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SHORT COMMUNICATION

A simple and cost effective method to detect adulteration in ghee with vegetable oils through microscopic examination of sterols

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Abstract: Plant sterols together called as phytosterols, and animal sterols mainly cholesterol differ from each other in their crystal shapes, apart from other differences in terms of chemical structure, melting points, etc. When viewed under the microscope, after their isolation from the samples in purified state, phytosterols crystals appear hexagonal in shape while those of cholesterol acquire parallelogram shape. But the mixture of phytosterols and cholesterol shows the crystal structure with re-entry angle (Swallow's tail).On the basis of this characteristic crystal shape, detection of vegetable oils in milk fat up to a level of 15 percent could be confirmed with this simple cost effective method . However, this approach cannot be applied for the detection of body fats in milk fat because of the existence of common sterol (cholesterol) in them.

Keywords: Ghee adulteration, Microscopic examination, Sterols, Vegetable oils

Lipids represent one of the most important constituents of milk and milk products. In India, milk fat is mostly consumed in the form of ghee (clarified butterfat). Due to its short supply and more demand, expensiveness (costing 3 to 4 times as much as edible vegetable oils) and variable chemical composition, ghee falls prey to adulteration by the unscrupulous traders in the market. The commonly used adulterants include vegetable oils and fats, animal body fats, mineral oils, etc. Detection of foreign fats in milk fat is a very complex phenomenon, almost comparable with the detection of Pacific water in a sample of Atlantic water. No single test is available to detect all types of adulterants in ghee. Several methods (Kumar et al. 2002 , Boghra et al. 1981, 2004, Molkentin , 2007, Gutierrez et al. 2009, Amrutha Kala et al. 2016 , Rani et al. 2016, Aparnathi et al. 2019, Kumar et al. 2019, Shinde et al. 2020) have been developed in the past to detect the adulteration in ghee. These methods were mostly based on chemical parameters like fatty acid composition and the physico-chemical constants. But few attempts have been made to detect the adulteration on the basis of minor components such as sterols, cis-trans isomers, poly unsaturated fatty acids(PUFA) etc. (Molkentin, 2007, Gutierrez et al. 2009 , Rani et al. 2016, Zychowski et al. 2016, Aparnathi et al. 2019, Kumar et al. 2019, Nurseitova et al. 2019, Khorsandmanesh et al. 2020, Shinde et al. 2020). All these methods require the use of sophisticated instruments like GC-MS, HPLC, which are very costly and require lengthy preparatory steps for the analysis of the fatty acids and sterols as markers for detecting the adulteration with vegetable oils. Sterol profile determination was found more efficient than fatty acid analysis (Rachna and Nath, 2008, Zychowski et al. 2016, Nurseitova et al. 2019,2021, Khorsandmanesh et al. 2020, Shinde et al. 2020).

Sterols represent the major constituent of the unsaponifiable matter and range from 0.24 to 0.50 percent in butter fat, 0.03 to 0.14 percent in body fats and 0.03 to 0.50 percent in vegetable oils. Plants and animal fats have different types of sterols. Animal fats have cholesterol as the characteristic sterol while plant fats have phytosterols, which include β-sitosterol, stigmasterol, campesterol, brassicasterol etc. (Bailey, 2005, Christie, 2014, De, 2019, McSweeney et al. 2020).

On the basis of microscopic structure of sterols, plant fats can be differentiated from milk fat, while body fats cannot be distinguished from milk fat because both body fats and milk fat have cholesterol as the common sterol. Phytosterols and cholesterol differ from one another in a number of properties like crystal shape, Resolution factor (R_f) value, melting point, etc (Gurr et al. 2008, Fox, 2012; Christie, 2014, De , 2019, McSweeney et al. 2020). Therefore, in the present study, a simple method of

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microscopic examination of characteristic structure of sterols has been used as a criteria for checking purity of milk fat suspected with the presence of vegetable oils .

Milk used for the preparation of ghee samples was collected from the Institute's cattle yard. Cow milk was a mixture of the milk obtained from the herd of Karan Swiss, Karan Fries, Sahiwal and Tharparkar breeds. Buffalo milk used was also the herd milk from Murrah breed only. Cows and buffaloes were maintained under identical conditions of feeding and management. Soon after the collection of milk, it was warmed to 40^0C and separated into cream, using mechanical cream separator. The cream was pasteurized at 77^0C for 5 minutes, cooled to room temperature and then kept in a refrigerator (5 to 10^0C) for 3 to 5 hours for ageing. Butter was prepared under standard conditions (9^0C in summer and 13^0C in winter) by churning the cream using hand churn.

The vegetable oil was added to pure ghee (buffalo as well as cow) at the butter stage on the basis of its fat content at 5, 10 and 15 % levels. The butter samples admixed with the adulterants were clarified on direct flame in a stainless steel vessel under continuous stirring at temperature of 120^0C/flash and finally filtered through Whatman No. 4 filter paper. Simultaneously, pure ghee sample (control) was also prepared under similar conditions from the same lot of butter.

Detection of vegetable oils in ghee samples was carried out according to standard methods (IS:3508, 1966 and IDF, 1965). The method involves saponification of fat sample followed by precipitation of sterols with alcoholic digitonine solution. Sterol digitonides were then acetylated using acetic anhydride followed by saponification to obtain the crystal form of sterols to be viewed microscopically. The method, in brief, was as follows:

Accurately, 15 g of the fat samples were weighed in a 250 ml conical flask and saponified after adding 10 ml of the potassium hydroxide solution (66.7%, w/v), 20 ml of ethanol (95 to 96%, v/v) and 2 to 3 glass beads. Then 60 ml of the distilled water and 180 ml of ethanol (95 to 96%, v/v) were added followed by the addition of 30 ml of the alcoholic digitonine solution (1%), shaking and cooling. The flask was placed in a refrigerator at about 5°C for about 12 hours followed by filtration through Whatman No.1 filter paper. The precipitates of sterol digitonide thus obtained were washed with water at about 5°C until the filtrate stopped foaming, followed by washing once with 25 to 50 ml ethanol (95 to 96%, v/v) and then finally with 25 to 50 ml diethyl ether, and dried in an oven at 102 ± 2°C for 10 to 15 min.

To about 100 mg of the dried sterol digitonide precipitate obtained, 1 ml of acetic anhydride was added and heated in a glycerol bath maintained at 145°C until the precipitate had dissolved. Heating was continued for 2 minutes, followed by cooling to about 80°C. Then 4 ml of ethanol (95 to 96%, v/v) was added, mixed, heated slightly and filtered through a small medium speed filter paper impregnated with ethanol. The filtrate obtained was heated and brought to gentle boiling. While still boiling, drop-by-drop of 1 to 1.5 ml of distilled water was added carefully until the steryl acetate was just about to precipitate but still remained in the solution.

A few drops of ethanol (95 to 96%, v/v) were added to dissolve again any precipitated sterol acetate and allowed to cool in the air for 2 hours and finally in ice water for 30 minutes. The crystals of steryl acetate formed on cooling were filtered on a fast speed filter paper (Whatman No. 4) and rinsed with 1 ml of ethanol (80%, v/v). The crystals thus obtained were redissolved by heating in 1 ml of ethanol (95 to 96%, v/v) and subsequently allowed to cool first in air for 15 minutes and then in ice water for 5 minutes. The fresh crop of crystallized sterol acetate was again filtered as described above. Again it was redissolved, crystallized and filtered to get the third, occasionally the fourth or fifth recrystallization. The crystal cake was dried on the filter paper first in the air (about 30°C) and then at 102°C ± 2°C in drying oven for 10 to 15 minutes.

About 10 mg of the sterol acetates purified as above were taken in a test tube followed by the addition of 1 ml ethanol (95 to 96%, v/v) and 1 or 2 drops of potassium hydroxide solution. The tube was heated on a boiling water bath until the boiling began and steryl acetate had dissolved. This solution was then transferred to a 125 ml separating funnel with the help of 10 ml distilled water. The sterols were extracted with 25 ml of diethyl ether. The ether layer was washed with 3 to 5 ml portions of distilled water and evaporated to dryness. The residue was dissolved in 10 ml of ethanol (80%, v/v). Then drop of the clear solution was placed on a microscope cover slip, waited until the crystallization started at the periphery of the drop, then the cover slip was inverted and laid on a microscope slide and examined under microscope at about 200 X linear magnification.

If the sterol crystals are found to have only the form of a parallelogram with an obtuse angle (100°), which is characteristic for cholesterol, the fat sample is considered to be free from vegetable fat. However, if some of the sterol crystals show the elongated hexagonal form with an apical angle (108°), which is characteristic for phytosterols or if some of the crystals have a re-entry angle (Swallow's tail), which is characteristic for mixtures of cholesterol and phytosterol, the fat sample is considered to contain vegetable oils /fat.

The sterol crystals, obtained by saponification of fat samples followed by alcoholic digitonization, acetylation and subsequent saponification, were viewed under microscope for their characteristic shapes.

Results on the microscopic examination of the sterol crystals of standard cholesterol, cholesterol isolated from pure buffalo ghee9Buffalo and cow) , standard phytosterol (stigmasterol),

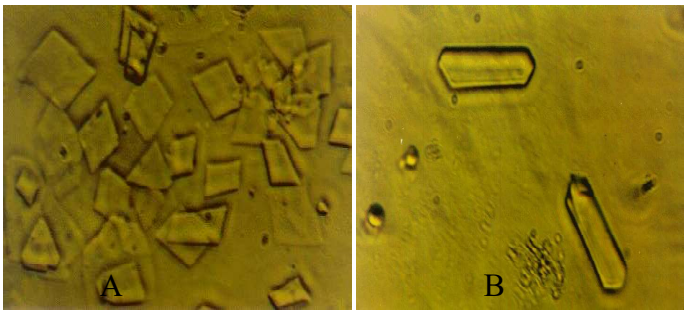

Fig. 1 Micrograph showing the sterol crystals of pure buffalo ghee (A) and groundnut oil (B)

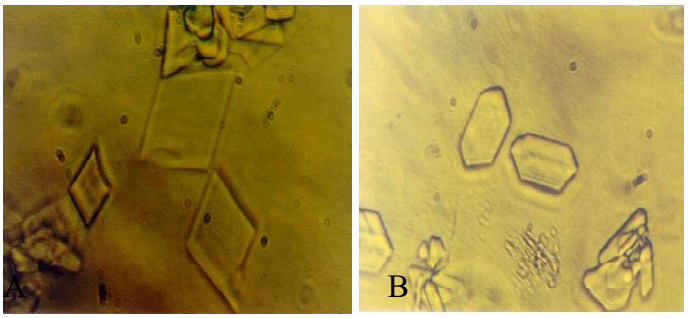

Fig. 2 Micrograph showing the crystals of standard cholesterol (A) and standard phytosterol (B)

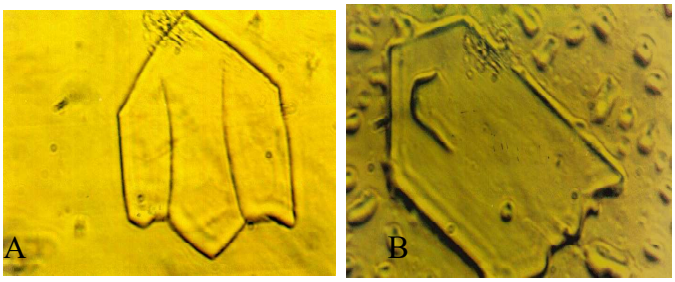

Fig. 3 Micrograph showing the sterol crystals of ghee adulterated with 15 percent groundnut oil (A) and a mixture of standard cholesterol and standard phytosterol in equal proportion (B)

phytosterols of pure groundnut oil, mixture of standards of cholesterol and stigmasterol, and sterols of buffalo ghee adulterated with groundnut oil at 15 percent level are depicted in Figs. 1to 3

Crystals of pure standard cholesterol and those isolated from pure buffalo ghee showed the characteristic parallelogram structures, while those of standard phytosterol and groundnut oil showed the characteristic hexagonal structures. On the other hand, crystals of mixture of standard cholesterol and phytosterol, and those of adulterated ghee samples showed a characteristic crystal structure with re-entry angle (Swallow's tail).

In the present study, the ghee samples adulterated with vegetable oils up to 10 percent level failed to show the expected type of crystal structure with re-entry angle (Swallow's tail), whereas the ghee sample adulterated with vegetable oil at 15 percent level exhibited these very clearly. The results obtained in the present study are supported by findings of Den Herder (1955) also who studied the detection of adulteration of butter with foreign fats on the basis of sterol structure , and indicated that if the sterol crystals show only the parallelogram, which is characteristic for cholesterol, the milk fat is considered to be free from vegetable oils and fats. Whereas, if the sterol crystals show the shape of hexagonal form, which is characteristic for phytosterols or if some of the sterol crystals have a re-entry angle (Swallow's tail) which is a characteristic for mixtures of cholesterol and phytosterols, the milk fat is considered to be adulterated with vegetable oils and fats. However, he further observed that crystals showing re-entry angle (Swallow's tail) are observed only when the percentage of phytosterol in a mixture of cholesterol and phytosterol exceeds 8 percent, which corroborated our findings. Therefore, adulteration of ghee samples with 15 percent groundnut oil could be confirmed by using this simple parameter.

Conclusion

In the present study, microscopic examination of sterol crystals from pure ghee showed a shape of parallelogram similar to the one shown by pure cholesterol, while those from vegetable oil (groundnut oil) showed hexagonal form similar to the one shown by pure phytosterols. On the other hand, sterol crystals isolated from ghee samples adulterated with groundnut oil (15%) showed a characteristic crystal structure with re-entry angle (Swallow's tail), as shown by the mixture of pure cholesterol and pure phytosterol. Therefore, adulteration of ghee samples with 15 percent groundnut oil could be confirmed with this simple cost effective method. The presence of natural sterols in milk fat and the detection of adulteration with animal fat are the limiting factors of this method. Therefore, it can be inferred that microscopic examination of sterols structure can be used as a simple and cost effective tool for detecting added vegetable oils in ghee.

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