Determination of Free Sterol Content of various Brands of Eggs, Ghee/Oils, using Immobilized Enzyme Column in a Flow System

[1]M. ANWAR AND [2]M. MASOOM-YASINZAI
[1]*Department of Chemistry, University of Balochistan, Quetta, Pakistan*
[2]*Institute of Biochemistry, University of Balochistan, Quetta, Pakistan*

(Received 6th July, 1995, revised 25th November, 1996)

Summary: A flow system containing immobilized cholesterol oxidase column is applied to the analysis of various brands of eggs, ghee, oil etc., for the free sterol content. Eggs of domestic hens have significantly low content of free cholesterol as compared to cholesterol level in farmy eggs. The total sterol content of the ghee is lower as compared to oils, probably because of the loss of sterol during bleaching, blending and refining. The sterol content varies widely among different oils/ghee.

Introduction

Beta sitosterol, stigmasterol and cholesterol have got some importance relating to their dietary values. The latter is the precursor for some steroidal hormones. It can also serve the purpose of insulating cover over the structures through which nerve impulses are conveyed. But, if it exceeds the physiological limits then it may cause human cardiac diseases [1]. Our aim in this study is to report the sterol content in various brands of eggs and oils/ghee; the common food products of daily usage.

High performance liquid chromatography, Gas chromatography [2,3] and enzymatic methods [4], are used for the quantitation of free sterol in particular cholesterol. We have used immobilized cholesterol oxidase mini-reactor column in a flow system for the determination of free sterol content in these samples.

Results and Discussion

A calibration graph for cholesterol plotted using cholesterol standards covering the range 0 - 0.25 mg/ml. This calibration graph was then used for the quantitation of sterol content of samples analysed. For comparing the oxidation of various sterols by cholesterol oxidase, a calibration or cholesterol, sitosterol, stigmasterol in the range 0 - 0.5 mg/ml was also plotted. The results are shown in Table-1. The egg yolk of domestic hens contain free cholesterol in generally lower amount compared to the egg purchased from various farms. Significant differences were observed in their free cholesterol values. The domestic eggs are again of two brands, one that are from the families providing their hens with some food (sample No. 1 and 2) while sample No. 3 was from the family totally leaving their hens free to search for their food. Unlike the two types of hens, the egg yolk of farmy hens were found rich in free cholesterol. The egg having double yolk has the highest proportion of free cholesterol (Table-2, sample No. 3), despite the fact that the same amount of yolk (1.0 g) was taken from the twin.

Determination of free sterols in the ghee/oils samples

The results are shown in Table-3. Oils show very high concentration of free sterols, even in those brands which claim the absence of cholesterol in their products. On the other hand it has been observed that ghee samples contain relatively less amount of free cholesterol as compared to oil samples. This may be attributed to the loss of cholesterol/sterol during the process of blending/bleaching in oil processing [5]. As these oils are of vegetable origin and sitosterol is the major sterol of plants, the higher values for oils may be the combined value for cholesterol and sitosterol as their is a slight difference in the response of cholesterol oxidase to cholesterol and sitosterol.

Eggs of domestic in comparison with farmy hens contain low cholesterol. Out of 3 samples 2 of them are taking partial food and have less physical exercise while the 3rd one has the highest physical exercise during its search for food. Both the nature

Table-1: Calibration values for sterols standards

Concentration (mg/ml)	B-Sitosterol (uA)*	RSD (%)	Stigmasterol (uA)*	RSD (%)	Cholesterol (uA)*	RSD (%)
0.2	0.090	1.60	0.080	1.70	0.10	1.90
0.4	1.180	2.00	0.170	1.90	0.20	1.80
0.6	0.360	1.60	0.350	2.00	0.40	1.50
0.8	0.540	1.70	0.520	1.80	0.60	1.70
1.0	0.706	2.00	0.680	1.60	0.80	2.00

*Mean of three readings.

Table-2: The determination of cholesterol in various brands of egg yolk.

	Sample No.	Cholesterol* (mg/ml)	RSD (%)
Domestic hens:			
	1.	4.08	1.90
	2.	4.04	2.00
	3.	3.52	1.70
Farmy hens:			
	1.	5.04	1.90
	2.	4.80	1.50
	3.	8.80	1.70

*Mean of six injections.

Table-3: Determination of free sterol in various samples of Ghee/Oils.

	Sample No.	Sterol* (mg/g)	RSD (%)
Oils:			
	1.	1.470	2.00
	2.	1.512	1.90
	3.	1.120	2.00
	4.	1.022	1.90
	5.	1.316	1.80
Ghee:			
	1.	0.378	1.90
	2.	0.686	2.00
	3.	0.532	1.70
	4.	0.798	2.00
	5.	0.450	1.80

*Mean of six injections.

of the food and physical exercise affects the cholesterol level. For surplus cholesterol deposition there are two kinds of subcutaneous tissues. One is called "less dense tissue" having the capability of releasing the contained cholesterol easily, while the second one is called "dense tissue" which has the capacity to hold the deposited cholesterol for long time till it has been called for service.

As given in Table-3, oils have more sterol in comparison with ghee. One of the possibility may be the loss of sterol during bleaching, blending, neutralization and deodorization stage of ghee processing [6,7]. Likewise stigmasterols are also reduced to a greater extent than beta sitosterol during physical refining [8]. The magnitude of the losses of minor components depends to a large extent on the refining conditions used. The preference should be given to those having the least sterol content although it needs further analysis of the exact type of sterol present by techniques such as HPLC and others. The immobilized column was used on the bench for over three months, during which period more than 300 analyses were performed. No noticeable change in activity of the enzyme column was observed.

Experimental

Cholesterol oxidase (EC 1:1,3.6 ex, pseudomonas species), cholesterol (ex. porcine), and Triton X-100 were obtained from Sigma USA. For free cholesterol determination samples were prepared as follows. 10 eggs from each brand were taken and their yolk carefully separated. In each case one gram of egg yolk was suspended in 20 vol. of chloroform/methanol (2:1) containing 0.9% (w/v) sodium chloride. After shaking well two layers were formed. The upper aqueous layer which contained most of the gangliosides, glycosylceramides and the lower layer which contained most of the non-polar lipids including cholesterol, was sampled. The solvent was evaporated on a rotavapour and the aqueous solution of the sterol residue with saline and surfactant were treated for solution preparation as follows. To a 100 ml volumetric flask containing the sterol was added 5 ml of isopropanol to dissolve the sterol. Then 4 ml of the Triton X-100 (optimized concentration of detergent) and 0.5 M sodium chloride was added. The contents were shaken well and incubated in hot water for a minute or so with stirring to get a homogeneous solution. The volume after cooling was made to 100 ml with deionized water. By this procedure clear solution of free cholesterol/sterols upto 100 mg/dl were obtained. At higher concentration the solution becomes turbid which was avoided due to its non-suitability for the immobilized enzyme column. Stock solution was diluted to prepared working solutions of the stock, with 0.1M phosphate buffer (pH 5.0), containing 4% Triton X-100. These solutions were kept at 4°C which were stable for several weeks.

The ghee and oil samples were also prepared in a similar manner. Different brands of

ghee and oils were obtained from provision stores. 1.0 gram of sample from each brand in 10 replicates was taken and processed according to the method as described for egg yolks. Cholesterol oxidase (25 units/0.5 g of controlled pore glass) was immobilized by cross-linking with glutaraldehyde [9]. The immobilized enzyme was packed in a column (2.5 x 25 mm). The conditions for the flow system containing immobilized cholesterol oxidase were optimized such as effect of detergent concentration, pH and temperature. The effect of Triton X-100 on the activity of cholesterol oxidase was checked covering the range from 1 - 10%. A maximum response was obtained at 4% of Triton X-100, which was used subsequently. The effect of pH was also checked on the immobilized cholesterol oxidase by using phosphate buffer (0.1 M) of various pH values as a carrier stream. The highest signals were obtained at pH 5.0. There appears to be a shift in pH optimum on immobilization of cholesterol oxidase on CPG, as the soluble enzyme shows a pH optimum 7.0. The effect of temperature on the activity of immobilized enzyme was investigated by flowing water at various temperatures around the immobilized enzyme column. There is an increase in signal with increase in temperature upto 50°C. After this experiment, the enzyme remained active for all subsequent studies, for which the column was maintained at 30°C.

Acknowledgement

The authors thank the University Grants Commission for providing funds for the project.

References

1. R. Nadar, F.C. John, M.S. Lawrence and G.T. John, *Analysis of Chemical and Lab. Sci.*, **18**, 429 (1983).
2. A.J. Sheppard, D.R. Newkirk, W.D. Hubbard and T.J. Osgood, *Assoc. Off. Anal. Chem.*, **60**, 1302 (1970).
3. D.R. Newkirk and A.J. Sheppard, *J. Assoc. Off. Anal. Chem.*, **46**, 54 (1983).
4. H.S. Huang, J.W. Khan, G.G. Guilbault, *Clin. Chem.*, **21**, 1605 (1975).
5. H.G. Michael and E.G. Rosemary, *Food. Chem.*, **1**, 43 (1992).
6. A. Johansson and I. Hoffmann, *J. Am. Oil Chem. Soc.*, **56**, 886 (1979).
7. T. Gutfinger and A. Letan, *J. Sci. Food Agri.*, **25**, 1143 (1974).
8. I.M. Dowad, S.P. Kochhar and B.J. Hudson, *ebenssem. Wiss. U. Technol.*, **17**, 155 (1984).
9. M. Masoom and A. Townshend, *Anal. Chim. Acta.*, **166**, 111 (1984).