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Simple chromogenic test for detection of adulterated milk with vegetable oil at village milk collection center- A preliminary study

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ABSTRACT

In the present investigation, a novel chromogenic test has been developed to ascertain the presence of vegetable oil in milk. This standardized protocol did not show any false-positive results in the genuine milk samples. Adulteration of milk with vegetable oil @ 1% level could be detected by this chromogenic test protocol. This said protocol is convenient to use in the rural dairy industry especially rural-based milk collection centers; as no costly instrument or trained manpower is required for this said test.

Keywords: Adulteration, Chromogenic test, Refined vegetable oil, Rural milk collection

The history of food fraud is as parallel as the history of food processing in the world (Hong *et al.* 2017). Among different food products, adulteration of milk and milk products is one of the major concerns encountered throughout the world and an alarming issue in the developing countries like India, Pakistan; but it is also a major problem for the developed country like Turkey (Barham *et al.* 2018, Karacaglar *et al.* 2019 and Ramani *et al.* 2019). The scientific reviews identified the possible reasons for milk adulteration as economic gains, demand and supply gap, complex nature of milk and lack of suitable field level detection test protocols (Luther *et al.* 2017 and Azad and Ahmed 2016).

Various scientific investigations proved that apart from water and whey, different hazardous chemicals like starch, chlorine, hydrated lime, sodium carbonate, formalin and ammonium sulfate are commonly added to milk (Poonia *et al.* 2016 and Azad and Ahmed 2016). Another problem encountered by the world's dairy industry is replacement of milk fat with vegetable oil either partially or fully (Karacaglar *et al.* 2019 and Hamed *et al.* 2019). Milk fat has always been a key target of adulteration due to its premium edible quality and economic benefits, as the milk's price is decided on the concentration of milk fat. Therefore, unscrupulous farmers or milk traders adulterate vegetable oil in milk or dairy products as a means to increase the profit (Hamed *et al.* 2019). Addition of vegetable oil into milk is very difficult to detect as both milk fat and oil's fatty acid make-up is very similar (Hansen and Holroyd 2019). Moreover, these type of adulteration cause major health hazards to the consumer (Karacaglar *et al.* 2019). Primarily, milk fat constants based analytical approaches like R. M. or P. values, B. R reading (Kumar *et al.* 2017) are frequently performed in dairy quality control laboratories or at milk reception docks, to check for the adulteration of milk with the vegetable oils. However, these tests are less sensitive to detect this type of adulteration (Hamed *et al.* 2019). Recent studies suggested that fraudulent milk traders are adding different blends of oils or different synthetic fatty acids to nullify fat constants based analyses approach for detecting the adulteration of milk fat with vegetable oil (Pathania *et al.* 2019). Therefore, vegetable oil detection in milk is a continuously challenging task for researchers. Advanced analytical instrument like differential scanning calorimeter (Upadhyay *et al.* 2016), fluorescence spectroscopy (Dankowska *et al.* 2015), MALDI-QTOF MS (Garcia *et al.* 2012), Raman spectroscopy (Karacaglar *et al.* 2019), electronic nose (Ayari *et al.* 2018), FT-IR reflectance spectroscopy (Antony *et al.* 2018), gas chromatography (Kala 2013 and Pathania *et al.* 2019) are now-a-days employed to ascertain the purity of milk fat. FT-IR spectroscopy coupled with chemometrics is proved as an efficient technique for the detection of vegetable oil in milk fat (Mehta *et al.* 2018), though the sensitivity of these techniques mainly depend on the type of foreign fat that has been adulterated and moreover these techniques fail to detect milk adulterated with <1% vegetable oil (Hansen and Holroyd 2019).

Milk, from farmers is generally collected in milk collection centers at village level and later sent to main dairies for processing (Luther *et al.* 2017). Most of the time it is hard to ascertain the quality of milk; specially milk

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adulterated with vegetable oil, due to lack of instrument facilities as well as trained manpower in rural area based milk collection centres. This makes it hard for rural-based small dairies or village based milk collection centers to maintain their quality by restricting the entry of vegetable oil adulterated milk.

It is evident from the above-cited literature that fast and reliable, low cost and easy to handle analytical approaches, for the detection of milk fat adulterated with vegetable oil are need of the hour for the world's dairy industry including developing as well as developed countries. (Garcia *et al.* 2012, Luther *et al.* 2017, Salve *et al.* 2018 and Hamed *et al.* 2019).

Hence, the present study was planned to develop a simple chromogenic test that could be easily performed at a village milk collection docks where milk is received and can segregate pure milk from adulterated milk with vegetable oil during receiving itself.

US pharmacopeia recently promoted to adopt non-targeted methods to detect food fraud more broadly and consistently (Xie *et al.* 2019). Therefore, we tried to exploit the antioxidant properties of oil to develop a very simple ABTS, (2'- azino-bis (3-ethyl benzothiazoline-6-sulphonic acid) based chromogenic test protocol to detect adulteration of vegetable oil in milk. The ABTS or 2, 2'-azino-bis (3-ethyl benzothiazoline-6-sulphonic acid), is a stable free radical; it reacts with Potassium persulphate (an oxidizing agent) to form a bluish green colour $ABTS^{\bullet++}$ complex (Schaich *et al.* 2015). Presence of antioxidants causes bluish green coloured ABTS radical complex to suppress or get reduced by donating the electron or by radical scavenging and inhabitation (Valantina and Neelamegam 2015). ABTS based test protocol is mainly used to determine the antioxidant activities in different foods (Puangbanlang *et al.* 2019). Till date no scientific report has been found that is stating the use of ABTS as a chromogenic material to detect vegetable oil adulteration in milk. Hence, our unique test protocol can be adopted at village level milk collection centers to efficiently segregate the pure milk from milk adulterated with vegetable oils and able to become an efficient weapon to combat the evil menace of adulteration of vegetable oil in milk.

MATERIALS AND METHODS

Collection of milk, vegetable oil and chemicals: Pooled bovine milk samples were collected from the local cattle-yard of Amreli. Thereafter skimmed milk was prepared in the laboratory of our institute. The emulsified oil was used to prepare milk with different fat percentages.

Refined vegetable oils (palm, groundnut, sunflower and cotton) used in the present study were procured from the local market of Amreli. However, a blend of all oil was prepared by mixing all the oils in the same quantities. Unrefined vegetable oils (groundnut and cotton) were procured from local oil processing plants of Amreli.

For the preparation of adulterated milk samples with different fat percentages; emulsified oil samples were mixed with skim milk at different percentages. For example, to prepare milk with 1% (w/v) fat, 1 g of emulsified fat was added to 99 ml of hot skimmed milk (60–70°C) and mixed for 1–2 min using a hand blender. So, the resultant milk had about 1% of fat. Pure milk or milk fat obtained from un-adulterated milk was used as control sample.

Preparation of chromogenic solution: 2, 2'-azino-bis (3-ethyl benzothiazoline-6-sulphonic acid) (ABTS) and Potassium persalphate (PPS) were procured from Sigma Aldrich Inc. St. Louis, USA. Methanol was procured from Loba chemicals, Gandhinagar, India.

Preparation of Potassium persulphate (*PPS*) *solution:* 1.892 g PPS dissolved was dissolved 50 ml of distilled water (DW).

Preparation of ABTS stock solution: 19.2 mg of ABTS was dissolved in 5 ml DW and thereafter 88 µl PPS solution was added and mixed the solution very gently. Thereafter the solution was put in an amber colour bottle in dark room for 16 hours.

Preparation of ABTS working solution: 2 ml. ABTS stock solution was dissolved in 50 ml methanol.

Extracation of milk fat: Milk fat from the milk sample was separated by centrifugation (5000 g, 10 min, 4°C) thereafter cream plug were removed carefully and put it in cream butyrometer and fat of the cream was determined using Gerber method mentioned in FSSAI (2015). After that the silicon stopper was removed from the stem side and the fat was taken out from the stem of the butyrometer using a pipette (FSSAI 2015).

Performance of chromogenic test: Two ml of fat sample was kept in a clean test tube. Thereafter 2 ml of ABTS working solution was added in that test tube and mixed; thereafter the colour was observed after five minutes of the completion of the test. The presence of bluish green colour indicated pure milk fat, however, absence of colour indicated adulterated milk fat.

RESULTS AND DISCUSSIONS

Standardization chromogenic test: For developing a chromogenic test, first and foremost priority was to standardize the effective concentration and quantity of chromogenic solution and reactant. So, in the initial phase of research we have tried different quantities of ABTS working solution (1 ml and 2 ml) and different quantities of milk fat (1 ml and 2 ml).

It was observed that whenever 1 ml of ABTS solution was allowed to react with 1 ml fat/oil sample, no colour difference was observed between milk fat and oil samples. However, a bluish green colour was observed soon after the addition of ABTS solution to the fat or oil samples but the colour did not persist. So, in the next phase of the study, we increased the quantities of fat samples and used 2 ml of fat/oil sample to react with 1 ml of ABTS solution.

Fig. 1 represents chromogenic difference between pure milk fat and pure oil samples. It was observed (Fig. 1) that for pure milk fat sample a bluish green colour ring developed at top of the fat but in case of oil samples no

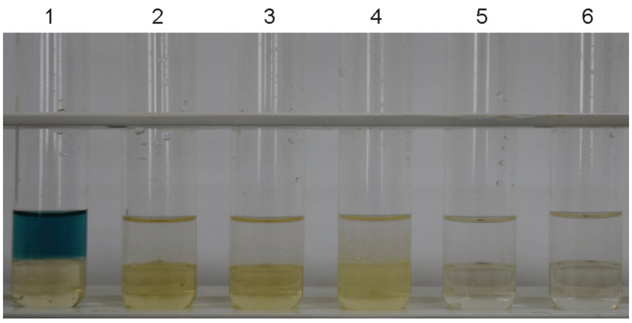

Fig. 1. Reaction between milk fat samples and chromogenic solution after 5 min of conductance of test. (1) Pure milk fat; (2) Pure Sun flower oil; (3) Pure Ground nut oil; (4) Pure Palm

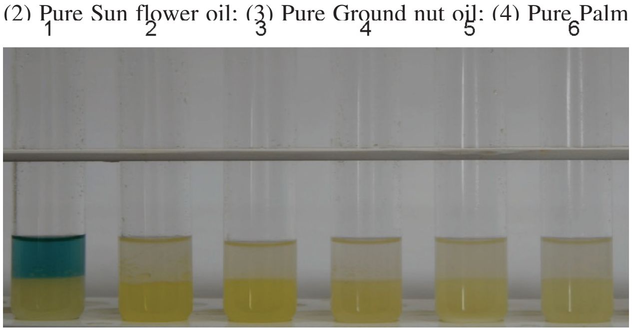

Fig. 2. Reaction between milk fat samples and chromogenic solution after 5 min of conductance of test. (1) Pure milk fat; (2) Pure milk fat + 3% Sun flower oil; (3) Pure milk fat + 3% Groundnut oil; (4) Pure milk fat + 3% Palm oil; (5) Pure milk fat + 3% Cotton oil; and (6) Pure milk fat + 3% Blends of oil.

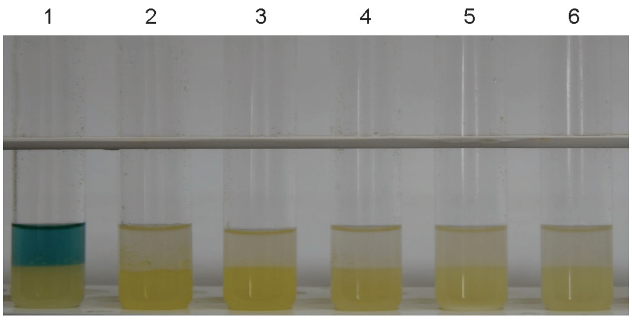

Fig. 3. Reaction between milk fat samples and chromogenic solution after 5 min of conductance of test. (1) Pure milk fat; (2) Pure milk fat + 1% Sun flower oil; (3) Pure milk fat + 1% Groundnut oil; (4) Pure milk fat + 1% Palm oil; (5) Pure milk fat + 1% Cotton oil; and (6) Pure milk fat + 1% Blends of oil.

colour was observed; however, the colour for the pure fat samples persisted five minutes after completing the reaction. Therefore, we used this protocol for the detection of oil in milk. It was observed from (Fig. 2) that for pure milk, fat sample a bluish green colour ring was observed at top of the fat, but in case of fat sample extract from 3% different oil adulterated milk were colourless within few seconds. The same protocol was repeated fifty times and the same result was observed in each and every time. Antioxidants are able to interact and inhibit the initiation or propagation of oxidizing chain reactions generated by reactive free radicals. In the presence of PPS solution ABTS-free radical; a bluish green coloured ABTS$^{\bullet++}$ complex is formed. However, this process is terminated due to presence of an efficient antioxidant. The efficacy of ABTS test mainly

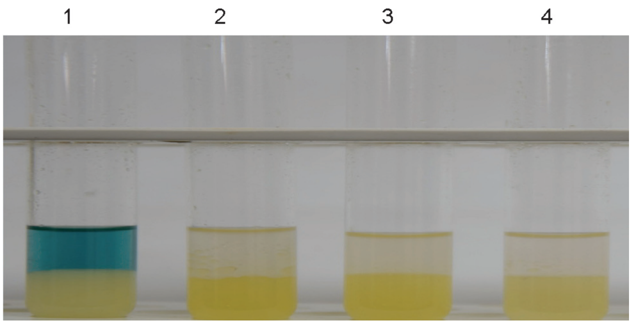

Fig. 4. Reaction between milk fat samples and chromogenic solution after 5 min of conductance of test. (1) Pure milk fat; (2) Pure milk fat + 1% Unrefined Groundnut oil; (3) Pure milk fat + 1% Unrefined Cotton oil; and (4) Pure milk fat + 1% blends (mix) of unrefined oil.

depends on the quantity of the antioxidant and type of antioxidant. Phenolic compound that are present in oil even after refining of oil (Mohdaly *et al.* 2017) act as both primary and secondary antioxidants by different mechanisms. So, the possible reason for this observation can be explained that whenever milk fat samples are adulterated with oil, the concentration of phenolic compounds might be increased.

Therefore, this certainly interepted or stopped the formation of ABTS$^{\bullet++}$ complex; that suppressed the bluish green colour or it turned to colourless. However, vegetable oil also contains carotenoids (alpha, beta and gamma carotenes), vitamin E (tocophoreols and tocotrienols), sterols (sitosterol, stigmasterol and campesterol), which are powerful water-soluble antioxidants (Yun *et al.* 2008). That also suppressed the bluish green colour or it turned to colourless; by stopping the formation of ABTS$^{\bullet++}$ complex.

In the next phase of the research, we used 1% oil adulterated milk. However, we observed that this said protocol was unable to detect 1% oil adulteration in milk. So, this protocol was not used for next phase of study. Therefore, in the next phase of the study, we used 2 ml of fat samples and 2 ml of ABTS solution. Fig. 3 represents chromogenic difference between pure milk fat and milk fat extracted from 1% oil adultrated milk. It was observed (Fig. 3) that for pure milk fat sample a bluish green colour ring was observed at top of the fat but in case of fat from 1% different oil adulterated milk samples became colourless within few seconds.

The same phenomena was observed for milk samples adulterated with 1% level of different blended oil. The bluish green colour of the pure milk fat sample remained after 5 min of completing the reaction. The same protocol was tried for detection of 1% different unrefined vegetable oil in milk and the same result was observed (Fig. 4). Thus, we repeated this protocol even 100 times in milk adulterated with refined or unrefined oil but same results were observed. It was observed that this said protocol was as effective to detect 1% level of vegetable oil individually and in the mixture or in blend.

However, the stock solution was stored for 15 days and the same protocol was repeated. We observed the same result as earlier discussed. Milk fat that contains a number of bioactive compounds namely, short-chain fatty acids, conjugated Linoleic acid, etc. is one of the premier edible fat. Adulteration of vegetable oil in milk or dairy products like butter, milk powder is encountered throughout the world (Garcia *et al.* 2012 and Hamed *et al.* 2019). Previously sophisticated instrument based (gas chromatography or MALD TOF-MS) methodologies have been adapted for detection of vegetable oil in milk or milk fat but most of these methods were mainly based on sophisticated and costly instrument based analysis (Garcia *et al.* 2012, Kala 2013 and Upadhyay *et al.* 2016); therefore rural-based milk collection center are unable to afford them to economical or trained manpower restriction. Dairy industry is one of the major pillor for world's economical growth. However, the dairy collection centers are mainly situated in rural areas where lack of modern analytical facility is predominant interference on routine quality analysis. Hence, inexpensive and easy to adopt test protocols are the need of time especially for milk collection centres.

In this complex situation this ABTS based test protocol will be able to segregate vegetable oil adulterated milk at the very initial level. This protocol was as efficient in ghee for the detection of 1% of vegetable oil adulteration. Presence of Palm oil in milk fat or ghee is very difficult to detect (Ramani *et al.* 2019). This test protocol was able to ascertain the presence of Palm oil in milk to the tune of 1%. Studies also suggested that presence of oil in milk fat at low level (1% level) is very difficult to detect. Although this ABTS based method was also able to detect even 1% level of oil individually or in mixture. So, this protocol should be used to ascertain the quality of both milk adulterated with refined as well as unrefined vegetable oil.

The present study was conducted to develop a simplex ABTS based chromogenic test to detect the presence of low price vegetable oil adulteration in milk. This assay did not involve any costly instrument or skilled manpower requirement, that is very much needful in the rural milk collection centers. The developed protocol for the detection of oil adulteration in milk was rapid and sensitive enough to be used for routine quality control analysis and was even able to detect adulteration of vegetable oil to the tune of 1%. So, this said methodology could be recommended to rural area based milk collection docks for earlier segregation of different vegetable oils (refined or unrefined) adulterated milk samples.

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