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Microwave-Based Biosensor for Determination of Cholesterol in Food

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A new method for determination of cholesterol in food has been developed based on dielectric properties of the cholesterol in food after enzymatic reaction at microwave frequencies in the range of 0.2-20 GHz. The dielectric properties of cholesterol solution, enzyme and cholesterol-enzyme reaction were measured using the open ended coaxial probe coupled with computer controlled software automated network analyzer at room temperature (25 °C). At frequency 4.98 and 10.9 GHz the dielectric loss difference was obtain by subtracting the dielectric after the reaction with dielectric of cholesterol oxidase alone at 1:2 ratios. The relative standard deviation of the reproducibility was calculated to be 1.85 % for frequency 4.98 GHz and 2.06 % for frequency 10.9 GHz at cholesterol concentration of 38.67 ppm. The limit of detection was calculated to be 2.48 ppm at frequency 4.98 GHz and 3.53 ppm at frequency 10.9 GHz. Result obtained from the developed method was compared with the standard method of cholesterol determination (BOHAC extraction-HPLC). The comparison result shows an excellent agreement between the developed method and standard method. This indicates the results obtained from both methods are comparable. The good linear correlations, suggest that microwave dielectric properties could be used in developing sensors to determine cholesterol in food.

Key Words: Cholesterol biosensor, Dielectric properties, Microwaves.

INTRODUCTION

Use of microwaves on food analysis is attractive because of the rapidity of treatment and/or measurement, ease of use and the fact that it can potentially be used on foods in optically opaque sealed containers. Many papers reporting investigations of the use of microwaves in characterizing the quality of various foods, including measurement of salt content, detection of bacteria in milk, milk's content in terms of groups of materials such as ionic compounds, fats and carbohydrates and proteins[1]. Cholesterol is a sterol with the molecular formula, $C_{27}H_{46}O$. Accumulations of cholesterol in the arterial wall results in narrowing of the arteries and increase the risk of coronary heart disease. Analysis of cholesterol in foods is important item performed in laboratory as the awareness over the effects of dietary cholesterol on heart disease and the obligatory nutritional labeling in the United States, thus led to the need for an accurate and efficient cholesterol determination technique. Biosensors have attracted great deal of interest due to their potential applications such in clinical diagnostics, food industries, environmental control and bioprocess monitoring[2]. Other methods of enzyme-based cholesterol biosensor were spectrophotometry, fluorometry[3], electrochemical method[4], chemiluminescence[5], electrochemiluminescence and quartz crystal acoustic wave[6]. Enzymatic reaction of cholesterol and cholesterol oxidase is as shown below:

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol Oxidase}} 4-\text{Cholesten-3-one} + H_2O_2$$

Microwave biosensor has not been studied as much as electrochemical biosensor which is electrode based. During past decade, microwave technology has become a well-established analytical tool. The microwave sensing method has recently gained considerable interest because it provides sensitive, simple and an expensive detection. Microwave sensing method has been widely used in agriculture and food industry. The successful use of microwave usually associated with the dielectric properties of the material.

Among the commonly used measurement methods for dielectric properties, the transmission line technique was reported to be most suitable for porous materials. The open-ended coaxial probe method associated with network analyzers or impedance analyzers is a useful technique to determine dielectric properties, *i.e.*, dielectric constant, dielectric loss factor and loss tangent, of materials, especially for liquid foods. The advantages of this method are easy to use, have a large bandwidth and has been commonly used within the food research group[1,7-9].

Most studies on the dielectric properties of foods has been carried out on solid foods such as meat, fruits, cheese and many more. For liquid foods, microwave heating rates have been reported for soy sauce. However, there is little information available on dielectric properties of liquid foods[8]. Up to now, minimal work has been done to study the dielectric changes as the mean to detect the present of certain ionic species for biosensor development.

EXPERIMENTAL

Cholest-5-en-3-ol (cholesterol) with purity of > 99 % (R & M) and cholesterol oxidase from *Brevibacterium* sp., > 50 units/mg protein (Sigma-Aldrich) were used without further purification. Ethanol, sodium phosphate monobasic and sodium hydroxide were of analytical grade obtained from R&M. Deionized distilled water was used throughout the experiments.

Network analyzer (Hewlett Packard 85070B, USA) with open ended coaxial probe (Agilent Technology, USA) was used to determine the dielectric properties of these materials. All measurements were made at room temperature (25 ºC). Care was taken to eliminate bubbles from the probe surface. The probe was washed with deionized distilled water and cleaned between each measurement. The testing probe was calibrated using a Hewlett-Packard 85070B dielectric probe kit which includes an open circuit (air) and a short circuit (gold-plated precision shorting block). Dielectric Probe Kit software (Model 85070A, Hewlett Packard, USA) was used to calculate the dielectric properties of the samples.

Dielectric properties measurement of related reagents: Dielectric properties each of reagents involved in this study were analyzed at room temperature between 0.2-20 GHz frequencies. The reagents were cholesterol, cholesterol oxidase, 4-cholestene-3-one, hydrogen peroxide and cholesterol-enzyme solution. 10 mL of each solutions were placed in 10 mL beaker, placed on a platform and then was raised up until the downward open-ended coaxial-line probe was completely immersed in the sample up to a depth of 30 mm[10]. Dielectric measurement was done when there were no air bubble between the probe and samples. When no bubble was observed between the probe and sample, dielectric property measurements at microwave frequencies were performed in 1 min. Three replicates were made on each sample. The probe was washed with water and wiped dry after each experiment.

Dielectric measurement of evaporated milk, low fat milk and full cream milk: The diluted solution of real samples is reacted with cholesterol oxidase (ratio 1:2) to analyse the product after the reaction. The data was measured by using the same network analyzer. The same samples were also analyzed by using HPLC method and the results were compared.

RESULTS AND DISCUSSION

Dielectric properties of the related reagents: Generally, ionic conductivity is a major contributing factor to the dielectric loss of liquid sample which occur at low frequency whereas polarity occurs when there are lone pair electrons within the molecule. Dipole orientation occurs at high frequencies. Oxygen unpaired electron in hydroxyl group in cholesterol molecule (Fig. 1) cause the cholesterol molecule to be polar. From theoretical molecular dynamic simulations, the hydroxyl group of cholesterol adds a contribution to the dipole potential[11]. Fig. 2a-b shows the dielectric properties of cholesterol measured by the network analyzer.

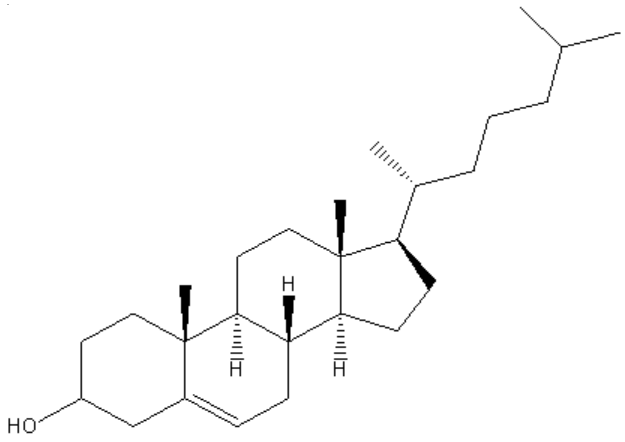
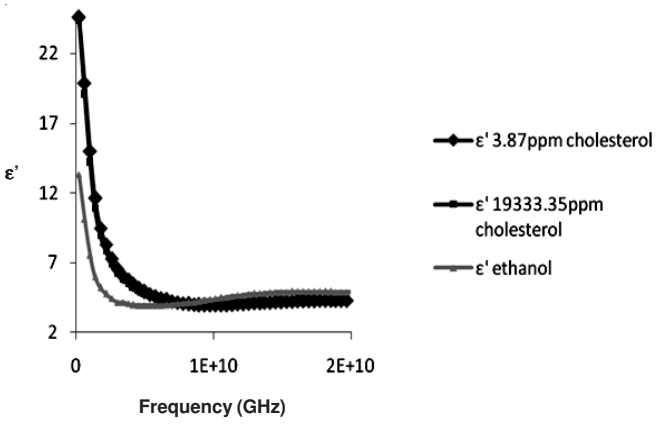
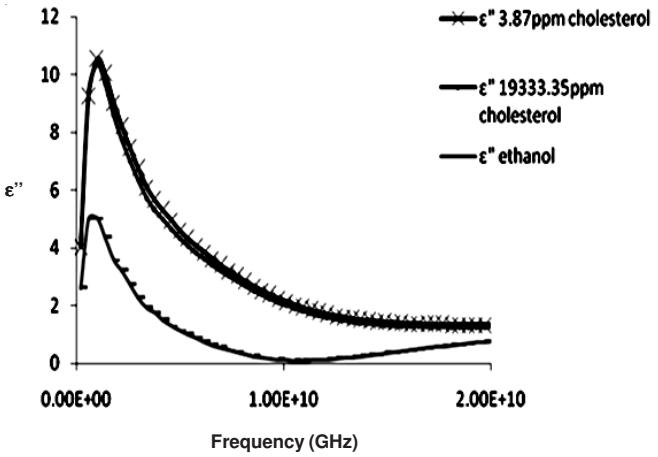


Fig. 1. Structure of cholesterol



(a)



(b)

Fig. 2. (a) dielectric constant, ε' and (b) dielectric loss, ε" of cholesterol and ethanol at frequency from 0.2-20 GHz at room temperature

Rao and Rao[12] reported that higher concentration of cholesterol will produce higher value of dielectric constant. However, from this study, both dielectric constant, ε' and

dielectric loss, ε" shows no significant different in each cholesterol concentration. This phenomenon may be due to dissolution of cholesterol in ethanol.

Fig. 3 presents the dielectric spectra of cholesterol oxidase. The dielectric constant, ε', decreases with frequencies whiles the dielectric loss, ε", can be divided into two regions *i.e.*, below 2.5 GHz and above 2.5 GHz. Ionic polarization usually dominates at frequency below 2.5 GHz. Dipole orientation of polar molecules in cholesterol oxidase solution dominates in the higher frequency (> 2.5 GHz). The high value of ε" at this frequency is mainly due to the large amount of polar species in cholesterol oxidase. These trends are the same with deionized water mainly because cholesterol oxidase was prepared by dissolution in deionized water. Mertz and Krishtalik[13] reported that water strongly affects the dielectric reorganization in the active site of the enzyme in solution. Since the cholesterol oxidase is surrounded with deionized water which is highly polarize aqueous, it may contribute to the dielectric response. Some parameter of water also controlled cholesterol oxidase activity[14]. Therefore, dielectric properties of cholesterol oxidase and water are almost the same.

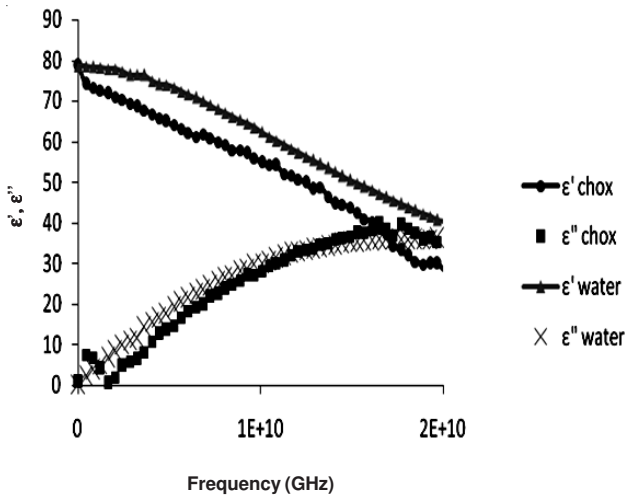


Fig. 3. Dielectric properties of cholesterol oxidase (chox) and water at frequencies 0.2-20 GHz at room temperature

Fig. 4 presents the dielectric properties of 10 ppm 4-cholestene-3-one. Dielectric constant, ε' decrease with increasing of frequency while dielectric loss, ε" increase with frequency up to 2.5 GHz and decrease at higher frequency. As shown in Fig. 5, the combination of carbons and hydrogen as in the hydrocarbon portion of a molecule is always non polar but because of the greater electronegativity of oxygen, the carbonyl group is polar. Effect of ionic portion of 4-cholestene-3-one can be seen at low frequency (below 2.5 GHz) whereas the effect of polar molecule can be seen at high frequency (above 2.5 GHz).

Hydrogen peroxide molecule is one of the polar molecules that are proximately alike to the H_2O molecule. The dielectric properties (Fig. 6) of hydrogen peroxide confirmed it is polar molecule and the dielectric constant is less than that of water at all temperatures[15]. Dielectric loss of H_2O_2 is increase with increasing of frequency. It can be observed that as a polar molecule, the dielectric loss is greater at high frequency.

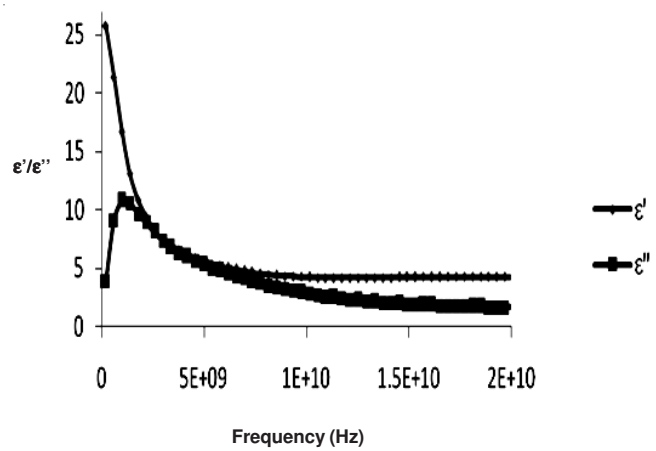


Fig. 4. Dielectric properties of 4-cholestene-3-one at frequencies 0.2-20 GHz at room temperature

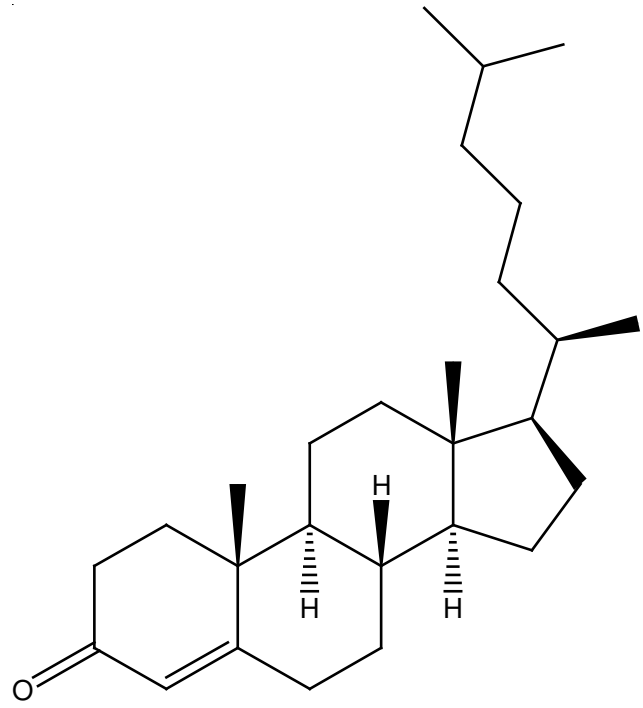


Fig. 5. Structure of 4-cholestene-3-one

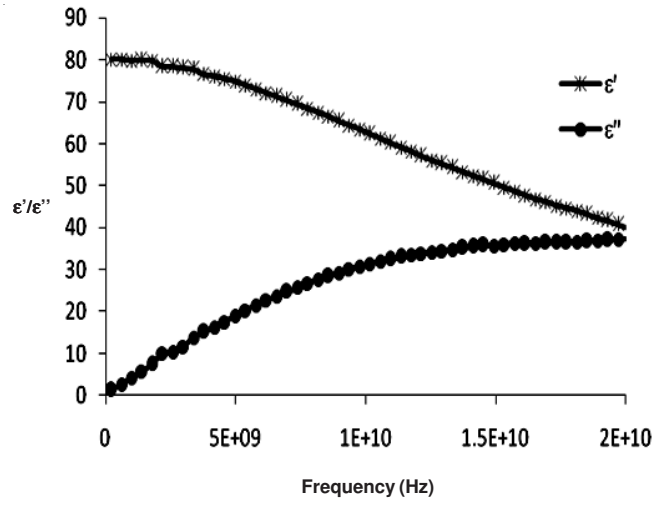


Fig. 6. Dielectric properties of hydrogen peroxide at frequencies 0.2-20 GHz at room temperature

Ratio study of cholesterol-enzymatic reaction: The dielectric properties of the enzymatic reaction (dielectric difference between product after reaction and cholesterol oxidase or difference between product after reaction and before reaction) were studied to get the best frequency and the optimum ratio between cholesterol and cholesterol oxidase for detection of ionic conduction and dipole orientation based on the differences of dielectric changes.

In this process, cholesterol is reacted with cholesterol oxidase in room temperature and the product of the reaction is 4-cholestene-3-one and hydrogen peroxide (H_2O_2). The enzymatic reaction is as the following:

$$\text{Cholesterol} + O_2 \xrightarrow{\text{ChOx}} 4\text{-Cholestene-3-one} + H_2O_2$$

In order to identify the best ratio of the enzymatic reaction, 4 ratios of enzyme (cholesterol oxidase): cholesterol was studied. The dielectric loss, ε" before and after reaction of ratio 1:1 (Fig. 7a), ratio 1:2 (Fig. 7b), ratio 2:1 (Fig. 7c) and ratio 3:2 (Fig. 7d) are shown as below. The entire graph show that after reaction with cholesterol oxidase, dielectric loss, ε" increase compared to dielectric loss, ε" before reaction but ratio 1:2 show the best resolution which is concentration of cholesterol inversely proportional with dielectric loss, ε". Three other ratios (which contain lots of cholesterol oxidase compared to cholesterol), the dielectric loss, ε" of each cholesterol are almost overlapping to each other and at low frequency (below 0.6 GHz) effect of ionic conductivity from excess of cholesterol oxidase can be observed. Effect of ionic conductivity which can be seen at frequencies below 2 GHz and dipole (H_2O_2) effect at frequencies above 10 GHz can been seen at all ratio.

Frequency study of cholesterol-enzymatic reaction: The frequency study was carried out for the product from the cholesterol enzymatic reaction at ratio 1:2 to identify the best frequency for detection of cholesterol using this system. The frequency study was divided into two regions, low frequency with range of 0.2-10 GHz and high frequency between 10 GHz and above.

The dielectric difference obtained by subtracting the dielectric after the reaction with dielectric of cholesterol alone and dielectric of cholesterol oxidase alone. Fig. 8a shows the relationship between dielectric difference and concentration of cholesterol at low frequency, whereas Fig. 8b shows the relationship between dielectric difference and concentration of cholesterol at high frequency. It can be concluded from these results that a proportional relationship between concentration of cholesterol and the dielectric loss difference, Δε" was observed based on subtraction with cholesterol oxidase alone.

From this observation, it can be deduced that at frequency 4.98 and 10.9 GHz the dielectric loss difference obtained by subtracting the dielectric after the reaction with dielectric of cholesterol oxidase alone at 1:2 ratio are the best experimental condition for the detection of cholesterol using this system.

Dynamic range: Fig. 9 illustrates the relationship between dielectric loss differences, Δε" and concentration of cholesterol for frequency 4.98 and 10.9 GHz for ionic conductivity and dipole orientation. The standard curve shows that the dielectric loss difference, Δε" increase proportionally with the concentration of cholesterol. The straight line obtained can be described by equation $y = 0.698 \ln(x) - 1.244$ and the correlation coefficient, R^2 was calculated to be 0.92 for frequency 4.98 GHz, where as for frequency 10.9 GHz the equation is $y = 0.491 \ln(x) + 19.47$ and the correlation coefficient, R^2 was calculated to be 0.927. The limit of detection was calculated to be 2.484 ppm at frequency 4.98 GHz and 3.531 ppm at frequency 10.9 GHz. LOD calculated in this work is better compared to Matharu *et al.*[16], which is 64.18 ppm but a bit high compared to Afsaneh and Fatemeh[17] which is 0.39 ppm. The high value for the correlation coefficient demonstrates good correlation between dielectric loss differences, Δε" and cholesterol concentration.

Reproducibility study: Fig. 10 shows the graph of reproducibility study of the product from enzymatic reaction. It indicated that microwave method is reproducible when used for measurement of cholesterol at concentration of 38.67 ppm. The dispersion of the distribution of test results (relative standard deviation) was calculated to be 2.06 % at frequency 10.9 GHz and 1.85 % at 4.98 GHz.

Application of the developed method on food sample: Samples used in this study are evaporated milk, fresh milk and full cream milk. Application of the developed method on real sample analysis is divided into two parts. The first part of the study was carried out based on extracted samples using BOHAC extraction procedure. The second part of the study was carried out without extraction procedure. The dielectrics of the samples were measured at frequency 10.9 and 4.98 GHz. The concentration of cholesterol in the food samples were calculated using calibration plot constructed in Fig. 9. This technique was validated using HPLC method based on extracted samples by using BOHAC extraction method. The results are presented in Table-1.

Based on the Table-1, the standard deviation for detection of cholesterol with the developed microwave method without extraction is high which is due to interference of other compound such as protein, calcium, water and carbohydrate in the milk that effect the dielectric loss[1]. Whereas detection of cholesterol with the developed microwave method with extraction procedure at frequency 4.98 GHz has shown a good agreement with the standard HPLC method (supported by *t*-test). This proves that the developed microwave method can be a low cost alternative way in measurement of cholesterol concentration.

Conclusion

In this study, a new microwave method for cholesterol measurement in food is developed based on cholesterol enzymatic reaction. This new method is based on dielectric changes measured by the open ended coaxial probe. The correlation between dielectric changes, frequency and cholesterol were successfully manipulated in order to develop microwave based cholesterol biosensor.

ACKNOWLEDGEMENTS

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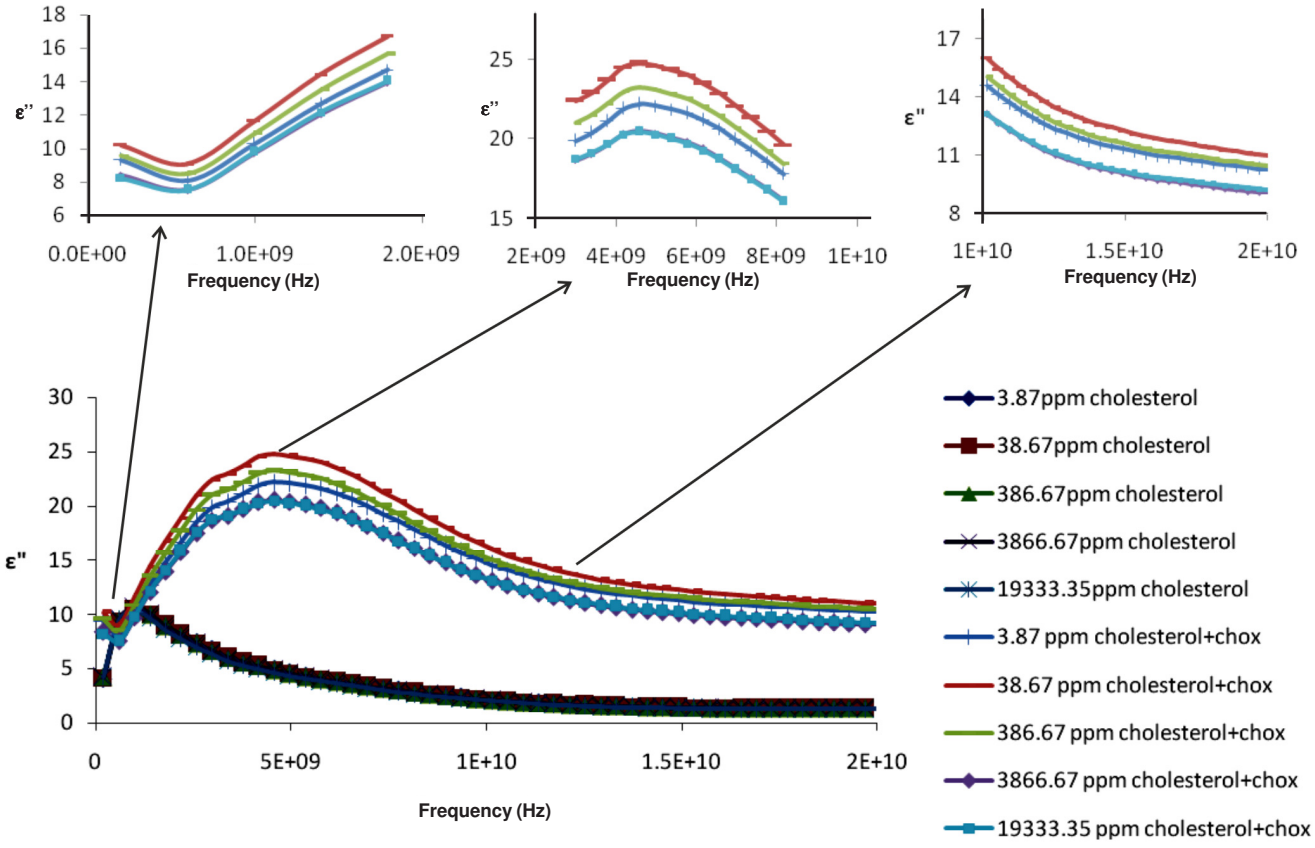
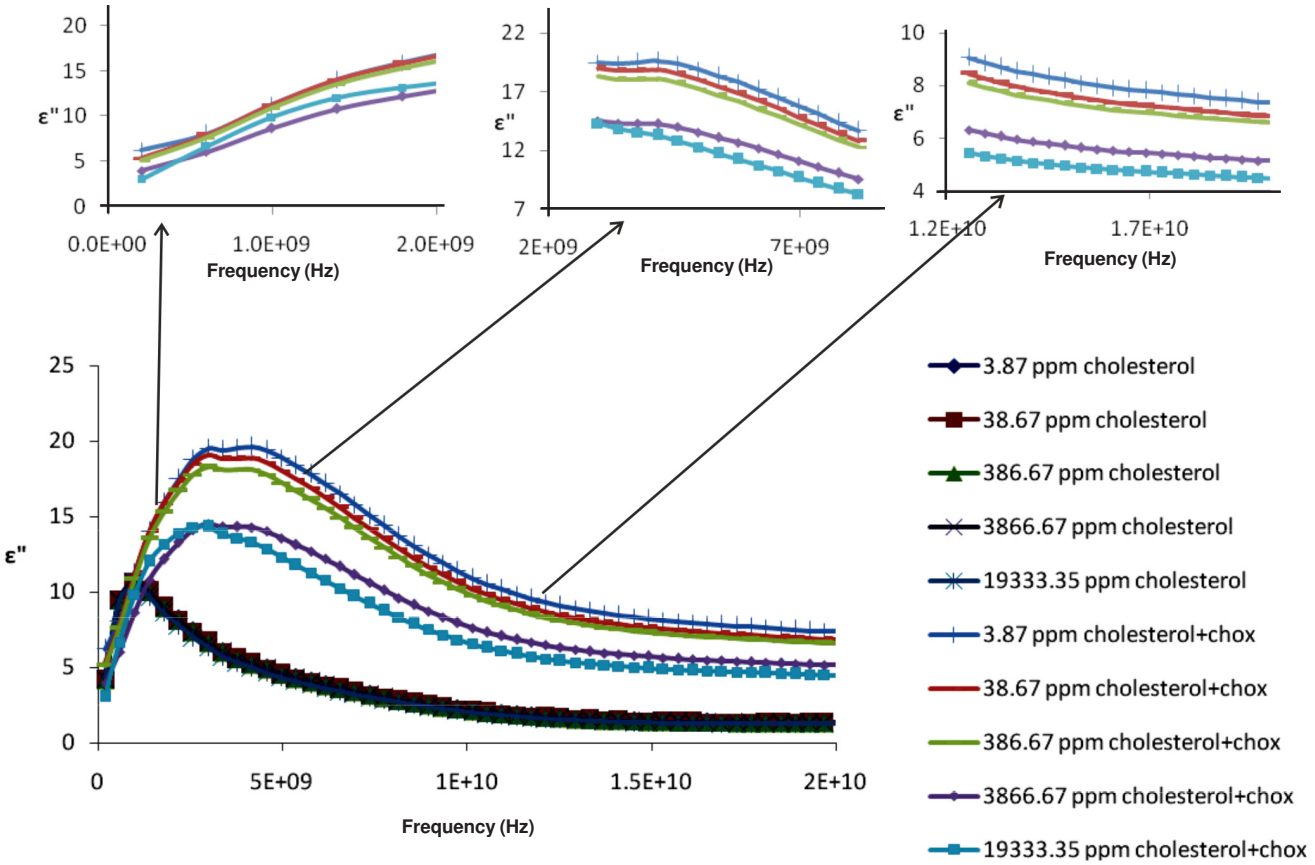

ε"
Frequency (Hz)
3.87ppm cholesterol
38.67ppm cholesterol
386.67ppm cholesterol
3866.67ppm cholesterol
19333.35ppm cholesterol
3.87 ppm cholesterol+chox
38.67 ppm cholesterol+chox
386.67 ppm cholesterol+chox
3866.67 ppm cholesterol+chox
19333.35 ppm cholesterol+chox

(a)


ε"
Frequency (Hz)
3.87 ppm cholesterol
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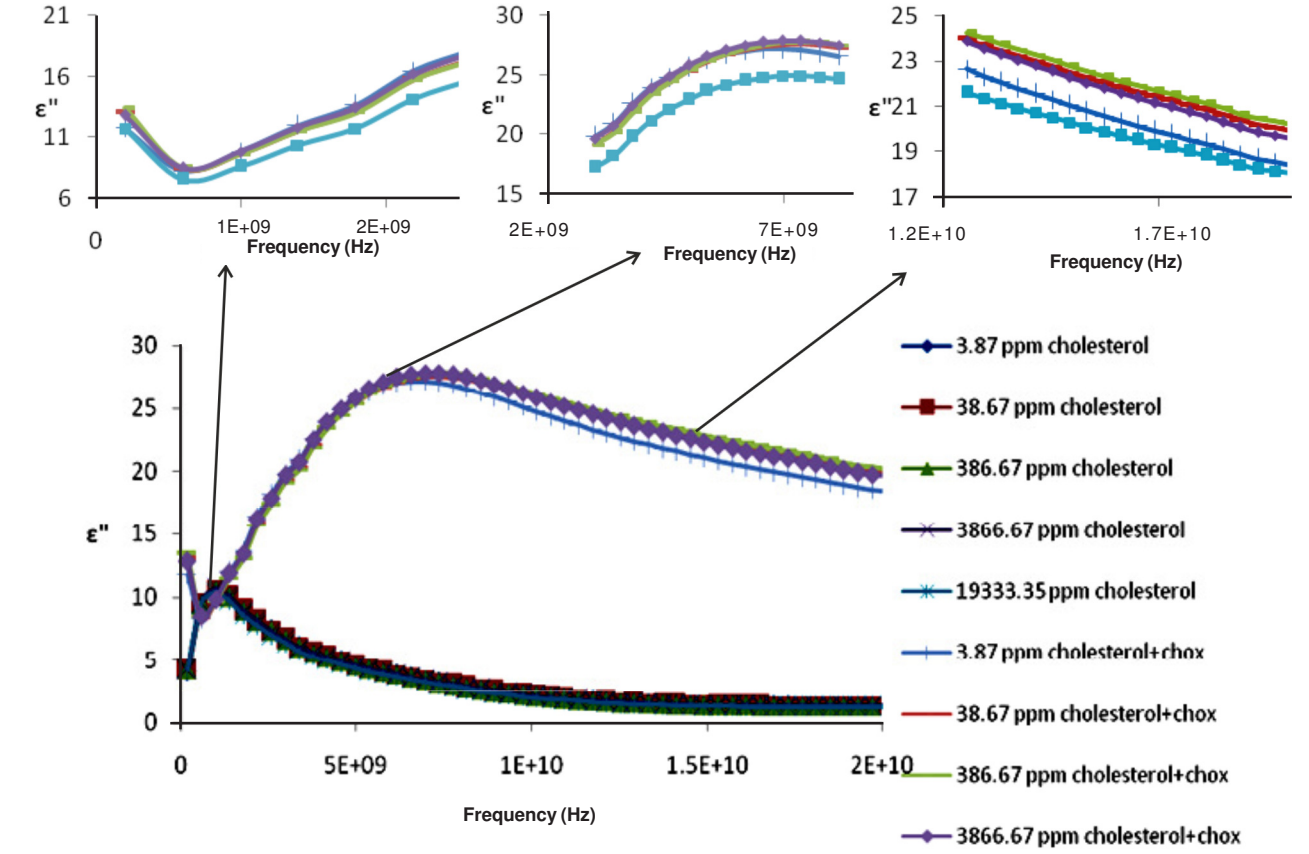
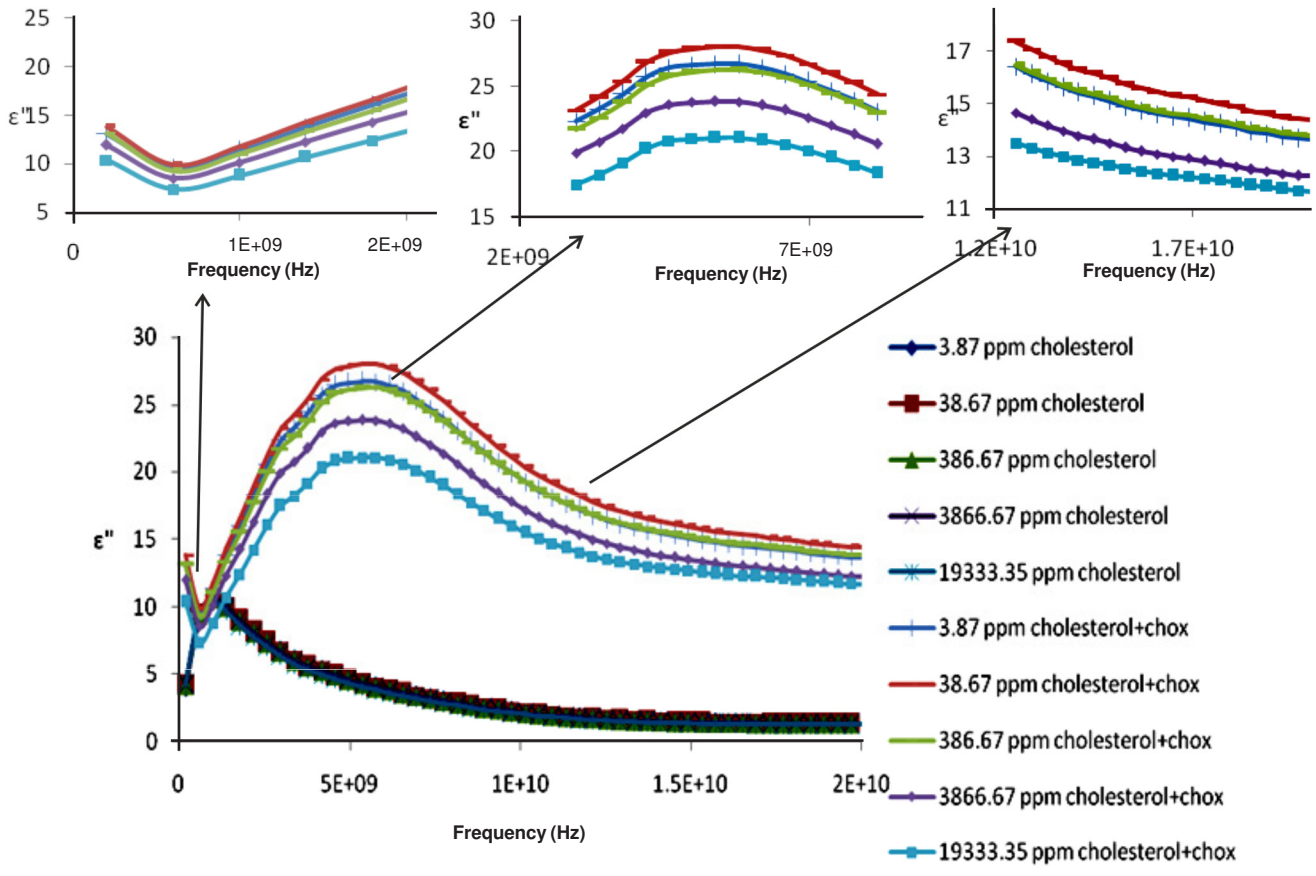
3866.67 ppm cholesterol
19333.35 ppm cholesterol
3.87 ppm cholesterol+chox
38.67 ppm cholesterol+chox
386.67 ppm cholesterol+chox
3866.67 ppm cholesterol+chox
19333.35 ppm cholesterol+chox

(b)



(c)

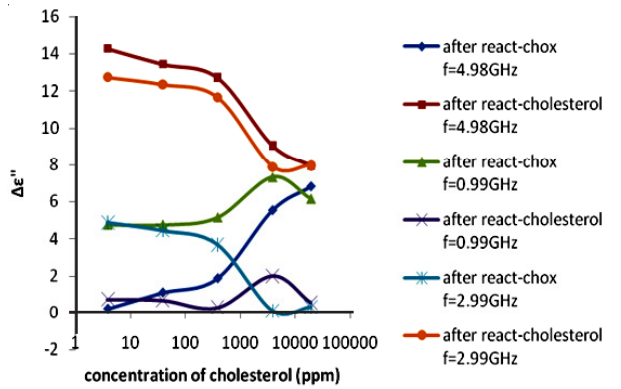


(d)

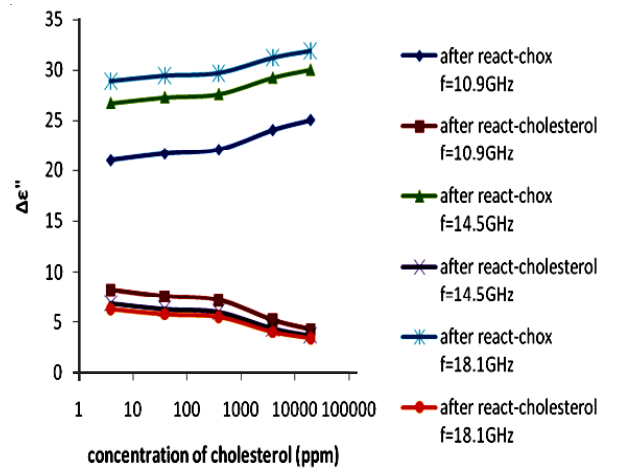
Fig. 7. Dielectric loss loss, e" at various concentration of cholesterol and cholesterol-enzyme at (a) 1:1 (b) 1:2 (c) 2.1 (d) 3:2 ratio

TABLE-1
CHOLESTEROL CONTENT IN FOOD SAMPLES BY HPLC METHOD AND MICROWAVE METHOD

Type of milk	Cholesterol conc. termined by HPLC (ppm)	Cholesterol concentration determined by microwave method without extraction (ppm)		Cholesterol concentration determined by microwave method with extraction (ppm)	
		4.98 GHz	10.9 GHz	4.98 GHz	10.9 GHz
Full cream milk	59.30 ± 5.74	0.02 ± 0.11	$5.23 \times 10^{13} \pm 1.84 \times 10^{12}$	40.96 ± 1.25	63.33 ± 5.74
Evaporated milk	33.80 ± 15.68	$7.3 \times 10^{-4} \pm 0.0001$	$5.97 \times 10^{16} \pm 4.22 \times 10^{16}$	43.74 ± 2.0	14.65 ± 5.71
Fresh milk	75.40 ± 16.75	$3.87 \times 10^{-5} \pm 5.6 \times 10^{-6}$	$4.66 \times 10^{13} \pm 4.74 \times 10^{12}$	81.22 ± 4.3	35.30 ± 2.8



(a)



(b)

Fig. 8. Dielectric difference Δε" at ratio 1:2, (a) low frequency (b) high frequency

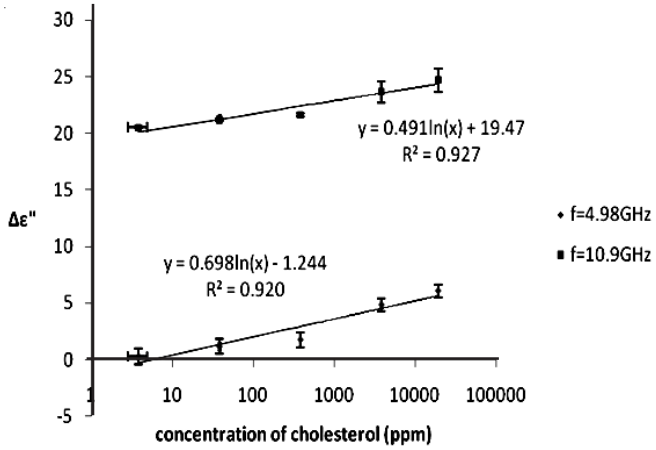


Fig. 9. Response curve of different concentration of cholesterol for frequency 4.98 and 10.9 GHz

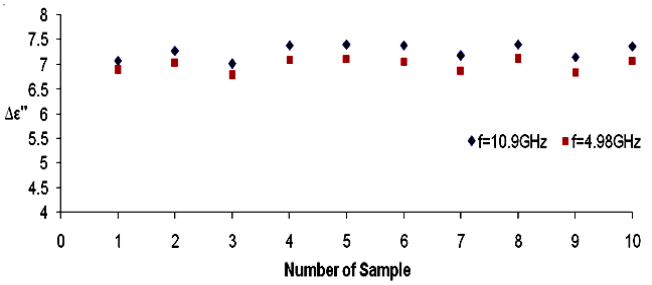


Fig. 10. Reproducibility study at concentration of 38.67 ppm cholesterol at frequency 4.98 GHz and 10.9 GHz

REFERENCES

1. A.C. Nunes, X. Bohigas and J. Tejada, *J. Food Eng.*, **76**, 250 (2006).
2. C. Dhand, S.P. Singh, K.A. Sunil, M. Datta and B.D. Malhotra, *Anal. Chim. Acta*, **602**, 244 (2007).
3. D.M. Amundson and A. Zhou, *J. Biochem. Biophys. Methods*, **38**, 43 (1999).
4. A.K. Basu, P. Chattopadhyay, U. Roychoudhuri and R. Chakraborty, *Bioelectrochemistry*, **70**, 375 (2007).
5. Y. Hung, C. Zhang and Z. Zhang, *Anal. Sci.*, **15**, 867 (1999).
6. W.C. Shih, M.C. Yang and M.S. Lin, *Biosens. Bioelectron.*, **24**, 1679 (2009).
7. A. Garcia, J.L. Torres, M. De Blas, A. De Francisco and R. Illanes, *Biosys. Eng.*, **88**, 343 (2004).
8. F. Tanaka, K. Morita, P. Mallikarjunan, Y.C. Hung and G.O.I. Ezeike, *J. Food Eng.*, **71**, 92 (2005).
9. Y. Liu, J. Tang and M. Zhihuai, *J. Food Eng.*, **93**, 72 (2009).
10. W. Guo, X. Zhu, H. Liu, R. Yue and S. Wang, *J. Food Eng.*, **99**, 344 (2010).
11. S.W. Chiu, E. Jakobsson and H.L. Scott, *J. Chem. Phys.*, **114**, 5435 (2001).
12. V.M. Rao and B.P. Rao, Role of Microwaves in the Measurement of Blood Parameters, The Journal of the Institution of Engineers (India) Electronics and Telecommunication Engineering Journal, **86**, 61 (2006).
13. E.L. Mertz and L.I. Krishtalik, *Proc. Natl. Acad. Sci. USA*, **97**, 2081 (2000).
14. E.T. Mc Guiness, H.D. Brown, S.K. Chattopadhyay and F. Chen, *Biochim. Biophys. Acta*, **530**, 247 (1978).
15. A.K. Lyashchenko, V.S. Goncharov and P.S. Yastremskii, *J. Struct. Chem.*, **17**, 871 (1950).
16. Z. Matharu, S.K. Arya, S.P. Singh, V. Gupta and B.D. Malhotra, *Anal. Chim. Acta*, **634**, 243 (2009).
17. A, Safavi and F. Fatemeh, *Biosens. Bioelectron.*, **26**, 2547 (2011).