KOH and after 1 hour, the unsaponified fraction was extracted with 2x10mL hexane. The extracts were combined and washed with 5mL distilled water. The extract was heated to dryness in a water bath (45^{o}C)

e. Analysis according to Queensland Health Science Institute [16]

The working standard solution were saponified using 2% alcoholic KOH. The unsaponified fraction was extracted with 4x10mL petroleum ether and all the extracts were later combined. The extract was washed with 0.5N NaOH and repeatedly with distilled water until neutral. Traces of moisture was removed by the addition of sufficient amount of anhydrous sodium sulphate. Finally, the extract was heated in water bath to remove the petroleum ether.

f. Spectrophotometric analysis

Preparation of colouring reagent:

The stock reagent was prepared by dissolving 10g of $FeCl_3.6H_2O$ in glacial acetic acid using a 100mL volumetric flask. Prior to use, the 1.0mL of the stock reagent was transferred into a 100mL flask and concentrated H_2SO_4 was added to volume.

Colour Reaction:

The dried extracts from (a), (b) and (c) were resuspended in 3mL glacial acetic acid, 2mL of $FeCl_3$ coloring solution was added and the resultant colour was read at 565nm (Pye Unicam UV1 Double Beam Scanning Spectophotometer). The absorption was compared against an external cholesterol standard and the cholesterol content was calculated using the following equation:

$$\text{Cholesterol (mg/100gram)} = \frac{\text{c x 20 x DF x W}}{\text{4 x 100}}$$

Where:

c = concentration of cholesterol (from standard curve); DF = dilution factor; W = weight of sample

g. Gas chromatographic method

Prior to analyses, all dried extract from (a), (b) and (c) were resuspended in 0.8mL petroleum ether. Analyses were performed on a HP 5890 Series II Plus gas chromatograph (equipped with split/splitless injector and electronic pressure control, EPC); detector, FID; using a capillary column (0.25mm x 25m length) coated with high-temperature phase 007-65HT (Alltech, USA). The GC conditions were as follows: Injection volume, 1.0µl; injector temperature, 300^{o}C; detector temperature, 350^{o}C; temperature programming: 65^{o}C – 200^{o}C (40^{o}C min^{-1}.) – 280^{o}C (10^{o}C min^{-1}). Carrier gas flow rate (He) at 1.6mL min^{-1}.

h. HPLC method

The dried extracts from (a), (b) and (c) were resuspended in 0.8mL isopropanol. The isocratic analysis (50% acetonitrile : 50% isopropanol) was performed using Waters Symmetry C18 column (4.6mm x 250mm) on a HPLC system, consisting of PU-980 Jasco HPLC Pump, Waters 480 UV-VIS detector, and Delta data processing software.

i. The precision of the measuring instruments

The precision was based on the accuracy of the measured cholesterol concentration against a known amount[17]. Based on repeated (4x) measurements coefficient of variation (CV) was calculated.

j. Determination of LOD and LOQ
The estimates were based on method proposed by an earlier investigators [17, 18] where LOD was reached when signal/noise (S/N) ratio is 3, while LOQ was defined as the point where S/N = 10.

Results and Discussion

The sensitivities if the spectrophotometer, gas chromatography and HPLC were tested using standard cholesterol solutions. The results are shown in Table 1. The precision of each measuring apparatus was defined by Dyson [17] as a set measurements with CV of less than 5%; if the CV is 10%, the precision of the instrument is considered as fair.

The most consistent equipment was found to be the HPLC, giving the lowest LOD and LOQ. Even at that low LOD, the response was clearly distinct (Fig. 1). In contrast, the LOD of GC was higher and less reproducible, as indicated by its higher CV. However the results were in general agreement with those obtained by Fenton & Sim [19]. Cholesterol was analysed without undergoing derivatisation [20, 21] and resultant peak obtained was sharp. TMS-derivatised cholesterol will interfere with linearity due to the deposition of silicon dioxide on the FID [22, 23]. The performance of spectrophotometer, was better than GC in terms of reproducibility, but it lacked sensitivity (LOD = 14 μg/mL) compared to the HPLC method. It was not as claimed by Bachman et al. [24]. The HPLC also returned the closest measured value of cholesterol (0.263 mg/mL), relative to the actual value of test standard (0.270 mg/mL). Based on these data HPLC was considered as the method of choice for cholesterol determination.

At higher concentration (Fig. 2) the response obtained were very precise and reproducible. The range of concentrations chosen for the serial dilution were related to the normal range of cholesterol found in food matrix. The response and linearity of detection of standards extracted from oil matrix were excellent for the HPLC analysis, relative to the neat standards (Fig 3).

The recoveries of standard cholesterol using all the three methods are shown in Table 2. Based on the results from Fig. 3 and Table 2, it is clear that the extraction method proposed by Bohac et al., [13] is superior to that of the other two. The recovered cholesterol are very close to the amount of added cholesterol. The Queensland method [16] are always retuning higher values (overestimation). As noticed by earlier investigators, cholesterol content can be affected by the extraction and analytical methods [5, 6]

Table 1 : Sensitivity of Measuring Instruments

Instrument	Actual Amount (mg)	Precision*		LOD (μg/mL)	LOQ (μg/mL)
		Measured (mg)	CV (%)		
Spectrophotometer	0.27	0.203 ± 0.032	5.76	14	15
HPLC	0.27	0.263 ± 0.013	4.94	0.08	0.60
Gas Chromatography	0.27	0.202 ± 0.048	23.76	4.00	13

*n = 8

Table 2 : Recovery of cholesterol (spiked) from oil matrix

Method	Actual Concentration (mg/mL)	*Extracted cholesterol (mg/mL)	CV (%)	Recovery (%)
1. Spectrophotometer				
Bohac	0.30	0.26	6.66	86.67
B & J	0.30	0.11	18.17	36.67
Queensland SE	0.30	0.38	18.42	126.67
2. HPLC				
Bohac	0.30	0.29	7.50	96.67
B & J	0.30	0.22	9.10	73.33
QSE	0.30	0.33	9.56	110
3. Gas Chromatography				
Bohac	0.30	0.25	28.57	83.33
B & J	0.30	0.18	51.23	60.00
QSE	0.30	0.44	34.38	146.67

* Mean from 8 extractions; Bohac = Bohac[13] , B&J = Beyer & Jensen[5]; QSE = Queensland Health Science Institute[16]

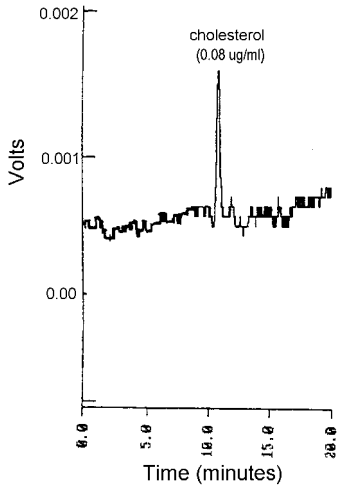

Fig. 1 : Detection of cholesterol at limit of detection of HPLC

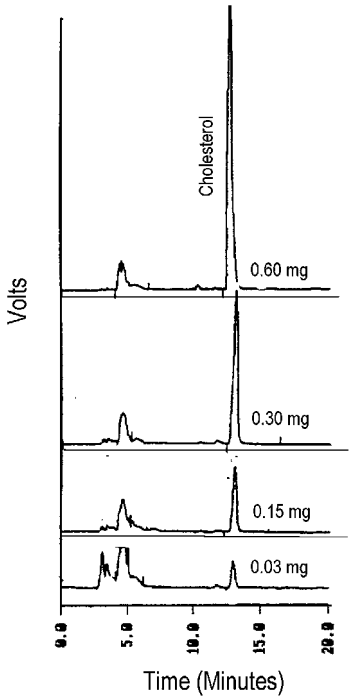

Fig. 2: Analysis of cholesterol at different concentrations.

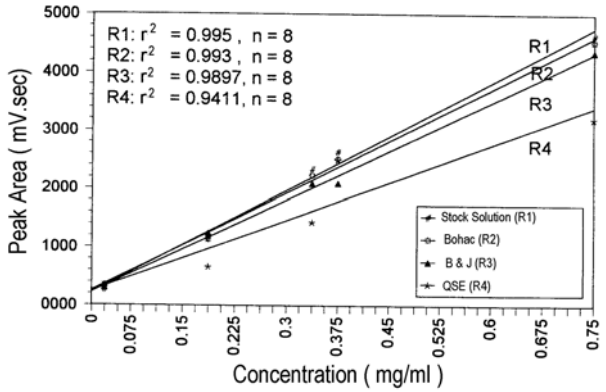

Fig. 3: Linearity of response from cholesterol extracts analyzed using HPLC method

Conclusion

The HPLC method was found to be the most convenient and consistent in giving highest sensitivity and accuracy for the determination of cholesterol from the models created in this investigation.

References

1 Sweeney, J. & Weihrauch, J. 1976. Summary of available data for cholesterol in food and methods for its determination. *CRC Crit. Rev. Food Nutr.* 8, 131 – 159.

2 Touchstone, J.C. 1986. CRC *Handbook of Chromatography*. CRC Press Inc., Florida, USA. pp 21-218.

3 Jiang, Z., Fenton, M. & Sim, J.S. 1991. Comparison of four different methods for egg cholesterol determination. *Poult. Sci*. 70(4), 1015 – 1018

4 Maxwell, R.J. & Schwartz, D.P. 1979. A rapid quantitative procedure for measuring the unsaponified matter from animal, marine and plant oil. *J.Am. Oil Chem. Soc.* 56(6), 636 – 635

5 Beyer, R.S. & Jensen, L.S. 1989. Overestimation of cholesterol content of eggs. *J.Agric.Food Chem.* 37, 917 – 920

6 Kritchevsky, D. & Dehoff, J.L. 1978. Sterol content of seafood as a function of analytical method. *J.Food Sci.* 43(6), 1786 – 1789

7 Folch, J.,Lees, M. & Sloane, G.H. 1956. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol.Chem.* 226, 497 - 509

8 Hubbard, W.D., Sheppard, A.J., Newkirk, D.R. & Osgood, T.J. 1977. Gas liquid chromatographic determination of cholesterol and other sterols in foods. *J. Assoc. Off. Anal. Chem.* 60(6), 1302 – 1305

9 Punwar, J.K. 1976. Gas liquid chromatographic determination of total cholesterol in multi-component foods. *J. Assoc. Off. Anal. Chem.* 58(4), 804-809

10 Sheppard, A.J., Newkirk, D.R., Hubbard, W.D. & Osgood, T. 1977. Gas liquid chromatographic determination of cholesterol and other sterols in foods. J.*Assoc.Off. Anal. Chem*. 60(6), 1302 - 1306

11 Oles, P., Gates, G. Kensinger, S., Patchell, J., Schumacher, T. & Silcox, A. 1990. Optimization of the determination of cholesterol in various food matrices. *J.Assoc. Off. Anal. Chem.* 73(3), 724 - 727

12 Bitman, J. & Wood, D.L. 1980. Comparison of two extraction of cholesterol in egg yolk. *Poult.Sci*. 59(7), 2014 – 2017

13 Bohac, C.E., Rhee, K.S., Cross, H.R. & Ono, K. 1984. Assessment of methodologies for colorimetric cholesterol assay of meat. *J.Food Sci.* 53(6), 1642 – 1693.

14 Elswyk, R.D., Schake, L.S. & Hargis, P.S. 1991. Research Note: Evaluation of two extraction methods for the determination of egg yolk cholesterol. *Poult. Sci.* 70(4), 1528

15 Guiochon, C.G. & Siouffi, A. 1979. Comparison of various systems for separation of free sterols by HPLC. *J.Anal.Chem.* 51(11), 1661 – 166

16 Queensland Health Science Institute (1995) Method QSE-CAM-004.

17 Dyson, N. 1992. *Chromatographic Integration Methods.* The Royal Society of Chemistry, Cambridge, United Kingdom. 1-67, 87-156.

18 Smith, J.S. 1994. Evaluation of analytical data. In: Nielsen, S.S. 1994. *Introduction to the chemical analysis of food.* Jones & Bartlett Publ., London. pp 53-59.

19 Fenton, M. & Sim, J.S. 1991. Determination of egg yolk content by on-column capillary gas chromatography. *J.Chromatogr.* 540, 323 – 329

20 Duncan, I.W., Culbreth, P.H. & Burtis, C.A. 1979. Determination of free, total and esterified cholesterol by HPLC. *J.Chromatogr.* 162, 281-292

21 Ballesteros, E., Gallego, M. & Valcarcel, M. 1996. Gas chromatographic determination of cholesterol and tocopherol in oils and fats with automatic removal of interfering triglyderides. *J.Chromatogr.* 719, 221- 227

22 Kovacs, M.I.P., Anderson, W.E. & Ackman, R.G. 1979. A simple method for the determination of cholesterol and some plant sterols in fishery-based food products. *J.Food Sc*. 44 , 1299 – 1300

23 Al-Hasani, S.M., Shabany, H. & Hlavac, J. 1993. Rapid determination of cholesterol in selected frozen foods. *J.Assoc. Off. .Anal.Chem.* 73(5), 817 – 820

24 Bachman, K.C., Lin, J.H. 7 Wilcox, C.J. 1976. Sensitive colorimetric determination cholesterol in dairy products. *J.Assoc. off.Anal.Chem.* 59(5), 1146-1149 ↵