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Raman spectroscopy based characterization of desi ghee obtained from buffalo and cow milk

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ABSTRACT

Raman spectroscopy bands (1006, 1156 and 1520 cm^{-1}) representing beta-carotenoids differentiated cow ghee from buffalo ghee. The band at 1080 cm^{-1} represented free cholesterol, the concentration of which was higher in cow ghee than in buffalo ghee. Vitamin D and conjugated linoleic acid (CLA) in both cow ghee and buffalo ghee was identified through their Raman bands; the former contained more CLA isomers. A partial least squares regression model was developed to predict cow ghee adulteration of buffalo ghee and unknown samples. Coefficient of determination, standard error of prediction and standard error of calibration values of 0.96, 0.101 and 0.105, respectively, confirmed the authenticity of the model. Unknown samples loaded into the model yielded values of 0 or 1, indicating pure buffalo ghee or cow ghee adulteration, respectively. Nine unknown samples were tested blind; the root mean square error in prediction was 0.02, confirming the accuracy of the model.

1. Introduction

Desi ghee is a type of liquid butter obtained from cow or buffalo milk by different extraction methods across the world and particularly in the Subcontinent by adding yoghurt culture. It is enriched source of lipid nutrients, fat soluble vitamins and essential fatty acids and has very low amount of polyunsaturated fatty acids (Sserunjogi, Abrahamsen, & Narvhus, 1998). It is composed of 62% saturated fats and is a rich source of conjugated linoleic acid (CLA) (Chinnadurai, Kanwal, Tyagi, Stanton, & Ross, 2013). CLA is a mixture of linoleic acid isomers containing two conjugated double bonds having different carbons in the fatty acid chain (Bernuy Del Carpio, 2010; Chinnadurai et al., 2013). Desi ghee has been reported to have antioxidant, antiatherogenic, anticarcinogenic, antidiabetic and antiadipogenic properties due to the enriched CLA content (Chinnadurai et al., 2013). It is also reported as improving immune function and altering body composition to treat obesity or build lean body mass, and helps preventing cancer and heart diseases (McCrorie, Keaveney, Wallace, Binns, & Livingstone, 2011; Whigham, Cook, & Atkinson, 2000).

Desi ghee obtained from cow or buffalo milk is popular for producing a variety of foods, cooking and dairy products. Due to its nutritional values, it becomes very important to label the nutritional parameters of desi ghee properly to ensure its quality and for the information of end users. Basic extraction methods of desi ghee employed in the Subcontinent including India and Pakistan have been described (Ganguli & Jain, 1972), and Sserunjogi et al. (1998) provided a comprehensive study of the origin, local names and its different products in different parts of the world. Recently, Ahmad, Saleem, Ahmed, and Mahmood (2018) reported the temperature effects on the composition of buffalo ghee and defined a temperature range for safe cooking where it does not lose much of its nutritious contents. Further, gas chromatography has been employed to study effects of storage on chemical stability of desi ghee (Andrewes, 2012).

Raman spectroscopy in last few decades has proved itself a unique, label free and nondestructive fingerprint tool for the characterisation of different edible oils (Ahmad et al., 2017; Baeten & Aparicio, 2000; Baeten, Hourant, Morales, & Aparicio, 1998; El-Abassy, Donfack, & Materny, 2009; Lopez-Diez, Bianchi, & Goodacre, 2003; Muik, Lendl, Molina-Díaz, & Ayora-Cañada, 2003; 2005; Yang & Irudayaraj, 2001; Yang, Irudayaraj, & Paradkar, 2005; Zhang et al., 2011), characterization of fats in milk (El-Abassy, Eravuchira, Donfack, von der Kammer, & Materny, 2011; Gallier, Gordon, Jiménez-Flores, & Everett, 2011; McGoverin, Clark, Holroyd, & Gordon, 2009; 2010; Meurens, Baeten, Yan, Mignolet, & Larondelle, 2005; Ullah et al., 2017b, c), butter adulteration

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(Nedeljković et al., 2016; Uysal, Boyaci, Genis, & Tamer, 2013) and its composition (Beattie, Bell, Borgaard, Fearon, & Moss, 2004) and characterisation of desi ghee as well along with different types of margarines/spreads for quality assurance (Ali, Nawaz, Saleem, Nurjis, & Ahmed, 2016). Recently, thermal effects on the molecular composition of desi ghee has been reported (Ahmad et al., 2018).

In addition to Raman spectroscopy, infra-red (IR) spectroscopy has also been used for the characterisation of biomolecular structures (Barth & Haris, 2009; Stuart, 2006; Türker-Kaya & Huck, 2017). Raman and IR spectroscopies are complementary techniques that probe vibrational transitions, but the selection rules incorporating Raman scattering and IR absorption are quite different. Raman spectra originates from inelastic scattering of light from the vibrating molecules whereas IR spectroscopy is based on the absorption of light by vibrating molecules. For acquiring Raman spectra, sample preparation is not very elaborate and samples can be in any state. IR spectroscopy, has recently been used for the characterisation of different types of ghee (Antony, Sharma, Mehta, Ratnam, & Aparnathi, 2018; Upadhyay, Jaiswal, & Jha, 2016). Similarly, ATR/IR spectroscopy has been used for interrogating samples directly from their surfaces. It is a label free newly emerging technique used for the analysis of sample surfaces. Recently, this technique has been employed for the detection of pig body fats in pure ghee (Upadhyay, Jaiswal, & Jha, 2018) and also for the detection of goat body fat in pure ghee (Upadhyay et al., 2016).

Raman spectra basically provide molecular signatures of the species inside the sample, providing qualitative analysis as well as quantitative outcomes; but this is possible only through applying chemometric techniques like principal component analysis (PCA) and PLSR. Further, results produced by Raman spectroscopy are comparable with other techniques (Qi & Berger, 2005; Strachan, Rades, Gordon, & Rantanen, 2007) and it offers advantage over other techniques as it takes measurements directly from samples and it is label free technique.

Using Raman spectra, this research characterised, identified and differentiated desi ghee obtained from cow and buffalo on the basis of presence and absence of unique nutritional ingredients such as β-carotene, vitamin D and CLA.

2. Materials and methods

2.1. Sample preparation of pure cow and buffalo ghee

There are four methods commonly used for the extraction of desi ghee: cream butter method, direct from cream, pre-stratification and indigenous milk butter method also called desi method (Ganguli & Jain, 1972). By adopting desi method, fresh cow and buffalo milk samples were taken boiled, cooled and after adding yoghurt culture left to incubate from eight to ten hours at room temperature to obtain dahi (curdled whole milk). Some water was added to the dahi and after churning it, desi butter with milk was produced in a clean metal vessel. The desi butter was then heated slowly at < 115 °C in an open pan under continuous stirring to allow evaporation of the water without charring the proteins. At 115 °C, desi ghee was fully separated from the rest of the ingredients of the butter (Sserunjogi et al., 1998) and was then stored at 4 °C for further experimentation. A K type thermocouple (Reotemp Instruments, USA) was used for the temperature measurements with an uncertainty of ±2 °C. The temperature used for separation of desi ghee from cream or butter is reported from 105 to 120 °C (Sserunjogi et al., 1998).

There are several traditional methods used to prepare desi ghee in different parts of the world. For example, in Egypt 2–4% salt is added in the butter while heating under continuous stirring and claimed that water content of resultant samna (local name of desi ghee) decreases by increasing the boiling point of the water in the ghee. Also in Ethiopia, maize flour is added during the extraction of ghee (Sserunjogi et al., 1998). Similarly, in our study, 1% wheat flour was added in the melted butter at temperature about 100 °C to separate ghee from residual solids present in the butter material. It is an ancient practice in subcontinent which helps caramelise nonfat butter solids. The quantity of flour added was very small, and does not have any effect on the molecular composition of desi ghee due to increased difference in density between fat and non-fat phase (Sserunjogi et al., 1998). These pure samples of both ghee types were directly used for recording Raman spectra and for subsequent analysis.

2.2. Sample preparation of adulterated ghee

To predict the adulteration of cow ghee in pure buffalo, a PLSR based chemometric model was developed. Ten adulterated buffalo ghee samples were prepared by mixing different concentrations of cow ghee into buffalo ghee (15, 20, 25, 30, 40, 50, 60, 70, 80, 90%). To make homogenised adulterated samples, both ghee types were weighed as per their concentration and pushed inside a test tube. The mixture in test tube was then heated at 50 °C for 10 min in an electrical oven and then mixed further with vortex mixture to make it fully homogenised. The adulterated samples were then refrigerated at 4 °C for further studies. In the present model, minimum level of adulteration was taken as 15%, i.e., the basis of the PLSR model. In addition, 9 ghee samples were kept hidden for blind testing of the developed PLRS model, out of which 3 were pure buffalo ghee samples and 6 were adulterated samples that were prepared by mixing 10, 35, 45, 65, 75 and 85% cow ghee into buffalo ghee.

2.3. Sample collection of vitamin D and CLA

For samples of vitamin D, D-Tres injection (SAMI Pharmaceuticals Pvt. Ltd.) which contains vitamin D3 (cholecalciferol) was used and for CLA, a product by Nature's Bounty (Bohemia, NY, USA) was used, both purchased from local market of Islamabad.

2.4. Acquisition of Raman spectra

Desi ghee solidifies at room temperature (25 °C); therefore, it was first melted slightly by warming the test tube container slightly. A small quantity of ghee was then placed in a semispherical cavity of 5 mm in diameter made on the surface of aluminium block, which does not produce any Raman scattering and immediately radiates heat if any is produced by focussing the laser on the sample. The sample holder was placed under the microscope of the Raman system (μRamboss: Micro Raman measurement system, Dongwoo Optron Co. Ltd, Korea) for the acquisition of spectra. The Raman system comprised a diode laser (532 nm) coupled to a microscope, producing Raman spectra from 600 to 1830 cm^{-1} with a spectral resolution of 10 cm^{-1}; however, Raman spectra from 750 to 1800 cm^{-1} were included in the analysis where maximum spectral variations are present. The Raman system operates in the back-scattering configuration. Furthermore, a microscopic objective of 100× was used to carefully focus just beneath the upper surface of the sample to acquire a good Raman scattering signal. Raman spectra were recorded by shining a laser power of 40 mW for an integration time of 7 s, and were recorded on different days and times keeping same laser power and integration time. To exclude experimental artifacts, 5 Raman spectra were recorded from each sample on three different days. In this way total 15 Raman spectra were recorded from each sample.

Laser power was optimised carefully to avoid photo-degradation (Amin et al., 2017; Bilal et al., 2015). Although all ghee samples were homogenised, Raman spectra were recorded from different positions on the sample surface.

Raman spectra from the samples of Vitamin D and CLA were obtained as described above.

2.5. Pre-processing of Raman spectra

The raw Raman spectra include noise, fluorescence background and spectral overlapping of nearby bands, which makes it difficult to visually identify peaks/bands of interest. To remove these unwanted components in the Raman spectra, all spectra were pre-processed in MATLAB (Mathworks, release 2014b) for smoothing/noise removing, baseline correction and vector normalisation so that molecular bands and functional groups could be identified correctly (Knief, 2010). All the spectra were smoothed using a Savitzky–Golay smoothing method (polynomial order 5, 13-point window) and vector normalised. The polynomial order and window size was optimised after several iterations so that the smoothing procedure did not affect the presence of less intense bands. A rubber band correction for baseline removal for all the spectra was carried out to remove the fluorescence background (Knief, 2010). In producing the Raman spectra of both ghee types, a continuous standard deviation was displayed along with the average to illustrate the spectra to spectra variations.

3. Results and discussion

3.1. Spectroscopic analysis of Raman spectra

The purpose of this study was to investigate differential spectral signatures associated with desi ghee obtained from cow and buffalo milk using Raman spectroscopy. Raman spectra of these species exhibit clear spectral differentiation in the pre-processed Raman spectra shown in Fig. 1, which shows normalised Raman spectra of desi ghee samples obtained from cow and buffalo milk and show clear differences in intensity as well as Raman shift. Most of the prominent Raman bands listed in Table 1 are taken from the literature and are dominated by the Raman spectra of CLA and vitamin D, as shown in Fig. 2. Raman spectra from cow and buffalo ghee shown in Figs. 1 and 2 are the average of three times repeatedly recorded spectra along with the standard deviation, plotted continuously along the average spectra as shaded region. It is plotted in this fashion to show the spectra to spectra variations at each Raman shift.

Fig. 1 shows difference plot of two species where the Raman spectra of cow desi ghee differs the most from buffalo samples at Raman bands 1006, 1080, 1156, 1178 and 1520 cm^{-1}. The difference was obtained after pre-processing of Raman spectra. All the pre-processing steps were same for all spectra used in study. The difference in spectra was obtained by subtracting pre-processed cow spectra from the buffalo spectra. It can be observed from Fig. 1 that there are number of other Raman bands that appeared in the Raman spectra of cow and buffalo that are common in two species. However, there may be small differences in the corresponding intensities that can be associated to the different concentrations of the same molecular group in both species. The variations in intensity between the Raman peaks of cow and buffalo desi ghee show different concentration of the same molecular structures and the shift in the Raman peak positions represents the variation in the molecular composition (Baeten et al., 1998; Gallier et al., 2011; Yang et al., 2005; Yang & Irudayaraj, 2001). Positive peaks above zero level in the difference plot (Fig. 1) indicate a relatively high concentration of a particular molecular structure in desi ghee obtained from cow milk, whereas a negative peak means a relatively higher concentration of same biomolecule in buffalo desi ghee.

The Raman band at 1080 cm^{-1} shows positive correlation and is assigned to free cholesterol (Ullah et al., 2017b), which is found to be higher in cow ghee as can be visualised in the difference spectra.

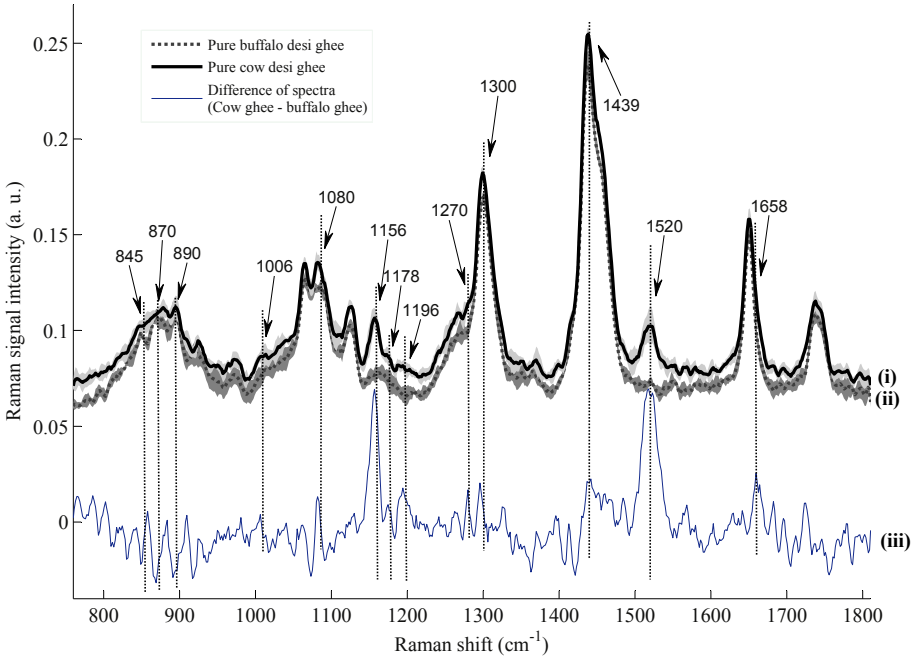

Fig. 1. Raman spectra (average of spectra recorded three times) of four buffalo ghee samples (i) and one pure cow ghee sample (ii) that were recorded on three different days from the same samples to exclude any experimental artifacts. In total, 15 Raman spectra of cow and 60 of buffalo were recorded and their averaged spectra is shown. The difference of Raman spectra (cow ghee − buffalo ghee) is also shown (iii).

Table 1
Raman spectral signatures of cow ghee, buffalo ghee, vitamin D and CLA.[a]

Raman shift (cm^{-1})	Molecular assignment	Reference	Present study			
			C	B	D	CLA
845	Saturated fats, ν (C1–C2), CH3 rk, ν (C–O) Complex broad table in liquid: 800–920 Vitamin D*	Ali et al. (2016) *In spectra of vitamin D	✓	✓	✓ *	–
870	Saturated fats, ν (C1–C2), CH3 rk, ν (C–O) Complex broad table in liquid: 800–920 Vitamin D*	Ali et al. (2016) *In spectra of vitamin D	✓	✓	✓ *	–
890	Saturated fats, ν (C1–C2), CH3 rk, ν (C–O) Complex broad table in liquid: 800–920 Vitamin D*	Ali et al. (2016) *In spectra of vitamin D	✓	✓	✓ *	–
970	Out of phase CCH wag vibration	Agbenyega et al. (1991)	✓	✓	–	–
1006	β-Carotenoids	El-Abassy et al. (2011); Ullah et al. (2017b)	✓	–	–	–
1063	Saturated fats Vitamin D*	Ullah et al. (2017b) *In spectra of vitamin D	✓	✓	✓*	–
1080	Free cholesterol Vitamin D*	Ullah et al. (2017b) *In spectra of vitamin D	✓	✓	✓*	–
1120	Vitamin D	In spectra of vitamin D	–	–	✓	–
1125	Out-of-phase C–C solid fat, saturated fats	Gallier et al. (2011)	✓	✓		
1156	β-Carotenoids	El-Abassy et al. (2011); Ullah et al. (2017b)	✓	–	–	–
1178	Saturated fatty acid	Czamara et al. (2015)	✓	–	–	–
1270	C=C *cis* unsaturation, unsaturated fatty acids, PC, PI and PS Unsaturated fatty acids	Gallier et al. (2011)	✓	✓	–	–
1300	δ(CH2) twisting unsaturation Vitamin D*	Ahmad et al. (2017); Gallier et al. (2011) *In spectra of vitamin D	✓	✓	✓*	–
1420	Saturated fats	Ullah et al. (2017b)	✓	✓	–	–
1439	Isomers of CLA** Vitamin D*	Meurens et al. (2005) **In spectra of CLA *In spectra of vitamin D	✓	✓	✓*	✓**
1450	Saturated fatty acid, Vitamin D*	Ullah et al. (2017b) *In spectra of vitamin D	✓	✓	✓*	–
1520	β-Carotenoids	El-Abassy et al. (2011); Ullah et al. (2017b)	✓	–	–	–
1650	Isomers of CLA	Ali et al. (2016); Meurens et al. (2005)	✓	✓	–	✓
1738	Triacylglycerol, glyceryl esters Vitamin D*	Ali et al. (2016) *In spectra of vitamin D	✓	✓	*✓	–

[a] Abbreviations are: C, cow; B, buffalo: D, vitamin D; CLA, conjugated linolenic acid; PC, phosphatidylcholine (lecithin); PI, phosphatidylinositol; PS, phosphatidylserine.

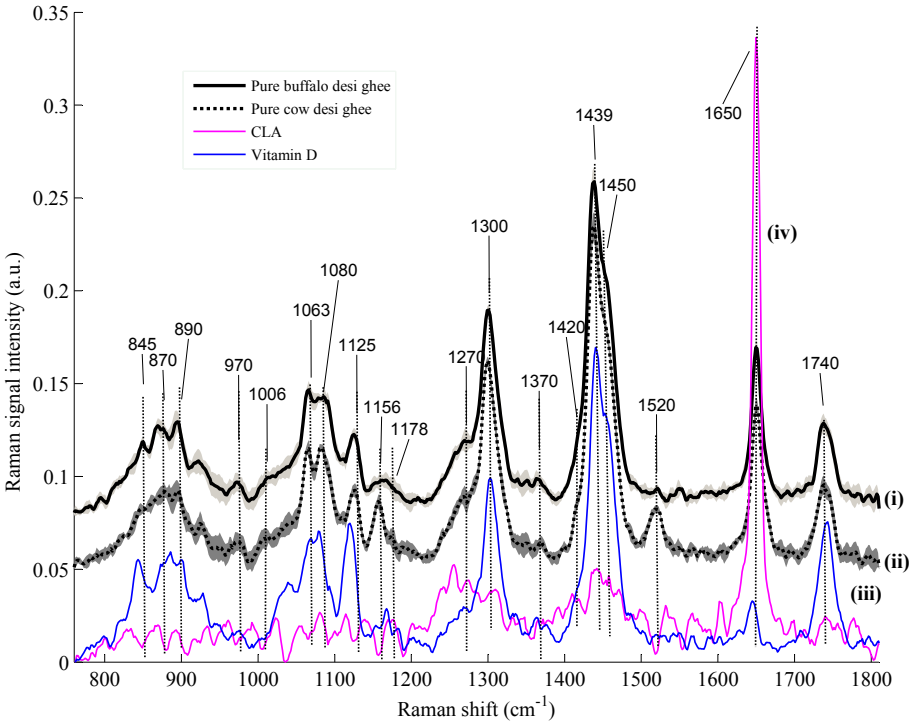

Fig. 2. Comparison of Raman spectra (average of spectra recorded three times) of (i) buffalo ghee, (ii) cow ghee, (iii) vitamin D and (iv) CLA.

This is in accordance with the literature values for cholesterol content of 275 mg 100 g^{-1} fat in buffalo milk and 330 mg 100 g^{-1} fat in cow milk (Haenlein & Wendorff, 2006).

Raman bands at 845, 870, 890 and 1178 cm^{-1} are attributed to saturated fatty acids. Raman band at 1178 cm^{-1} assigned to C–C stretching vibrations (Czamara et al., 2015) shows positive

correlation in difference plot of Fig. 1, which means concentration of this type of saturated fatty acids is more in cow ghee than in buffalo ghee. Similarly, Raman bands at 845, 870 and 890 cm^{-1} correspond to ν (C1–C2), CH3 rk, ν (C–O) complex broad table in liquid, 800–920 (Ali et al., 2016; Talari, Movasaghi, Rehman, & Rehman, 2015) show negative correlation, which indicates the concentration of these saturated fatty acids in buffalo ghee is more than in cow ghee.

Fig. 2 shows the comparison of Raman spectra of cow and buffalo desi ghee, vitamin D and CLA. Raman band at 845 cm^{-1} in ghee samples is assigned to saturated fats and vitamin D contents and Raman bands at 870 and 890 cm^{-1} are assigned to saturated fats (Ali et al., 2016) and appear due to CLA contents. The saturated fatty acids with Raman bands at 870 and 890 cm^{-1} were assigned to CLA isomers by comparison with CLA spectra. Heating of ghee during extraction can cause CLA oxidation, which may be the reason for low intensity of these bands in ghee samples.

From Fig. 2, the Raman band at 970 cm^{-1} is assigned to out of phase CCH wag vibration and represents vitamin D content (Agbenyega, Claybourn, & Ellis, 1991). The Raman band at 1063 cm^{-1} is assigned to saturated fatty acids and that at 1080 cm^{-1} to free cholesterol (Ullah et al., 2017b) and these bands also show signatures of Vitamin D. The Raman band at 1125 cm^{-1} is out-of-phase C–C solid fat and assigned to saturated fats (Gallier et al., 2011) and also shows vitamin D signatures in both types of samples. Similarly Raman bands at 1006, 1156 and 1520 cm^{-1} show strong positive correlation and represent β-carotenes (El-Abassy et al., 2011; Ullah et al., 2017b), which are absent in buffalo ghee and can be the potential biomarkers to differentiate cow desi ghee from buffalo. Animals are unable to synthesise β-carotenoids and obtain them from plants in the form of their food. The digestive system of animals converts carotenoids into different ingredients as human body converts them to vitamin A (Tang, 2010). Raman bands of carotenoids are absent from desi ghee obtained from buffalo milk because buffalo metabolise almost all β-carotene into fat soluble vitamin-A through their digestive system, which then passed on to milk, whereas β-carotene is retained in cow milk (Green & Fascetti, 2016; Nozière et al., 2006; Olson, 1989).

Raman bands at 1270 cm^{-1} indicates C=C *cis* unsaturated fatty acids (Gallier et al., 2011) and, in addition, signatures of vitamin D and CLA. Raman band at 1300 cm^{-1} shows unsaturated fatty acids (Ahmad et al., 2017) and also defines signatures of vitamin D. Raman bands around 1420 and 1450 cm^{-1} are assigned to saturated fatty acids (Ullah et al., 2017b) and also show fingerprints of vitamin D. The Raman band around 1439 cm^{-1} is a complex band and assigned to fingerprints of several molecular structures. Meurens et al. (2005) assigned 1439 cm^{-1} as the fingerprint for CLA isomers, but also shows signatures of vitamin D. Similarly, Raman band around 1650 cm^{-1} (cis-9–18:1, C=C stretching) is assigned to isomers of CLA (Ali et al., 2016; Meurens et al., 2005), which is also clear from CLA spectra with a broad peak at 1650 cm^{-1}. In difference spectra (Fig. 1), Raman spectral peak of CLA in the difference plot is slightly shifted to 1658 cm^{-1}; this depends upon the overall shape of both spectral peaks. Positive correlation at this Raman band position clearly indicates that cow ghee has more isomers of CLA than is the case for buffalo ghee. The Raman band at 1738 cm^{-1}

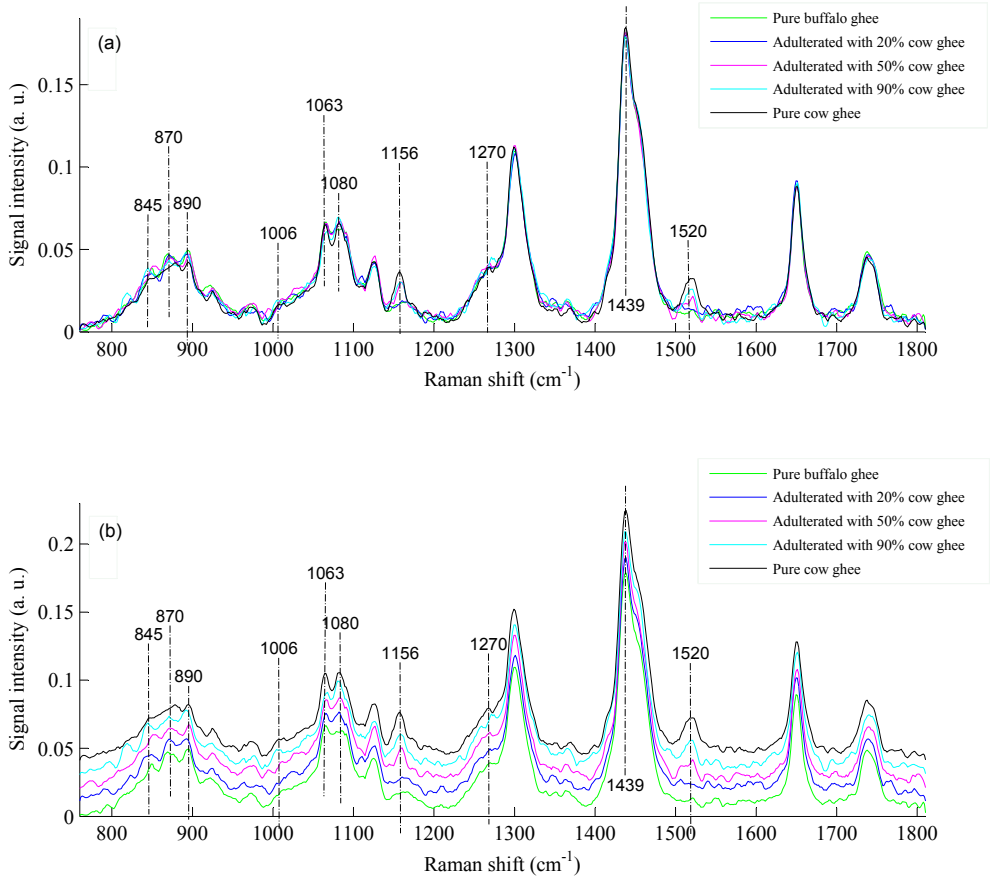

Fig. 3. Raman spectra with (a) common base line of cow and buffalo ghee along with adulterated buffalo ghee samples at a ratio of 20, 50 and 90% and (b) shifted base line on intensity axis of cow and buffalo ghee along with adulterated buffalo ghee samples at a ratio of 20, 50 and 90%.

is assigned to C=C stretch of ester group and labelled as saturated fatty acid (Ali et al., 2016) and also shows strong appearance of vitamin D content.

The presence of CLA in two types of species is evident in Fig. 2 and Table 1. It can be seen that CLA showed Raman spectra at 870, 890, 1080, 1270, 1439, 1450, 1582, 1610, 1738 cm^{-1} and the most prominent band at 1650 cm^{-1}. These bands associated with CLA are strongly correlated with the Raman bands originating from cow and buffalo desi ghee samples. Similarly, the presence of vitamin D in buffalo and cow desi ghee can be seen in Fig. 2 and Table 1. It is evident that vitamin D showed Raman bands at 842, 870, 890, 923, 1063, 1080, 1300, 1450, 1610, 1650, 1738 cm^{-1} and the most pronounced band at 1439 cm^{-1}. All these bands are closely associated with the Raman bands originating from cow and buffalo desi ghee samples.

In addition, the presence of vitamin D in both species is confirmed by Fig. 2 and Table 1 where Raman spectra of vitamin D shows strong similar band positions with Raman spectra of cow and buffalo ghee.

About 30–50% of population is suffering from deficiency of vitamin D (Lee, O'Keefe, Bell, Hensrud, & Holick, 2008), which in turn can cause cardiovascular diseases and rickets (Rajakumar, 2015; Wang et al., 2008). Therefore, the continuous use of desi ghee may be a dietary source of vitamin D.

3.2. Detection of adulteration

The presence and absence of β-carotenoids in desi ghee of cow and buffalo is predicated on their presence and absence in milk (Raynal-Ljutovac, Lagriffoul, Paccard, Guillet, & Chilliard, 2008; Ullah, Khan, Ali, Bilal, & Saleem, 2017a) has been utilised for detecting inter-species adulteration. In this study, buffalo ghee was adulterated with different concentrations of cow ghee. Raman spectra of adulterated buffalo ghee samples is shown in Fig. 3, which evidently shows that pure buffalo ghee does not show any appreciable β-carotene fingerprint. Fig. 3a shows the Raman spectra of pure and adulterated buffalo and cow ghee samples with common base line whereas Fig. 3b displays the Raman spectra with shifted base line of individual samples along the y-axis to illustrate their inter-differences due to adulteration.

Interspecies adulteration can be detected on the basis of β-carotenes, the free cholesterol band at 1080 cm^{-1} and the concentration of saturated fatty acids appearing at 845, 870 and 890 cm^{-1}. However, β-carotenes play major role in detection of adulteration. According to the authors' own observations (unpublished), the greenish-white colour of buffalo ghee is only changed by almost 25% adulteration with cow ghee. Therefore, the colour test can only be used for high adulteration values, whereas the technique employed in this study has detected

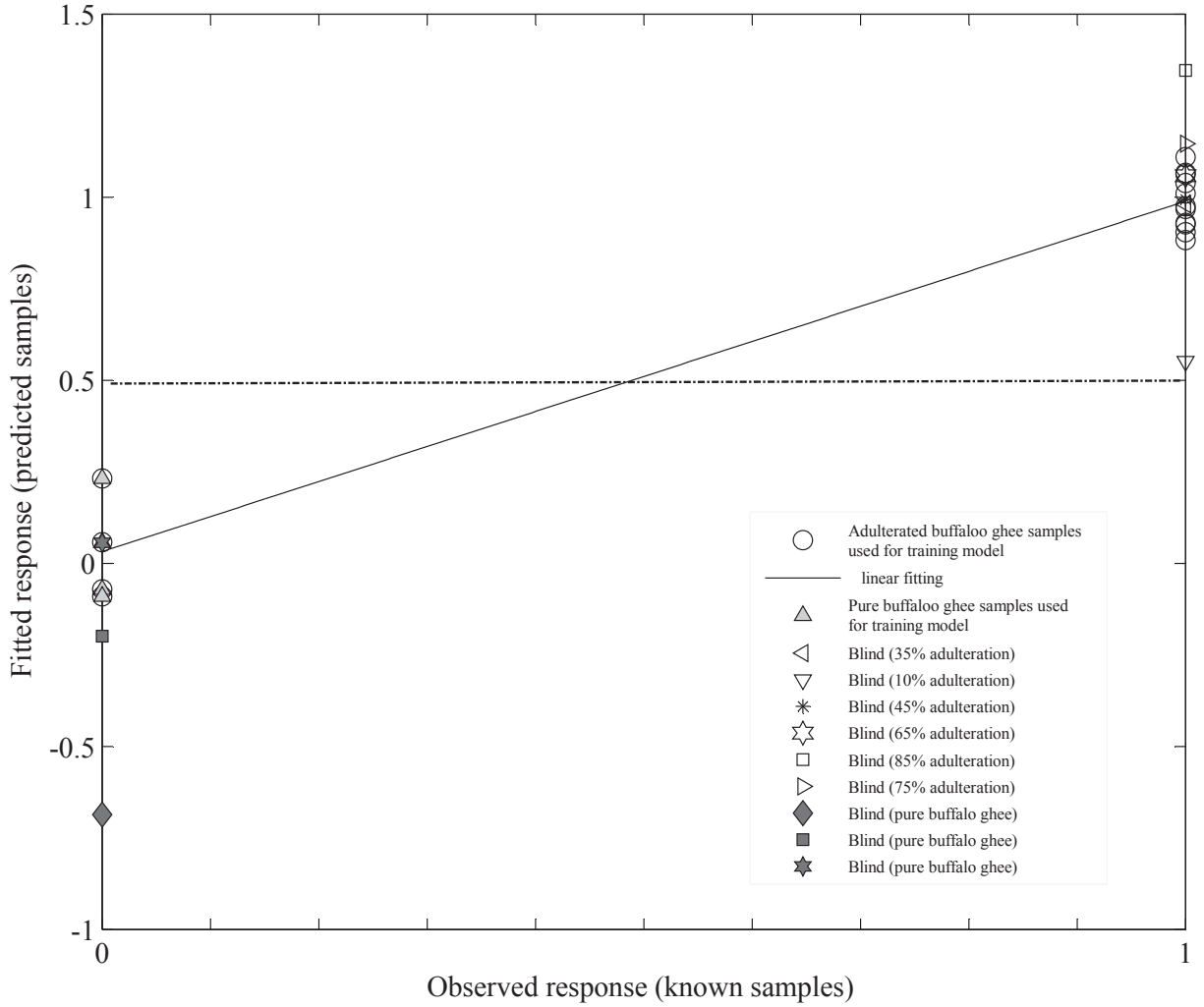

Fig. 4. Calibration curve (linear fit) produced by PLSR model based on the trainee data set (○, adulterated buffalo ghee; ▲, pure buffalo ghee) with blindly predicted samples (pure buffalo ghee; adulteration at: △, 10%; ◁, 35%; ✳, 45%; ☆, 65%; ▷, 75%; □, 85%).

around 10% adulteration with cow ghee in buffalo ghee, far better than the colour test.

Spectroscopic results in Fig. 3 show that when cow ghee is adulterated with buffalo ghee, the Raman bands at 1156 and 1520 cm^{-1} representing β-carotene started to evolve in intensity with increase of adulteration ratio. Similarly, a trend of rise in intensity can be seen at Raman peak position of 1080 cm^{-1}, which is assigned to free cholesterol (Manoharan, Baraga, Feld, & Rava, 1992) and it shows the rise in cholesterol with the rise in adulteration value of cow ghee containing more cholesterol than buffalo ghee (Haenlein & Wendorff, 2006). This confirms the fact that desi ghee obtained from cow milk contains more cholesterol than ghee obtained from buffalo milk. In Fig. 3, the Raman band 1270 cm^{-1} shows unsaturated fatty acids (Gallier et al., 2011) and an increase in its intensity can be observed with the increase of adulteration ratio of cow ghee, which has high content of unsaturated fatty acids compared with buffalo ghee. Raman bands at 870 and 890 cm^{-1} show more concentration of saturated fatty acids in buffalo ghee (Ali et al., 2016) and their intensity shows a decreasing trend with the increase of adulteration ratio by cow ghee.

These Raman spectral signatures appearing at 1080, 1156, 1178 and 1520 cm^{-1} discussed above can be used as visual biomarker to identify cow and buffalo ghee and are also helpful in labelling true nutritional contents that are very important from health point of view both for the producers and consumers. Along with β-carotenoids, free cholesterol at 1080 cm^{-1} and triacylglycerols at 1740 cm^{-1} appear with higher concentration in cow ghee whereas concentration of saturated fatty acids appearing at 845, 870 and 890 cm^{-1} is higher in buffalo ghee; this can, in addition, be utilised for the detection of adulteration of cow ghee in buffalo. However, β-carotene plays the major role in detection of adulteration. Further classification of adulterated samples is explained by partial least square regression (PLSR) model in Fig. 4.

3.3. Statistical analysis

The Raman spectra of adulterated ghee samples in Fig. 3 show that for small concentration of adulterants, it seems difficult to identify them with naked eye. Therefore, it is important to apply some statistical analysis on the Raman spectra to predict adulteration. For this purpose, a PLSR model was developed.

Both ghee samples contain similar chemical composition with little differences in component concentrations except β-carotenoids (identified by bands at 1006, 1157 and 1520 cm^{-1}) and cholesterol (identified by a band at 1080 cm^{-1}), which have maximum concentration in cow ghee. It is evident from Fig. 3 that with the increase of adulteration of cow ghee in buffalo, the intensity of spectral bands in of cow ghee starts to appear in adulterated samples. Therefore, due to adulteration of cow ghee in buffalo, the spectral signatures of cow ghee (β-carotenoids and cholesterol) in the adulterated ghee samples form the basis of detection of adulteration.

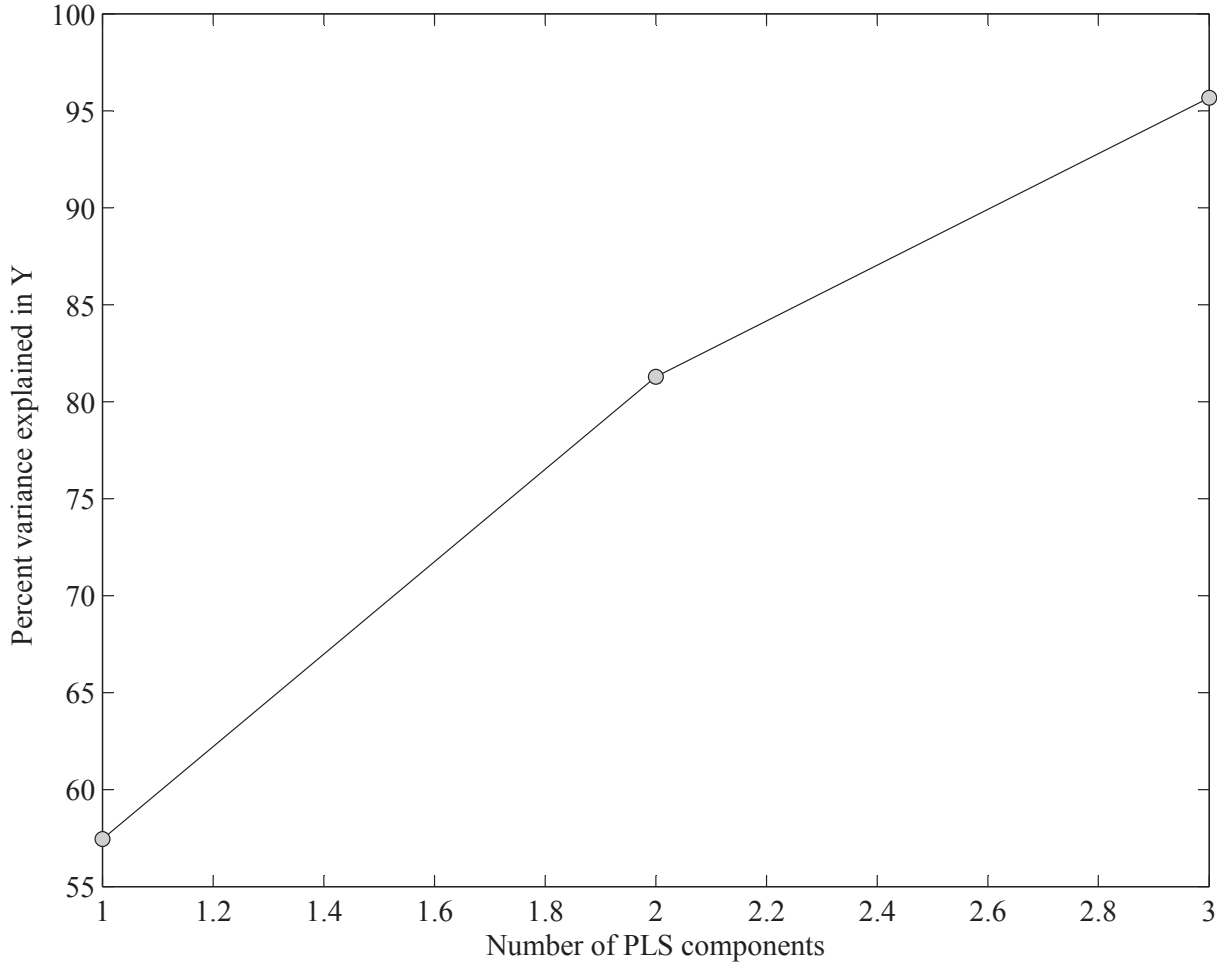

Fig. 5. Percent variance in the predicted samples used in training the PLSR model at different PLS components.

Partial least squares regression (PLSR) is a supervised statistical technique that bears some relation to principal components regression instead of finding hyperplanes of maximum variance between the response and independent variables. It basically determines a linear regression relationship by projecting the predicted variables and the observed variables to a new space. Because both observed variables and predicted variables are projected to new spaces, the PLS family of methods are also known as bilinear factor models.

In the present study, PLS regression model was developed based on a trainee dataset of 4 pure buffalo ghee samples, one cow ghee and 10 adulterated samples. To validate the accuracy of the model, 9 unknown samples were used, out of which 3 samples were pure buffalo ghee and were adulterated by cow ghee. PLS regression was implemented through built-in routine in MATLAB. Each trainee sample consists of 15 Raman spectra and the average of these spectra was used in the model. Based on the trainee dataset, PLS model produced regression coefficients at corresponding Raman shifts that provide the basis for the prediction of unknown samples (Thomas & Haaland, 1990), which is called the β-vector. The β-vector basically contains all the varying signatures of adulterants, which, when multiplied with unknown spectrum, yield a correlation factor around the fitting curve of the model.

The model was duly calibrated by using leave one out (LOO) cross validation method. Calibration curve produced by this model is shown in Fig. 4, which is the best fit over the trainee data set. It yields a coefficient of the determination (R^2) of 0.96 values that is statistically accepted for any model (Martens & Næs, 1984). R^2 explains how well the independent variable explains the dependent variable in a regression while another parameter coefficient of correlation (R) explains the relationship between the actual values of independent and dependent variables. The correlation coefficient can be positive or negative, but the coefficient of determination always yields a positive value, however, for both cases greater than 0.98 is accepted for a best fit model.

During development of the PLSR model, different principal components were utilised repeatedly for the trainee data sets and optimum number of components for the best possible prediction was found to be 3, where over fitting was avoided. The percent variance in data used for training the model for different principle components is shown in Fig. 5, which shows that with up to 3 PLS components, 96% variance was explained in the model. It basically explains percent variance in the data (Y) that is predicted during training the model.

The estimated mean square error in prediction (EMSEP) of the developed model is shown in Fig. 6, which shows that the error reduces to almost zero in prediction for using 3 latent variables (principal components). EMSEP is calculated for each principal component as a parameter of plsregress code. Therefore, the R^2 value of 0.96, the percent variance for different principal components and estimated mean squared error in prediction (EMSEP) for different latent variables evidently illustrated that 3 latent

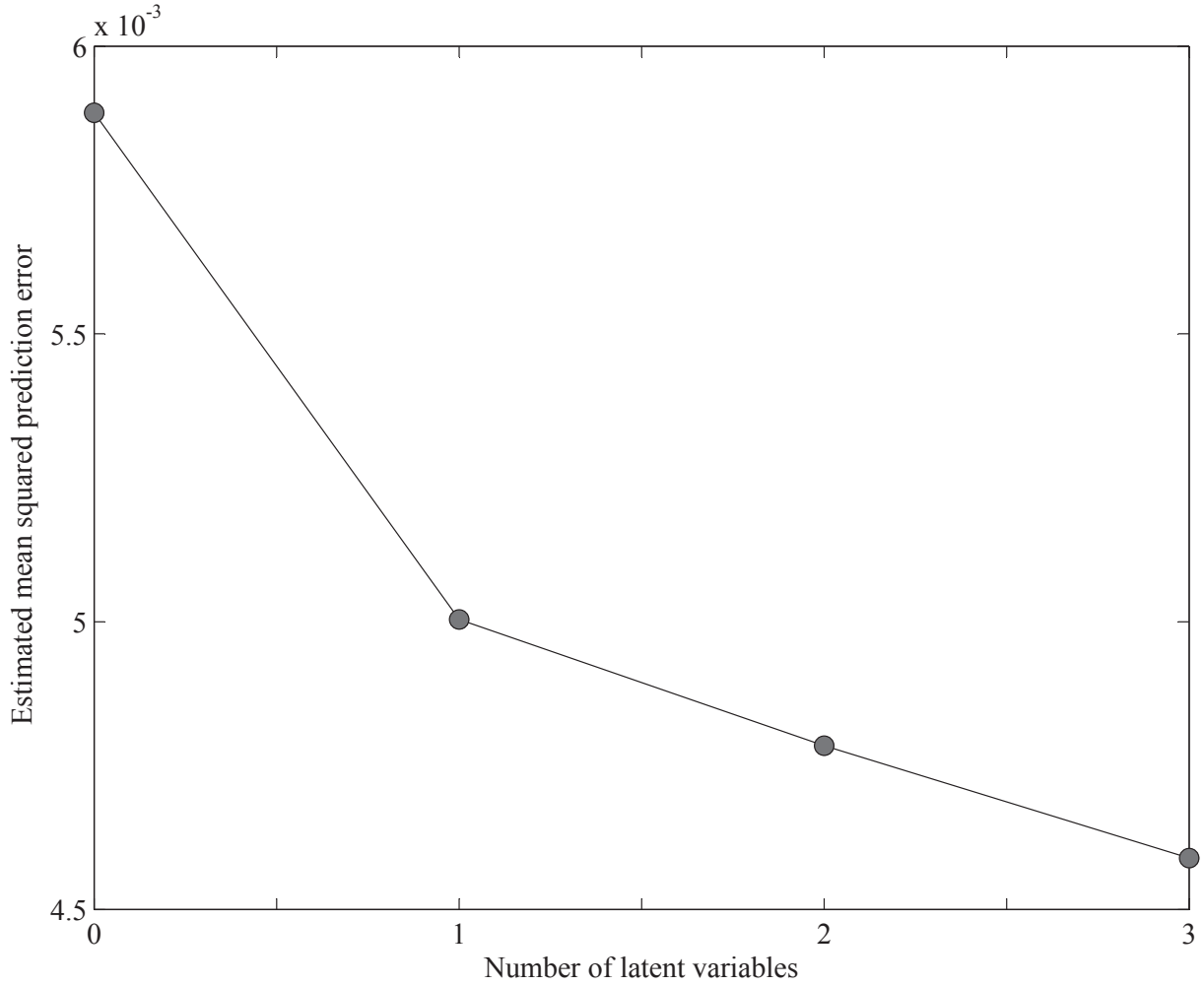

Fig. 6. Estimated mean squared prediction error for different PLS components.

variables (principal components) are sufficient to develop PLSR model for best prediction of unknown samples.

The quality of the calibration model was quantified by the standard error of calibration (SEC), standard error of prediction (SEP) and the correlation coefficient (R) between the predicted and measured parameter. A good model should have a low SEC, a low SEP, and a high R, but also a small difference between SEC and SEP (Jha & Ruchi, 2010). For the present model, the calculated values of SEP, SEC and correlation coefficient (R) are 0.101, 0.105 and 0.98, respectively, confirming the validity of the model. In addition, the ratio of SEP/SEC is 0.96, which is much less than the value of 1.3 reported as upper limit for best fit PLS model (Cantor, Hoag, Ellison, Khan, & Lyon, 2011). Standard error in prediction (SEP), standard error in calibration (SEC) and correlation coefficient (R) may therefore set a criterion to avoid overfitting of the PLSR model for selected latent variables.

The trained PLSR multivariate model was used to blind test 9 unknown samples. The spectrum of unknown sample is loaded by this multivariate model and compared with the regression curve (β-vector) generated by the model and yields a numerical value that represents the correlation between the loaded spectra and the regression curve. This value is then displayed at its proper location on Fig. 4 to show its correlation with regression curve. In Fig. 4, trainee adulterated buffalo ghee samples are marked by green circles and pure buffalo ghee samples are marked by filled yellow circles, whereas adulterated blindly predicted samples are marked with different colours and shapes and pure blindly predicted samples are marked as magenta filled squares.

Root mean square error (RMSE) for blindly predicted samples is also a good measure of how accurately the model predicts the response (blind samples) and its low values indicates the accuracy of model. In the present model, RMSE in predictions for 9 blindly predicted samples was calculated to be 0.05, which shows the accuracy of the model.

4. Conclusion

The potential of Raman spectroscopy has been utilised for the characterisation, identification and differentiation of desi ghee obtained from cow milk and buffalo milk for the first time. Raman bands at 1006, 1156 and 1520 cm^{-1} defines the signature of β-carotene and that at 1080 cm^{-1} of free cholesterol. β-Carotene was used as a biomarker to differentiate between both types of ghee samples. Desi ghee obtained from cow milk is rich in β-carotene, but is absent from buffalo ghee. Desi ghee obtained from buffalo milk is a good source of vitamin A when metabolised in human body. Additionally, both species are anti-oxidants and protect human body against cancer and obesity. However, cow ghee has a higher concentration of free cholesterol than does buffalo ghee. In addition, Raman spectra of Vitamin D and CLA isomers confirm their presence in both ghee types and the Raman spectral signatures showed that cow ghee has more isomers of CLA than the buffalo ghee samples used in the present study.

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