

## Sterol and Squalene as Indicators of Adulteration of Milk Fat with Palm Oil and Its Fractions

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### ABSTRACT

Milk fat, one of the expensive fatty matters, might be sometimes adulterated with other edible fats, particularly palm oil or its fractions, which can be a major problem for the dairy industry, especially in Iran. The aim of this study was to investigate the adulteration of milk fat based on some minor components present in unsaponifiable matters, namely, sterols and squalene. Different concentrations of palm oil and its fractions (0, 1, 2, 5, 10, 20, 50, and 100%) were added to pure milk fat. Sterol and squalene contents were determined by the application of GC and HPLC, respectively. The experimental data showed that  $\beta$ -sitosterol was the main phytosterol in palm oils and it could be a good indicator for detection of adulteration by palm oil as low as 5%. The result also indicated that squalene was not identified in milk fat, so, it can be considered as a good indicator to show the presence of palm oil as low as 1%. According to the results, it might be concluded that sterols and squalene could be used as important indicators to find the presence of palm oil and its fractions in milk fat.

**Keywords:** Dairy industry, Edible oils, Food monitoring, Phytosterol.

### INTRODUCTION

There are two notable adulterations in edible oils and fats, specifically: (1) Admixing cold press oil with refined one and (2) Substitution of more high-priced oils and fats with economical one (Jee, 2002). Dairy products due to their high nutritional value are desirable for a wide range of people for a healthy diet (Hilding-ohlsson *et al.*, 2012). Adulteration of costly oils and fats such as milk fat caused a critical complication because of cost-effective advantages by replacement with less expensive oils (e.g. mostly palm oil because of its similarity to milk fat) without marking the product properly. Due to the high price

of milk fat, there is a great appeal to adulterate the milk fat with oils with similar fatty acid and sterol profiles. Food monitoring laboratories tried to uncover such deception by using cost-effective and fast method of distinction (Precht, 1991). The adulteration has become quite complicated. The condition is more complex when the milk fat is adulterated by the addition of a vegetable oil such as palm oil that is inexpensive and readily available. Palm oil is composed of many chemical components where some might be used as indicators to detect the adulteration (Kim *et al.*, 2015). Crude palm oil is rich in minor components such as carotenoids, tocopherols, tocotrienols, sterols, phospholipids, triterpene alcohols, squalene,

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aliphatic alcohols, and aliphatic hydrocarbons. The major components of interest are the carotenes, tocopherols, tocotrienols, sterols, and squalene (Zou *et al.*, 2012). Therefore, it is necessary to use suitable advanced methods to detect adulteration. Many methods have been developed to detect fat adulteration, and some of them have been officially recognized. Generally, extensive survey of the literatures revealed that several methods have been developed in the past based on parameters like fatty acid composition and the physicochemical properties like refractive index, melting point and iodine value to detect the adulteration in milk fat, but they are not practical to detect adulteration anymore because they are labor and time consuming and involve several manual operations and on the other hand the adulterers have become quite professionals. In order to detect milk fat adulteration, it is possible to use both major and minor components as the detection tools. The presence of phytosterols in milk fat might drive to the conclusion that oils or fats of vegetable origin might have been added (Clemente and Cahoon, 2009). The most effective ways to detect the presence of foreign fats in milk are by determining the fatty acid composition, the triacylglycerol profile, and the different fractions of other minor lipid constituents, mainly from the unsaponifiable fraction.

This paper discusses methods for the detection of adulteration of milk fat with an emphasis on unsaponifiable matters, because palm oil has particular components that are absent in milk fat. Several methods have been used to check the purity of milk fat. There are limited data in the literature concerned with minor components for the assessment of milk fat authenticity. To the best of our knowledge there are no published records regarding detection of milk fat adulteration by palm oils using squalene and sterols content. Papers about the squalene content of milk fat are few; almost no research has been reported in this subject. Therefore, methods for the detection using

statistical analysis of milk fats adulteration with an emphasis on chromatographic methods based on sterols and squalene using GC and HPLC has been suggested.

The aim of this study was to investigate the adulteration of milk fat based on some minor components present in unsaponifiable matters, namely, sterols and squalene.

## MATERIALS AND METHODS

The experimental materials consisted of Refined, Bleached, Deodorized (RBD) Palm Oil (PO 100%), Palm Olein (POO 100%), and Palm Stearin Oil (PSO 100%), which were obtained from Behshahr Industrial Company. Milk Fat (MF 100%) was obtained from Vizheh Company. All chemicals and solvents used were of analytical grade from Merck. The PO, POO, and PSO were kept in darkness at 4°C and melted at 60°C prior to use. Milk fat was extracted from cream by heating in water bath at temperature of 40°C for 15 minutes, then, separated into two phases. The upper phase was filtered using anhydrous sodium sulfate, obtaining purified samples.

### Preparations of Adulterated Milk Fat Samples

Milk fat, palm, palm olein, and palm stearin oils were melted at 50°C until a clear liquid phase was obtained. Milk fat containing 1, 2, 5, 10, 20 and 50% (w/w) of palm, palm olein and palm stearin oils were prepared separately.

Milk fat, palm, palm olein and palm stearin oils have been marked with MF, PO, POO and PSO respectively. Also the amount of these oils added to milk fat has been shown by numbers 1, 2, 5, 10, 20 and 50 (For example, POO5 represents milk fat containing 5% palm olein). The samples were stored for further analysis in sealed vessels at 4°C.

### Determination of Unsaponifiable Matter (Sterols and Squalene).

Minor components analyses are fundamental for detailed characterization of oils and fats. The nonsaponifiable matters of the oils were isolated and quantified by the saponification of the oils with methanolic potassium hydroxide followed by the extraction of the nonsaponifiables with diethyl ether according to AOCS official method (AOCS, 1997). Sterol fractions were scrapped off the TLC plates to be analyzed by GC.

### Determination of Sterol Concentration by GC

Quantitative analysis of sterols was carried out by Agilent model 7890B. The GC instrument used was equipped with FID detector and capillary HP 5 column (30 mL, 0.32 mm ID, 0.25  $\mu$ m FT). Inlet and detector temperature were kept at 300 and 320°C, respectively, and the oven temperature was programmed at 265°C for 40 minutes holding time according to ISO method. (ISO 2014).

### Determination of Squalene by HPLC

The oil sample (0.5) was vortexed vigorously with 20 mL of methanol:acetone (7:3) in a 25-mL ground-glass stoppered test tube for 1.5 minutes and stored at -20°C for 24 hours. The samples were rapidly filtered through a coarse filter paper. The solvent was evaporated in a rotary evaporator at 40°C and the residue dissolved in 5 mL of acetone, which was subsequently injected to the HPLC as follows:

The concentration of squalene was determined by Agilent HPLC model 1220 Infinity Gradient LC with DAD equipped with (4.6 $\times$ 250 mm, 5  $\mu$ m) column, solvents were acetonitrile:acetone (60:40), flow rate 1 mL min<sup>-1</sup>, isocratic stop time 20 minutes, temperature TCC was 30°C according to

method carried by Nenadis and Tsimidou (2002).

### Statistical Analysis

Experiments for determination of sterols and squalene by GC and HPLC were carried out in triplicate, and the results were expressed as mean $\pm$ Standard Deviation (SD). The obtained results were treated with statistical analysis using the program Statistical Package for Social Science (SPSS 22). The results were calculated and analyzed by a paired sample t-test. Duncan's multiple range tests were used to compare differences between sample means. Level of significance was defined at (P< 0.05).

## RESULTS AND DISCUSSION

### Sterol Content

Sterols are compounds that occur naturally in plants, animals, and fungi. Sterols are very useful components to detect almost all types of vegetable oils in dairy products. Cholesterol is the predominant sterol in milk fat, and phytosterols are present in trace amounts in milk fat. Sterols are the major constituents of the unsaponifiable fraction of palm oil, which might also be affected by refining procedures and storage conditions (Sambanthamurthi *et al.*, 2000). The common vegetable oil sterols are also found in palm oil products. As indicated by Siew (1990), crude palm oil contains 210-620 ppm of sterols and the results showed that all treatments had mainly  $\beta$ -sitosterol, and campesterol, with small quantities of stigmasterol, with cholesterol only a minor component (Siew, 1990). Therefore, in the cases that fatty acid determination is not the suitable detection method, it is possible to use phytosterols as the detection agents, as discussed by Phillips *et al.* (2002), Verleyen *et al.* (2002), Ntakatsane *et al.* (2013), Oguntibeju *et al.* (2009), and Gordon, (2002). Palm oil contains different kinds of



phytosterols as presented in Table 1. The sterol profile of oils can identify the origin of the oils, indeed much more than their fatty acid compositions. The content of selected sterols is widely accepted as one of the most important indicators for the detection of adulterated milk fat. In this work, the detection of sterols revealed the added palm oil in all the mixture formulations; therefore, it could be a good indicator for adulteration detection in milk fat. The analytical results of sterol analysis of different milk fat samples are presented in Table 1.

The presence of plant sterols in milk fat indicates that the product was adulterated. Determination of cholesterol in animal fats and oils is an important topic in the food industry, since high amounts of cholesterol in foods are closely related to cardiovascular

disease risks. Cholesterol has also been commonly used to detect mixtures of animal oils in vegetable oils. Usually, the cholesterol content in milk fat is between 204.3 and 382.4 mg 100 g<sup>-1</sup> (Precht, 2001). Adanyi and Varadi (2003) observed that cholesterol content in butter was between 200 and 250 mg/100g.  $\beta$ -sitosterol is a typical vegetable sterol and detected in milk fat due to the animal feed (about 0.31%) while campesterol and stigmasterol were not detectable in pure milk fat. Molkentin (2006) reported that the cholesterol content in butter fat was 302.6 mg 100 g<sup>-1</sup>. Since milk fat is high in cholesterol, adulteration with foreign oils will be detectable through the cholesterol content. Table 1 presents the changes in sterol content of palm oil and its fractions when adulterated with other oils or fats. Gee (2007) showed that fractionation and

**Table 1.** Sterol content (%) in milk fat samples containing different amounts of palm oil, palm olein, and palm stearin.<sup>a</sup>

Samples <sup>b</sup>	Sterols (%)			
	Cholesterol	Campesterol	Stigmasterol	$\beta$ -Sitosterol
Milk Fat (MF)	99.67± 0.00a	ND	ND	0.31± 0.00m
Palm Oil (PO)	0.26± 0.02o	22.87± 1.08c	14.19± 0.05c	62.65± 1.16a
Palm Olein (POO)	0.22± 0.00o	27.49± 0.01a	14.77± 0.06b	57.5± 0.05c
Palm Stearin (PSO)	0.14± 0.02o	26.02± 0.4b	15.51± 0.01a	58.3± 0.4b
PO1	99.67± 0.00a	0.11± 0.01g	0.08± 0.00k	0.11± 0.02m
PO2	99.44± 0.01bc	0.15± 0.01g	0.09± 0.01jk	0.31± 0.01m
PO5	98.06± 0.05f	0.41± 0.01efg	0.37± 0.04gh	1.12± 0.02m
PO10	97.72± 0.13g	0.46± 0.02efg	0.39± 0.04gh	1.42± 0.11jk
PO20	95.29± 0.13k	0.96± 0.16e	0.66± 0.05e	3.04± 0.07g
PO50	85.50± 0.1n	3.07± 0.1d	1.77± 0.1d	9.63± 0.1d
POO1	99.58± 0.05ab	0.13± 0.02g	0.09± 0.00jk	0.16± 0.03m
POO2	99.40± 0.07c	0.14± 0.01g	0.11± 0.01jk	0.34± 0.04m
POO5	98.98± 0.00d	0.21± 0.01g	0.14± 0.04ijk	0.65± 0.05lm
POO10	98.08± 0.11f	0.27± 0.1fg	0.27± 0.02hi	1.37± 0.2jk
POO20	96.42± 0.02i	0.89± 0.1ef	0.46± 0.1fg	2.21± 0.02hi
POO50	89.06± 0.04l	2.87± 0.11d	1.65± 0.03d	6.38± 0.1f
PSO1	99.01± 0.12d	0.12± 0.06g	0.14± 0.05ijk	0.7± 0.00lm
PSO2	98.6± 0.04e	0.18± 0.00g	0.15± 0.03ijk	1.05± 0.00kl
PSO5	97.56± 0.1dg	0.38± 0.2efg	0.19± 0.01ijk	1.83± 0.3ijkl
PSO10	97.37± 0.1h	0.53± 0.01efg	0.23± 0.02ij	1.84± 0.1ij
PSO20	96.10± 0.02j	0.76± 0.11efg	0.54± 0.00ef	2.57± 0.1gh
PSO50	87.99± 0.05m	2.98± 0.3d	1.64± 0.2d	7.36± 0.1e

<sup>a</sup> Values within a column with different letters are significantly different ( $P < 0.05$ ). Each value in the table represents the means±SD from triplicate. <sup>b</sup> Abbreviation: MF, PO, POO, and PSO means Milk Fat, Palm Oil, Palm Olein, and Palm Stearin Oil; milk fat has 1, 2, 5, 10, 20, and 50% palm, palm olein, and palm stearin oil. Numbers in the symbols for treatments show the percent of the oil that was added to milk fat.

refining process could change the composition of the sterols content in the oil. The data reported in this research (Table 1) corresponds well with the work of Bonnie and Choo (1999) who reported that commercial red palm olein had higher sterol content than refined, bleached and deodorized palm oil. Examples of GC chromatogram of sterols in milk fat and one of the analyzed samples are in Figures 1.

As a result, among all sterols,  $\beta$ -sitosterol at a level of 5% in all the mixture could be a good indicator, but others like campesterol, stigmasterol and cholesterol at a level of 50% and above could be indicator for detection because, from the results, it was clear that the content of campesterol in all the mixture was below 1%.

The C30 hydrocarbon squalene is available at about 200-500 ppm in crude palm oil (Zou *et al.*, 2012). Abdul Gapor and Hazrina (2000) reported squalene as high as 421-979 ppm in some crude palm oils and 184-791 ppm in refined palm oils. These levels are generally higher than that of other vegetable oils, except olive oil (Singer *et al.*, 2008). The squalene contents in pure milk fat and mixtures of milk fat with 1, 2, 5, 10, 20 and 50% of palm oil and its fractions are shown in Table 2.

Squalene might be considered as a good indicator for palm, palm olein, and palm stearin oils since milk fat has no mentionable squalene content, as Jensen (2002) and Fox and co-workers (2007) reported the content of milk fat squalene as trace. The results showed significant statistical differences between all treatments and pure milk fat ( $P < 0.05$ ). The presence of squalene is well detectable at 1% addition. Using this method, the adulteration of milk fat by 1% addition of palm oil and its fractions can be detected. The results proved that the squalene contents in the sample show a significant difference in all the mixtures of adulterated samples. Squalene determination saves time and allows the determination of milk fat adulteration. This useful advantage makes it ideal for quality control reasons. In conclusion, squalene determination serves as a parameter in identifying and detecting milk fat adulterations. Examples of HPLC

chromatogram of squalene in milk fat and one of the samples are shown in Figure 2.

## CONCLUSIONS

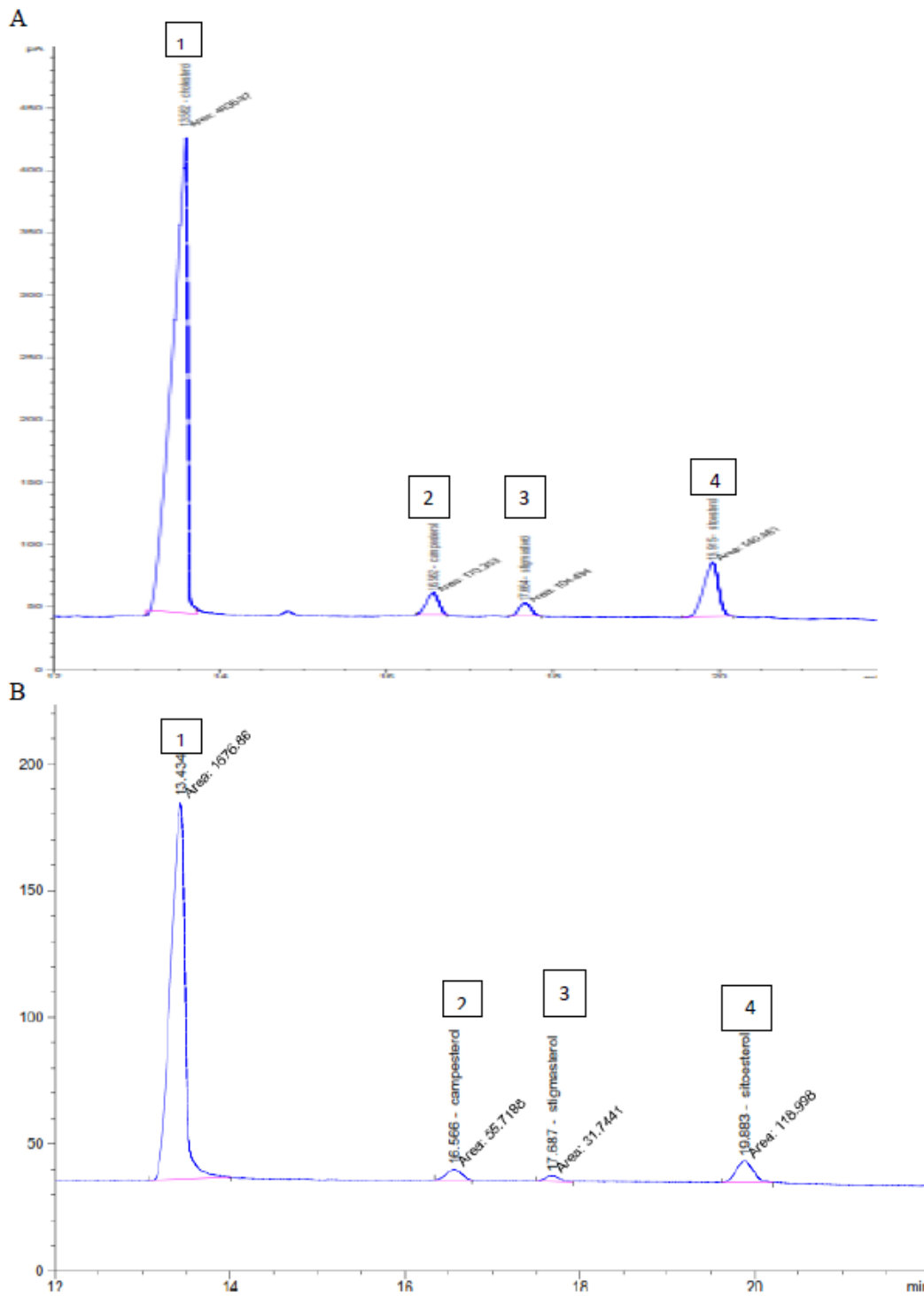
In this study, we aimed to develop an analytical method to detect milk fat adulteration with palm oil and its fractions. Sterols and squalene were employed as means for the detection of adulteration in oils and fats. Although fatty acid

**Table 2.** Squalene content (ppm) in milk fat samples containing different amounts of palm oil, palm olein, and palm stearin.<sup>a</sup>

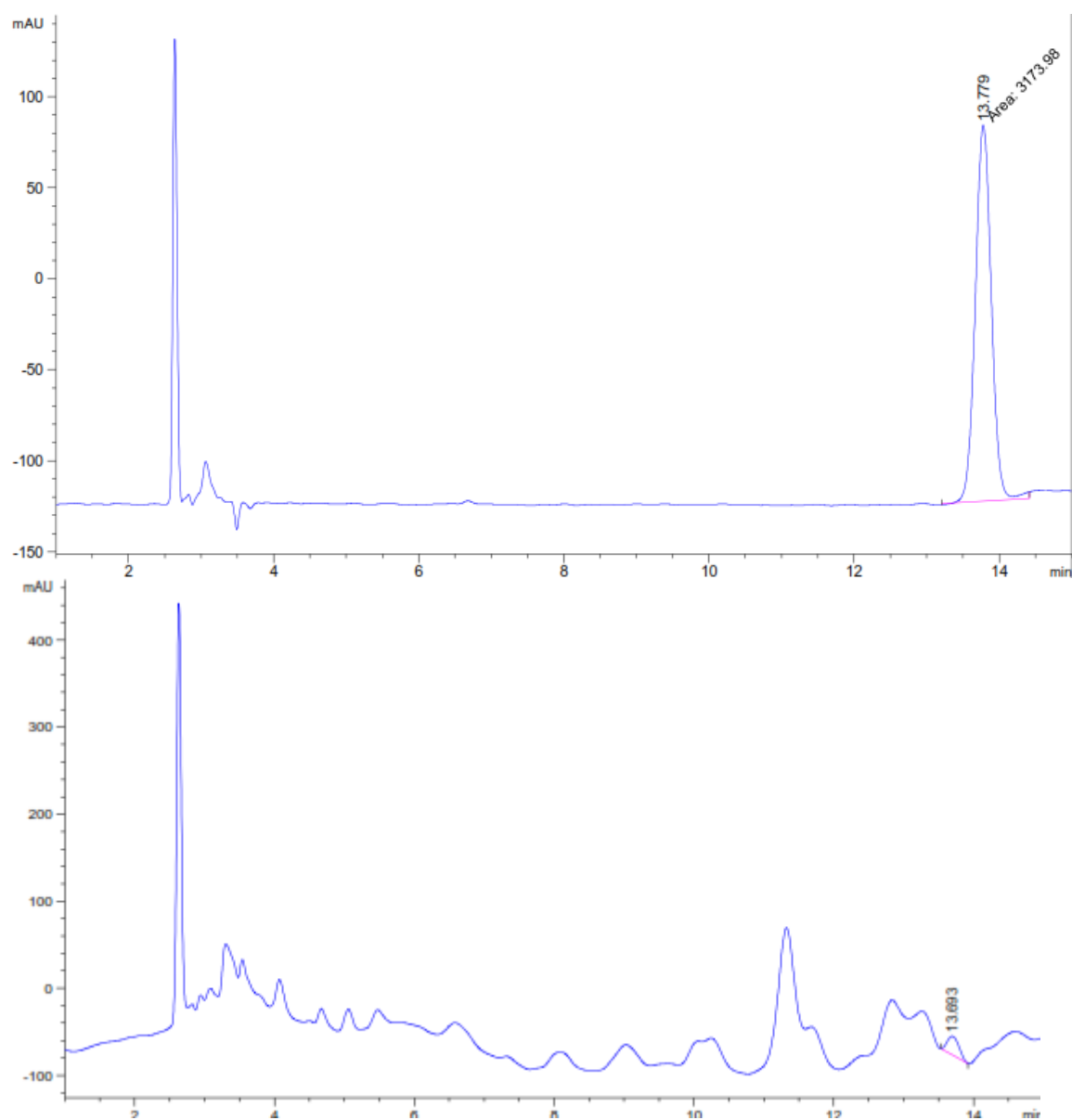
Sample <sup>b</sup>	Squalene (ppm)
Milk fat	ND
Palm oil	a 273.8± 2.93
Palm olein	194.75± 1.83b
Palm stearin	179.19± 0.44c
PO1	14.2± 0.02k
PO2	14.56± 0.1k
PO5	14.73± 0.07k
PO10	22.48± 0.8i
PO20	145.33± 2.52d
PO50	178.92± 0.84c
POO1	10.08± 0.04i
POO2	10.34± 0.15i
POO5	10.81± 0.04i
POO10	19.46± 0.33j
POO20	121.39± 0.64g
POO50	135.27± 1.01e
PSO1	6.50± 0.03m
PSO2	6.84± 0.03m
PSO5	7.09± 0.02m
PSO10	16.28± 0.05k
PSO20	103.53± 0.92h
PSO50	127.73± 0.23f

<sup>a</sup> Values within a column with different letters are significantly different ( $P < 0.05$ ). The values are expressed as mean±standard deviation. ND means Not Detectable.

<sup>b</sup> Treatments symbols are the same as Table 1.



**Figure 1.** GC chromatographic analysis of sterols in: (A) Standard and (B) Milk fat containing 50% palm olein (POO50).. (1)= Cholesterol; (2) Campesterol; (3)= Stigmasterol, (4)=  $\beta$ -sitosterol. Squalene Content



**Figure 2.** HPLC chromatographic analysis of squalene: (A) Standard and (B) Milk fat containing 10% palm oil (PO10).



composition has been employed for detection, the low concentration of adulterated oil or fat cannot be detected by this method. Sterols were analyzed by GC while squalene analyses were carried out by HPLC. The results of our studies indicated that sterol analysis with an additional squalene content examination can confirm if milk fat was exposed to adulteration. The presence and level of adulteration can be determined by comparison of milk fat and mentioned oil compositions. The presented analytical methods can be successfully applied to confirm milk fat adulteration.

Based on this preliminary investigation, the usefulness of this approach could be tested for other oils in the future. Moreover, industries and certified analysis institutions can use this method to monitor multiple samples simultaneously. This method is more useful for analysis of milk fat content, and we believe that it can serve as a database for monitoring and inspection. However, further study is necessary to analyze the sterols and squalenes in the milk fat products in the market.

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## استرول و اسکوالن بعنوان شاخصه های شناساگر تقلب صورت گرفته در چربی شیر توسط روغن پالم و فراکسیون های آن

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### چکیده

چربی شیر بعنوان یکی از با ارزش ترین اجزای چرب ممکن است گاهی اوقات توسط سایر چربی ها مورد تقلب واقع شود خصوصا توسط روغن پالم و فراکسیون های آن که میتواند مشکل عمده ای در صنایع لبنی به ویژه در کشور ایران باشد. هدف اصلی این تحقیق بررسی تقلب چربی شیر بر پایه ترکیبات جزئی غیر صابونی شونده از جمله استرول ها و اسکوالن است. روغن پالم و فراکسیون های آن (اولئین و استارین) در غلظت های (۰، ۱، ۲، ۵، ۱۰، ۲۰، ۵۰ و ۱۰۰ درصد) جایگزین چربی شیر خالص شد. میزان استرول و اسکوالن به ترتیب توسط کروماتوگرافی گازی و کروماتوگرافی مایع با کارایی بالا سنجش شد.  $\beta$ -سیتوسترول در میان استرول ها یک شناساگر خوب محسوب می شود به طوریکه تا حداقل میزان ۵٪ غلظت روغن پالم قابل شناسایی و پایش بود. از طرف دیگر به دلیل عدم یافتن اسکوالن در چربی شیر هر گونه جایگزینی آن با انواع



روغن پالم تا حداقل میزان ۱٪ قابل پایش است. بر اساس نتایج بدست آمده می توان ذکر نمود که استرول ها و اسکوالن شناساگرهای مهمی جهت حضور پالم و مشتقات آن در چربی شیر محسوب میشوند.