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On the Colorimetric Method for Cholesterol Determination in the Laboratory Media

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ABSTRACT

This study aimed at the evaluation of sensitivity and reproducibility of the modified o-phthalaldehyde colorimetric method in determination of cholesterol in the *Lactobacillus* specific laboratory media; (de-Man Rogosa Sharpe medium). The results indicated that at the range of 0-100 mg L⁻¹ cholesterol concentration, the quantity of measured cholesterol was affected by the type of cholesterol. Application of the method for water soluble cholesterol yielded less quantity of cholesterol in comparison to the same situation for free alcoholic cholesterol. Also, the method did not present substantial reproducibility. Therefore, cholesterol containing MRS solutions cannot be accurately distinguished using this method.

Key words: Cholesterol, lactobacillus specific medium, o-phthalaldehyde method

INTRODUCTION

In recent years Lactic acid bacteria have drawn attention as a natural means of alleviating hypercholesterolemia (Fazeli *et al.*, 2010; Ahire *et al.*, 2012). Reduction of cholesterol, in the cholesterol added laboratory media is considered as an indication for the selection of bacterial strains with cholesterol assimilation property (Gilliland and Walker, 1990; Lin and Chen, 2000). In this regard, cholesterol measurement in the cholesterol added deMan-Rgosa-Agar (MRS) (Selective media for Lactobacilli) before and after the complete growth of examined lactobacillus strains has been carried out by some researchers as a typical approach (Gilliland and Walker, 1990; Lin and Chen, 2000; Liong and Shah, 2005a). However, respecting the cholesterol experiment and the source of the used cholesterols, there are differences in the methods proposed. A natural source of plasma cholesterol and a colorimetric method based on cholesterol-O-phthalaldehyde reaction was used by some researchers (Gilliland and Walker, 1990; Lin and Chen, 2000). This method had been originally described for the measurement of human blood plasma cholesterol; saponification of plasma lipids by alcoholic KOH followed by extracting unsaponifiable material in hexane, drying the extract under nitrogen and color development using o-phthalaldehyde reagent in acidic media constitute the principles of the given method (Rudel and Morris, 1973). Later, this methodology went through modification by others. Higher proportion of the tested sample was used against the

experimental solutions by Liong and Shah (2005a) and Lin and Chen (2000). In addition, in the modified method, described by Liong and Shah (2005a) utilization of water soluble cholesterol (polyoxyethanyl cholesteryl sebacate) as the source of cholesterol as well as lower incubation temperature are the different points from that of the original method. This method was well referenced by the authors in their later studies (Liong and Shah, 2005b, c) but it was scarcely discussed for reproducibility and precision.

This study aimed to evaluate the efficacy of modified colorimetric method for cholesterol determination using O-phthaldehyde as the reagent for the measurement of polyoxyethanyl cholesteryl sebacate in aqua medium.

MATERIALS AND METHODS

Polyoxyethanyl cholesteryl sebacate (Sigma) and alcohol soluble cholesterol (Panreac-Spain) were used as the tested cholesterol. Several preparations of the standard solutions at the concentrations of 1, 2, 5, 7, 10, 20, 30, 50, 75 and 100 mg L⁻¹ of both types of cholesterol were prepared in double distilled water or pure ethanol (Merck). Standard solutions were also prepared for water soluble cholesterol in sterilized MRS (Merck). The concentration of standard solutions was chosen based on the reported cholesterol reducing capability of lactobacilli when they grow in 70 to 100 mg L⁻¹ containing media. Each standard solution underwent the whole procedure of the experiment. A solution of o-phthaldehyde (Sigma) 0.5 mg mL⁻¹ in glacial acetic acid was used as the reagent (Rudel and Morris, 1973). The procedure described before was followed for determination of cholesterol (Liong and Shah, 2005b); one milliliter of the tested solution was added with 1 mL of 33% w/v potassium hydroxide and 2 mL of absolute ethanol, mixed for 1 min and incubated at 37°C for 15 min. After cooling, 2 mL of distilled water and 3 mL of hexane layer were added and mixed for 15 min one milliliter of hexane layer was removed and transferred into a tube and evaporated under nitrogen. The dried material was dissolved in 2 mL of o-phthalaldehyded reagent. After complete mixing, 0.5 mL of sulfuric acid (12 N, Merck), was added and the mixture was mixed for 1 min. After 10 min, absorbance was read at 550 nm (Genway-model 6800). For the evaluation of reproducibility of the method, Inter assay coefficient of variation (Cvs) for the measurement of polyoxyethanyl cholesteryl sebacate in distilled water and MRS during 6 consequent days (n = 3×4) was determined. Intra assay Cv for the measurement of 50 mg L⁻¹ polyoxyethanyl cholesteryl sebacate was investigated by ten replicates of the measurement through (n = 10×1) a day. As the cholesterol is susceptible to oxidation the determined concentrations were prepared freshly on the day of use o-phthaldehyde was prepared freshly before spectrophotometric measurement. Minitab 16 was used for statistical analysis.

RESULTS

The standard curves of alcoholic cholesterol and water soluble cholesterol are shown in Fig. 1. The curves were different for the tested cholesterols. Equation "a" and Equation "b" were depicted from the calibration curves using water soluble and alcohol soluble cholesterols, respectively.

- Water soluble cholesterol(mg L⁻¹) = [absorbance (_{550nm})- 0.00152]/0.000772
- Free alcohol soluble cholesterol (mg L⁻¹) = [absorbance (_{550nm})-0.0065]/0.00115

Standard curves of water soluble cholesterol in distilled water and MRS have been presented in Fig. 2. Fitted lines of standard curves were set as R² = 98.7 and R² = 98 for distilled water and

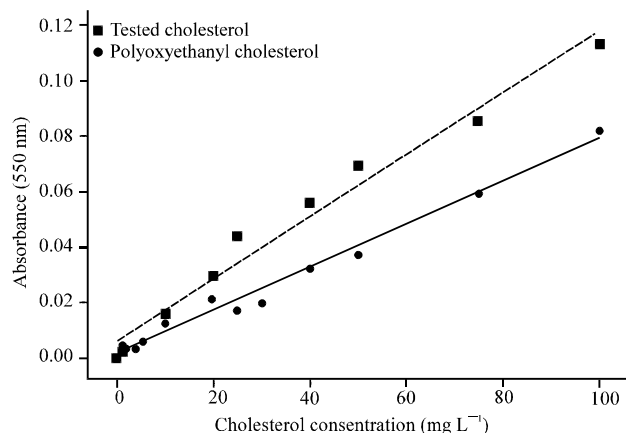


Fig. 1: Comparison of typical standard curves for tested cholesterol: solutions of cholesterol in ethanol ($R^2 = 97$) and of polyoxyethanyl cholesteryl sebacate in distilled water ($R^2 = 98.7$) examined by colorimetric method. Each data presented as the mean of 6 replicates obtained from 2 independent experiment runs

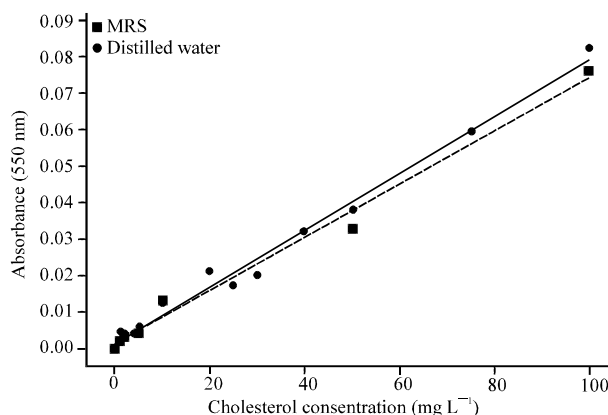


Fig. 2: Comparison of typical standard curves for polyoxyethanyl cholesteryl sebacate in MRS and in distilled water examined by colorimetric method

Table 1: Reproducibility of the method for determination of water soluble cholesterol in MRS and distilled water for 6 consequent days

Adjusted concentration (ppm)	Mean measured cholesterol (ppm)	SE mean	StDev	Variance	% Coefficient of variation
10	14.31**	3.40	9.79	95.81	68.40
	16.03	4.21	11.13	123.83	69.44
50	46.31	4.83	16.01	256.19	34.56
	43.25	4.14	16.08	171.00	37.24
100	104.10	5.31	16.79	281.96	16.13
	102.81	7.45	21.00	443.77	20.49

Absorbance measured in 1-cm (sample-light path) at 550 nm; **Measured cholesterol in distilled water; ***Measured cholesterol in MRS

MRS, respectively. Standard deviation and coefficient of variation values obtained from the repeated measurements were higher in MRS from that of distilled water during 6 consequent days (Table 1). As shown in the Table 1, for the tested concentrations, the difference in measured

cholesterol, resulted from the repeated measurements was about 10-20 mg L⁻¹. Intra assay Cv for 10 replicates of measurements of 50 mg L⁻¹ water soluble cholesterol in distilled water and MRS were obtained as 22.08 and 11.21%, respectively which was close to Inter assay Cv values. The mean percentage recovery of water soluble cholesterol was obtained as 83%.

DISCUSSION

According to Fig. 1, at the range of tested concentrations, this method yielded better results for free alcoholic cholesterol than water soluble cholesterol. Such difference can result in up to 40 mg L⁻¹ more cholesterol concentration, if water soluble cholesterol be used for preparation of standard curve. Esterified residue in polyoxyethanyl cholesteryl sebacate may restrict the extraction procedure and reduce the cholesterol concentration in the spectrophotometric sample. However, in terms of using water soluble cholesterol in the laboratory media, selection of bacterial strains with cholesterol assimilation properties could be feasible because such evaluation is designed based on the relative reduction of cholesterol by microorganisms. In the original method, cholesterol in alcoholic solutions were used for preparation of standard curve at extremely higher concentrations (Rudel and Morris, 1973). In another study, cholesteryl hemisuccinate as a type of water soluble cholesterol was compared against free alcoholic cholesterol as the standard cholesterol. It was shown that water soluble cholesterol can be detected identical to free alcoholic cholesterol examined through both Killiani-Zak and Libermann-Burchard methods (Klein *et al.*, 1974). However, tested cholesterol solutions in that study were also prepared at higher concentrations than that used in the present study. We used lower cholesterol standard solution because studies in which such modified colorimetric methods were used for assessing cholesterol reducing potential of lactobacilli, reported about 10 to 100 mg L⁻¹ cholesterol reduction in their experiments.

Based on data represented in Fig. 2, standard curves of water soluble cholesterol in distilled water and MRS are suitably overlaid, showing that at the used standard concentrations, recovered cholesterol values from MRS can be representative of their solutions in distilled water. However, standard deviation and coefficient of variation values obtained from the repeated measurements were higher in MRS than distilled water (Table 1). It can be inferred that using this method, the difference between bacterial strains with cholesterol assimilation potential in this range, is not distinguishable.

It should be mentioned that when MRS (free of cholesterol) was tested as the blank, noticeable absorbance (0.4 at 550 nm) was observed. Instead, the absorbance of blank samples containing distilled water or alcohol was less than 0.03. This could be resulted from hydrophobic amino acids and peptides, extracted as un saponifiable material, participating in colorimetric reaction by o-phthalaldehyde in acid media. This might introduce a source of error and decrease the reliability of the method.

CONCLUSION

Overall, the results of cholesterol measurement using modified colorimetric method are affected by utilization of polyoxyethanyl cholesteryl sebacate as the control cholesterol. Also, the method does not yield substantial precision when the difference in cholesterol concentrations among the samples is lower than about 10-20 mg L⁻¹. Therefore, when investigation of cholesterol reduction ability of lactic acid bacteria in MRS is of concerned, this method dose not presents an accurate discrimination between bacterial strains.

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REFERENCES

- Ahire, J.J., A.A. Bhat, J.M. Thakare, P.B. Pawar, D.G. Zope, R.M. Jain and B.L. Chaudhari, 2012. Cholesterol assimilation and biotransformation by *Lactobacillus helveticus*. Biotechnol. Lett., 34: 103-107.
- Fazeli, H., J. Moshtaghian, M. Mirlohi and M. Shirzadi, 2010. Reduction in serum lipid parameters by incorporation of a native strain of *Lactobacillus plantarum* A7 in mice. Iran. J. Diabetes Lipid Disorders, 9: 1-7.
- Gilliland, S.E. and D.K. Walker, 1990. Factors to consider when selecting a culture of *Lactobacillus acidophilus* as a dietary adjunct to produce a hypocholesterolemic effect in humans. J. Dairy Sci., 73: 905-911.
- Klein, B., N.B. Kleinman and J.A. Foreman, 1974. Preparation and evaluation of a water-soluble cholesterol standard. Clin. Chem., 20: 482-485.
- Lin, M.Y. and T.W. Chen, 2000. Reduction of cholesterol by *Lactobacillus acidophilus* in culture broth. J. Food Drug Anal., 8: 97-102.
- Liong, M.T. and N.P. Shah, 2005a. Acid and bile tolerance and cholesterol removal ability of *Lactobacillus* strains. J. Dairy Sci., 88: 55-66.
- Liong, M.T. and N.P. Shah, 2005b. Optimization of cholesterol removal, growth and fermentation patterns of *Lactobacillus acidophilus* ATCC 4962 in the presence of mannitol, fructooligosaccharide and inulin: A response surface methodology approach. J. Applied Microbiol., 98: 1115-1126.
- Liong, M.T. and N.P. Shah, 2005c. Production of organic acids from fermentation of mannitol, fructooligosaccharide and inulin by a cholesterol removing *Lactobacillus acidophilus* strain. J. Applied Microbiol., 99: 783-793.
- Rudel, L.L. and M.D. Morris, 1973. Determination of cholesterol using ophthalaldehyde. J. Lipid Res., 14: 364-366.